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A(2B) adenosine receptor coupled activation of mutant CFTR: cAMP and cPLA(2) signaling.

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A_{2B} ADENOSINE RECEPTOR COUPLED ACTIVATION OF MUTANT CFTR: cAMP AND $cPLA_2$ SIGNALING

by

BRYAN R. COBB

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Medical Genetics

Name of Candidate Bryan R. Cobb

Committee Chair Eric J. Sorscher

Title A_2 Adenosine Receptor Activation of Wildtype and Mutant CFTR: cAMP and Cytoplasmic PLA_2 Signaling

The work reported in this dissertation involves two separate studies designed to *1)* characterize the signaling pathways used by adenosine (Ado) and A_{2B} Ado receptors to regulate the cystic fibrosis transmembrane conductance regulator (CFTR) activity, and *2)* utilize native signaling pathways to functionally enhance the activity of CFTR and surface localizing CFTR mutants. Also included in this work is a comprehensive review regarding the molecular biology of Ado receptors. Ultimately, the intent of these studies is to better understand CFTR regulation, which may lead to studies that improve the treatment of individuals with Cystic Fibrosis (CF).

In the first study, A_2 receptor signaling pathways were examined. Stimulation with Ado activated CFTR and Cl^- secretion and the apical release of arachidonic acid (AA). Inhibiting cytoplasmic phospholipase A_2 ($cPLA_2$) activity blocked Ado-stimulated halide efflux in Cos-7 cells transiently expressing CFTR, Calu-3 cells that endogenously express CFTR, and Cl^- secretion in vivo across the murine nasal mucosa. Ado alone activated ΔF508 CFTR, while activation of G551D CFTR required Ado stimulation combined with AA and phosphodiesterase (PDE) inhibition. Our studies implicate both cAMP/protein kinase A (PKA) and AA/$cPLA_2$ signaling pathways in A_2 receptor regula-

tion of CFTR and indicate that Ado can be used in strategies to activate mutant CFTR molecules that cause disease.

In the second study, we further explored regulation of CFTR by PDEs. Earlier experiments have demonstrated a role for the PDE inhibitor (PDEi) milrinone in activating CFTR across the nasal mucosa of mice and in Cl^- efflux studies using airway cell lines. However, few studies have examined clinically used PDEis, particularly in the context of polarized airway cell monolayers. Several clinically used PDEis were evaluated, including papaverine, theophylline, rolipram, milrinone, cilostazol, and sildenafil. Each PDEi (excluding theophylline) acutely activated Cl^- secretion in Calu-3 cells, and several of these compounds also activated R117H CFTR transiently expressed in CF airway cells. Pretreatment with papaverine, cilostazol, and to a lesser extent rolipram increased the magnitude and duration of low dose Ado-stimulated short circuit current (I_{sc}) in CFTR-expressing airway cell monolayers. Cumulatively, these studies suggest that PDEs provide tonic regulation of CFTR and that PDEis may represent an additional strategy to maximize wildtype and mutant CFTR activity.

DEDICATION

This dissertation is dedicated to my father, who has equiped me with the skills to succeed by his example and who has provided a beacon so that I can find my way, support so that I can persevere, and confidence in me so that I believe in myself. It is also dedicated to my mother, who envisions the achievement of my desired accomplishments so that they will come to fruition, who has empowered me with a spiritual, guiding awareness, and who has recognized, enabled, and nurtured my gifts.

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I could not have succeeded in completing the work presented in this dissertation without Dr. John Paul Clancy, friend and mentor. Dr. Clancy's enduring patience, intelligence, supportiveness, and guidance combined with his infectious good nature have been a source of motivation. Dr. Eric J. Sorscher has also played a significant role in the development of my educational and professional career and served as my mentor while I studied under his guidance.

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LIST OF ABBREVIATIONS

5'AMP	5' adenosine monophosphate
5-LO	5-lipoxygenase
8-PT	8- Phenyl theopylline
AA	Arachidonic Acid
ADA	Adenosine deaminase
Ado	Adenosine
AKAP	A Kinase Anchoring Protein
ATP	Adenosine triphosphate
BAPTA-AM	1,2-*bis* (o-Aminophenoxy)ethane-N,-tetraacetic Acid Tetra (acetoxymethyl) Ester
CAP70	CFTR associated protein of 70 KDa
CBAVD	Congenital bilateral absence of the vas deferens
CCPA	2-chloro-N^6-cyclo-pentyladenosine
CF	Cystic Fibrosis
CFTR	Cystic fibrosis Ttransmembrane conductance regulator
CGS	(2-[[4-(2-carboxyethyl)phenethyl]-amino]Ado-5'-*N*-ethlcarboxamide
CHO	Chinese hampster ovary
CNS	Central nervous system
COX	Cyclooxygenase

$cPLA_2$	Cytoplasmic phospholipase A_2
CPX	8-cyclopentyl-1,3-dipropylxanthine
CPZ	Chlorpromazine
DAG	Diacylglycerol
DHA	Docohexanoic acid
DMSO	Dimethylsulfoxide
DOPE	Propane-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTAP	*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
EBP50	Ezrin binding protein of 50 KDa
EC_{50}	Concentration giving half-maximal response
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GPCR	G-protein coupled receptors
IBMX	3-iso-butyl-1-methylxanthine
IC_{50}	Concentration giving half-maximal inhibition
IL	Interleukin
IP_3	Inositol Phosphate 3
$iPLA_2$	Calcium-independent phospholipase A_2

I_{sc}	Short circuit current
MAP	Mitogen activated pathways
NADPH	Nicotinamine adenine dinucleotide phosphate
NECA	5'-*N*-ethylcarboxamidoadenosine
ORCC	Outwardly rectified chloride channel
PDE	Phosphodiesterase
PDEi	Phosphodiesterase inhibitor
PDZ	Postsynaptic density
PKA	Protein Kinase A
PKA	Protein Kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMN	Polymorphonuclear leukocytes
R-domain	Regulatory domain of CFTR
R-PIA	N^6 –cyclopentyladenosine > (-)-N^6-(R-phenylisopropyl)adenosine
SH3	Proline-rich Src homology 3
$sPLA_2$	Secretory phospholipase A_2
SPQ	6-methoxy-*N*-(3-sulfopropyl-quinolinium)
TNF	Tumor necrosis factor
TRL	Threonine, arginine, and leucine ordered amino acids
VIP	Vasointestinal peptide

WT	Wildtype

INTRODUCTION

BACKGROUND

Clinical manifestations of cystic fibrosis. Cystic fibrosis (CF) is an autosomal recessive disease characterized by defective electrolyte transport. Abnormalities in CF have been described in the epithelia of several organs and tissues, including the airways, airway submucosal glands, pancreatic and bile ducts, gastrointestinal tract, sweat glands, and male reproductive ducts (135). Lung histopathology and function are often normal at birth; however, bronchiolar mucous obstruction, bronchiolar wall inflammation, and bacterial colonization are characteristically seen in the CF airways. The pathophysiological consequences that follow are believed to stem from repetitive cycles of bacterial infection and endobronchitis, contributing to a progressive deterioration in pulmonary function.

In addition to the airways, many of the other epithelia affected in CF are characterized by abnormally thick and dehydrated secretions, which appear to cause obstruction resulting in organ dysfunction. For example, the pancreatic ducts are obstructed by inspissations, resulting in parenchymal tissue degeneration, fibrosis, and exocrine glandular dysfunction. Reduced bicarbonate transport combined with a decreased volume of pancreatic fluid contributes to pancreatic enzyme insufficiency. Exocrine pancreatic dysfunction is seen in ~ 85% of CF patients. Without treatment, enzyme deficiency results in protein and fat maldigestion and severe malnutrition.

In the gastrointestinal tract, the cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in variable quantities throughout the large and small intestines. Accumulation of mucous secretions combined with goblet cell hyperplasia in the crypts of liberkuhn are characteristic of CF. Dehydrated intestinal contents combined with abnormal mucous secretions are thought to predispose patients to bowel obstruction and meconium ileus, which is a presenting symptom in 10-20% of CF newborns. Other intestinal complications outside of the newborn period include the distal ileal obstructive syndrome (DIOS) and rectal prolapse (which is seen in ~20% of CF children). The bile ducts of CF patients can also be obstructed, producing gall bladder disease and elevations in liver function enzymes. These clinical findings are associated with eosinophilic concretions observed in histological sections of the bile ducts. Other complications from hepatobiliary obstruction include esophageal varices, portal hypertension, splenomegaly, and occasionally hepatic failure.

Greater than 95% of males with CF are infertile due to structural alterations in the vas deferens and seminal vesicles that result in azoospermia. Reproductive tract secretions are often characterized by reduced fructose concentrations and increased acidity (45, 131). Congenital bilateral absence of the vas deferens (CBAVD) is also common in CF patients of certain genetic backgrounds (discussed later in this document). Reduced fertility has also been noted in females with CF and may be related in part to abnormal cervical mucous composition.

In the sweat gland, defective salt absorption leads to characteristically detectable "salty sweat" (> 60 meq), which represents the traditional gold standard test for diagnosing CF. The ducts of the sweat gland normally function to reabsorb Na^+ and Cl^- ions

across the water impermeable epithelium. In CF, absent apical Cl^- conductance in the ducts prevents Cl^- ions from being reabsorbed. Demands for electroneutrality keep Na^+ ions from being reabsorbed through epithelial Na^+ channels. As a result, lumenal salts fail to be reabsorbed and concentrate in sweat. Clinical manifestations include a predisposition to hyponatremic dehydration.

Despite the multi-organ involvement of the disease, respiratory failure is the primary cause of morbidity and mortality in >90% of CF patients, with a median survival age of 31 years. Current hypotheses suggest that, in the CF airways, defective electrolyte transport results in alterations in airway-surface liquid volume, salt content, and/or mucus composition, leading to thick and tenacious mucus secretions that impair mucociliary clearance. The resulting airway microenvironment is conducive to chronic bacterial colonization and infection by specific bacterial pathogens, including *Pseudomonas aeruginosa, Haemophilus influenza, Staphylococcus aureus,* and *Burkholderia cepacia* (134). These infections, coupled with the vigorous inflammatory host response, lead to progressive airway destruction and death in the vast majority of CF patients.

The cystic fibrosis transmembrane conductance regulator (CFTR). CF is caused by mutations in the gene that encodes CFTR. The gene was identified in 1989 by a combination of linkage analysis studies and chromosome walking techniques (101, 102). CFTR is composed of 1480 amino acids and is a member of the ATP-binding cassette (ABC) family by virtue of two conserved transmembrane domains (TMDs) that anchor the protein to the cell membrane and two cytoplasmic nucleotide binding domains (NBD-1 and NBD-2) that bind and hydrolyze ATP (52). ABC protein family members function

in ATP-dependent movement of macromolecules through the cell. CFTR is a unique member of this protein family in that it possesses a regulatory domain (R-domain) within the predicted structure. CFTR functions as a cAMP-regulated Cl^- channel at the surface of polarized epithelial tissues where it is expressed, including airway submucosal glands and surface airway epithelial cells (8, 33, 66).

Both ATP binding and hydrolysis at NBD-1 and NBD-2 and phosphorylation of the regulatory domain (R-domain) by PKA activates CFTR. In addition to functioning as a cAMP-regulated Cl^- channel, CFTR has also been shown to be a regulator of other ion conductance pathways, including modulation of the outwardly rectifying Cl^- channel (ORCC) and the epithelial Na^+ channel (ENaC) (106, 125). Defective ion transport, including absent cAMP-stimulated Cl^- secretion and hyperreabsorption of Na^+, are hallmarks of the CF bioelectric phenotype in the airways.

CFTR has also been implicated in regulating ATP permeability (98, 106). ATP release has been shown to be associated with cAMP activation of CFTR and changes in extracellular Cl^- concentrations, similar to Cl^- concentrations reported in the airway-surface liquid (57). CFTR may potentiate ATP release by stimulating a separate ATP channel. Released ATP in turn can activate P_2 purinergic receptors, controlling membrane ion permeability through Ca^{2+}-dependent Cl^- channels. CFTR-dependent ATP release has been shown to contribute to whole cell volume recovery after hypotonic challenge (17). These studies point to a role for CFTR as a salt concentration sensor that is hypothesized to be an important mechanism for maintaining the appropriate solute concentration in the airway-surface liquid. Abnormalities in CFTR-dependent ATP release

could, therefore, represent a significant physiological difference between CF and non-CF airways, contributing to the pathogenesis of CF.

CFTR and lung disease. Despite the well-studied molecular and electrophysiological aspects of CFTR, the link between defective ion transport and CF-related lung disease is not well understood (41, 67, 94, 97, 135). There are several hypotheses that relate defects in ion transport to lung disease. Two fundamentally differing hypotheses regarding airway fluid absorption have recently been proposed. Described by Welsh and Boucher, these hypotheses are referred to as the "high salt hypothesis" and the "low volume hypothesis," respectively (82, 140). Both are based on the notion that the primary defect involves the CFTR's role in salt absorption across the human airway epithelia.

The basis of the high salt hypothesis stems from reports that the CF airway-surface liquid salt concentration is abnormally high as a result of absent CFTR-dependent Cl^- transport. Since a paracellular Cl^- shunt pathway in the absence of an apical conductance has not been identified, it is felt that absent transcellular Cl^- transport coupled with demands for maintaining electroneutrality prevents Na^+ reabsorption. As a result, fluid at the luminal surface becomes hypertonic for salt. High salt concentrations have been shown to inactivate endogenous antimicrobial peptides such as defensins and lysozyme (42, 115, 140). Inhibition of these antibacterial factors could provide a selective survival advantage for specific bacterial species. In contrast, the underlying tenet of the low volume hypothesis is that absent CFTR results in *1*) hyperreabsorption of Na^+ due to upregulated ENaC activity, *2*) absorption of Cl^- via trans or paracellular Cl^- permeability pathways, and *3*) concomitant water absorption that may be paracellular, resulting in re-

duced airway-surface liquid volume. This cascade, in turn, impairs mucociliary clearance. Several in vitro studies support one or the other hypothesis but differ in experimental methodologies such as tissue culture conditions (82, 140).

Other hypotheses have been attributed to the pathological consequences of CFTR mutations, including functions outside a role in ion transport. One model suggests that CF airway epithelia is predisposed to *Pseudomonas aeruginosa* infection due to receptor-substrate interactions between CFTR and specific components of the bacteria. In this hypothesis, CFTR is a receptor for *Pseudomonas*, and mutations in CFTR result in inefficient or absent binding to the cell surface (76, 93, 95, 96). The outer core oligosaccharide of lipopolysaccaride has been proposed as a component of bacteria important for binding to CFTR. In CF airways, hypersusceptibility to *Pseudomonas* infection has been suggested to involve an inability to bind and clear this bacterial pathogen and might explain the susceptibility to bacterial colonization.

Other studies suggest that an abnormal inflammatory response may represent the primary defect. A distinguishing feature of the inflammatory response in CF is that cells characteristic of acute inflammation produce a chronic inflammatory response (134). CF lungs from infants frequently show signs of exaggerated inflammation with elevated interleukin-8 levels and reduced IL-10 levels, despite the failure to detect bacterial pathogens. This suggests that the excessive neutrophil dominated infiltration of CF airways is due (at least in part) to a mechanism independent of infection (5, 15). Following recruitment from the peripheral circulation, neutrophil activation in the tissues results in polymorphonuclear (PMN) degranulation and the release of multiple pro-inflammatory and

injurious mediators, including cytokines such as IL-6 and IL-8 (16), myeloperoxidase, elastase, and free radical products.

Classes of mutations in CF. Currently, there are 1,001 described CFTR mutations and 208 sequence variations that result in CF (http://www.genet.sickkids.on.ca/cftr/). These mutations are classified into five distinct classes (Fig. 1) (44).

Class I mutations lead to defective CFTR biosynthesis (i.e., G542X) (44). These include insertions or deletions leading to frameshifts, nonsense mutations, or splice site abnormalities that produce premature termination signals. Consequently, patients carrying these mutations cannot synthesize full-length protein. Class I mutations encompass nearly half of all known mutations and are found in approximately 10% of CF patients.

Class II mutations are the most common and involve a defect in the maturation and trafficking of CFTR. These include the most common CFTR mutation, ΔF508 CFTR (29). ΔF508 CFTR refers to a deletion in amino acid (phenylalanine) at position 508 of the protein. Class II mutations are characterized by improper folding in the endoplasmic reticulum, failure to transport to the golgi for glycosylation and maturation, and severely reduced levels of mutant protein at the cell membrane. The majority of protein produced is targeted for degradation in the proteosome. Various maneuvers, including growth at low temperatures, treatment with 4-phenyl butyrate, dimethylsulfoxide (DMSO), or glycerol, can partially overcome the trafficking defect (9, 18, 22, 31, 103-105, 109, 142). Surface localized ΔF508 CFTR retains cAMP-dependent activation, but the level of activity retained is controversial (54, 74).

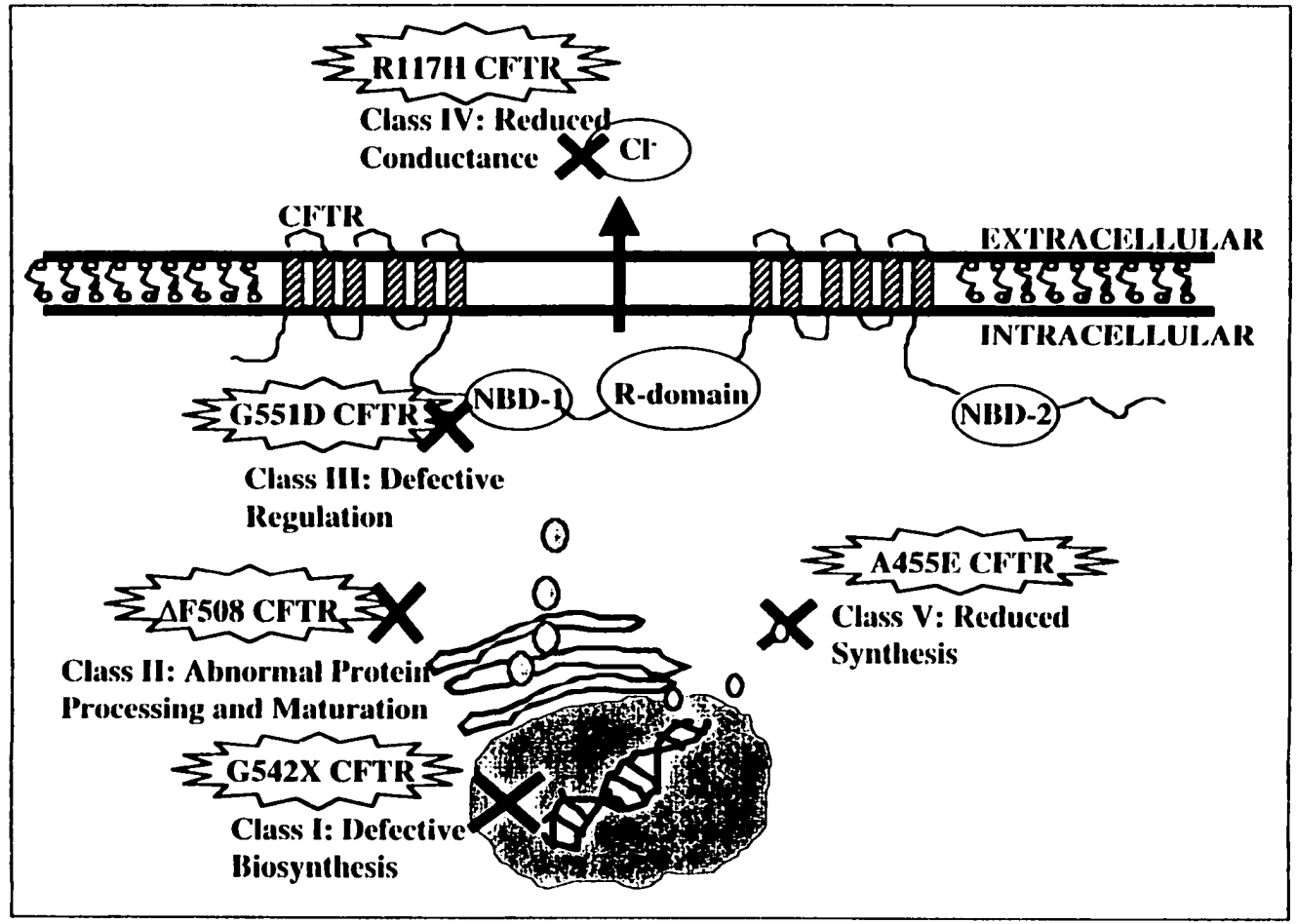

Fig. 1. Five classes of CFTR mutations. This figure illustrates CFTR at the cell membrane and depicts the various stages during protein biosynthesis. "X" indicates the stage at which the biosynthetic defect takes place, and an example of each class of mutation is shown. It is felt that class I mutations (but not the others) result in the inability to produce surface localized protein. NBD-1 (2) = nucleotide binding domain 1 (and 2), R-domain = regulatory domain.

Class III CFTR mutations are normally synthesized, mature properly, and are localized to the cell surface but are characterized by defective regulation. G551D CFTR isthe most common member of this class and the second most common disease-causing CFTR mutation. It is found in ~5% of CF patients. The G551D mutation occurs in NBD-1 of CFTR, disrupts ATP binding and hydrolysis, and leads to extremely low level Cl^- channel activity (48).

Class IV mutations have reduced Cl^- conduction. Cumulatively, these mutations are found in <5% of CF patients. Examples include R117H, R334W, and R347P CFTR, all of which encode missense mutations that replace an arginine with an aromatic amino acid. Patch clamp analysis has shown that, while all three of these mutant CFTR proteins can be activated, the single channel Cl^- conductance of each is reduced. It has been speculated that many of these mutations can retain partial function in vivo, as most are associated with pancreatic sufficiency (69, 89). Clinical manifestations may also be influenced by transcription efficiency for some of these defective alleles. For example, the genetic background in which the R117H CFTR mutation occurs can have profound effects on disease presentation (38). Three different phenotypes based on splicing efficiency of exon 9 determine the severity of the clinical manifestations. However, lung disease in these patients is generally indistinguishable from the CF population in most instances.

Class V mutations are characterized by a decrease in the abundance of functional CFTR. These mutations involve alternative splicing or affect the promoter of *cftr*. The phenotype is variable and depends on the amount of mRNA transcript and CFTR that is produced. Many of these rare mutations are also characterized by pancreatic sufficiency.

One of these, A455E CFTR, is unique in that it predicts a mild pulmonary disease course (111).

CFTR mutations that are characterized by defective channel regulation (class III) or conductance (class IV) but localize normally to the cell membrane may serve as appropriate targets for strategies to augment CFTR activity. Additionally, mutants that can be manipulated pharmacologically to increase protein expression (class I, II, and V) or can be made to overcome processing defects (class II) and lead to increased of surface CFTR may also be appropriate targets for strategies to maximize CFTR function. One goal of this dessertation is to characterize a novel signaling pathway to activate CFTR and determine whether signaling pathways native to airway cells can be used to improve the function of wildtype (wt) and clinically significant mutant CFTR molecules.

REGULATION OF THE CFTR PROTEIN

CFTR can be regulated by many different mechanisms. Phosphodiesterases, phosphatases, and PKA activity all play a role in regulating CFTR by modulating the phosphorylation status of the R-domain (10, 23, 32, 92). Membrane-associated phosphatases dephosphorylate the R-domain and inactivate the channel. Phosphodiesterases, also negative regulators of channel activity, bind and hydrolyze cAMP and limit PKA activation. These types of regulatory control of CFTR (phosphorylation of the R-domain) are the primary focus of this dissertation. CFTR can be regulated by other mechanisms as well. Protein:protein interactions between CFTR and associated scaffolding proteins have been shown to be important in CFTR regulation (19, 49, 63, 127). Proteins that interact with CFTR through both the amino and carboxy terminus have been

shown to couple CFTR to PKA activity through compartmentalized localization of protein complexes at the cell membrane. Finally, CFTR can also be self-regulated by domain:domain interactions within the protein, and these interactions can play a role in channel gating (88).

Phosphorylation and dephosporylation of CFTR. CFTR activity is thought to be regulated predominantly by PKA-dependent phosphorylation of the R-domain, which activates ion transport following conformational changes in the protein (1, 32, 92). Although PKA-dependent phosphorylation of CFTR is thought to be critical for channel activity; the specific intracellular signaling pathways that modulate CFTR are not fully defined. For example, it is not clear whether PKA activates CFTR by stimulating channels already inserted in the membrane or by increasing the number of CFTR molecules at the cell membrane. A recent series of experiments demonstrated that PKA activation enhances membrane trafficking of CFTR by a mechanism that is independent of CFTR activation or syntaxin 1A association (19).

Regulation of CFTR by phosphorylation of the R-domain is complex. To determine the role of the R-domain phosphorylation sites and CFTR channel activity, a study was performed that involved mutagenesis of four prominent PKA-dependent phosphorylation sites located in the R-domain, which resulted in loss of PKA-activated transepithelial ion transport. However, another study showed that ,even with all 10 predicted PKA sites mutated on the R-domain, PKA-activation of CFTR was retained, suggesting that PKA might regulate CFTR independent of phosphorylation sites on the R-domain (20). Winter and Welsh demonstrated that unphosphorylated R-domain does not equate to in-

hibition of channel activity, but rather phosphorylation of the R-domain stimulates the interaction of ATP with another domain resulting in increased channel activity (139). Finally, PKA may not be the only kinase important in regulating CFTR. A recent study demonstrated that PKC is required for maximal activation of CFTR by PKA (55), suggesting that differences in PKC activity may result in variability of the responsiveness to PKA activation.

PKA-dependent phosphorylation of the R-domain can also be regulated by phosphatases such as PP2A, PP2B, and PP2C, which have been shown to inactivate CFTR-related channel activity (14, 75). Different isotypes have different effects in vitro on the magnitude of the decreased open probability of CFTR, suggesting that multiple phosphatases may be necessary to achieve complete deactivation of channel activity. Phosphatases have been shown to participate in the formation of a multiprotein complex that includes CFTR (143). For example, PKA and PP2B have been shown to interact with the protein kinase A anchoring protein 79 (AKAP79) (28). In another study, β_2 adrenergic receptors were shown to form dynamic complexes with both PKA and phosphatases (112). Together, these studies suggest an important negative regulatory role for phosphatases, limiting CFTR activity in various model systems.

PDEs limit CFTR activity by hydrolyzing cAMP. As part of an endogenous response to cAMP-dependent stimuli, phosphodiesterases (PDEs) play a crucial role in regulating intracellular cAMP concentrations. They encompasses 11 kinetically distinct families, with each family having multiple isotypes as a result of genetic heterogeneity and multiple alternative splicing mechanisms (34). Isotypes are distinguished based on

both enzymatic characteristics and inhibitor profiles. The catalytic domain is highly conserved and represents the inhibitor and substrate (cAMP and cGMP) binding sites. "Cross-talk" between cAMP and cGMP signaling pathways exist in that PDE2-specific PDEs are activated by cGMP, while PDE3-specific PDEs are inactivated by cGMP but both selectively cleave cAMP (119). This suggests that many factors in the local environment may contribute to cAMP signaling and that the level of complexity is increased due to the integrated cAMP and cGMP pathways.

PDEs can be membrane targeted and anchored through mechanisms that include *1*) PDZ binding domains such as PDE6, *2*) proline-rich SH3 binding domains (PDE4), *3*) amino terminal transmembrane domains (PDE3), and *4*) prenyl groups (PDE6) as part of their structure (81, 90, 107). The nature of the interactions between PDEs and the cytoskeleton or intracellular membranes in terms of stringency of the interaction, the specificity of targeting, and the functional significance of membrane localization are not well understood. However, anchoring of PDEs would result in permissive restrictions on the diffusion gradients of cAMP signaling. Therefore, determining whether specific PDE isotypes are responsible for regulating CFTR by carefully investigating multiple isotype-specific PDE inhibitors (PDEis) may be helpful in better understanding this type of endogenous regulation.

Previous studies have investigated the use of PDEis as potential activators of CFTR. Milrinone, a PDE3-specific PDEi, has been shown to activate CFTR in human airway cell lines and CFTR-dependent ion transport in the mouse nose and trachea (59, 61). Follow-up studies revealed that combining milrinone with direct adenylate cyclase stimulation (with forskolin) stimulated halide efflux from an immortalized ΔF508 CFTR

nasal polyp cell line (CF-T43 cells) (58). Although milrinone improved ion transport in the nose of non-CF humans and mice, milrinone alone or combined with isoproterenol (a β_2 adrenergic receptor agonist) was shown to have no effect on Cl^- transport across the nasal mucosa of CF mice and humans with either ΔF508/ΔF508 CFTR or ΔF508/G551D CFTR genotypes (117).

Although these studies represent important findings providing evidence that PDEis can augment wt and mutant CFTR activity, other PDEis may be more useful in this regard. No studies have systematically investigated PDEis as activators of CFTR using polarized epithelial cell monolayers. The mechanism of action of PDEis has also been questioned since several studies have documented that increases in cAMP produced by PDEis are not associated with parallel increases in chloride permeability (21, 58, 59). One of the aims of the first manuscript was to determine whether PDEis could be used as part of strategies to activate the most common surface localizing mutant CFTR molecule (G551D CFTR). The aims of the second manuscript expanded upon this application, investigating the ability of various clinically important PDEis in experiments to *1*) activate CFTR in a polarized epithelial cell line (Calu-3 cells); *2*) correlate cAMP concentration with stimulated Cl^- secretion; *3*) determine if PDEis can activate mutant, surface localized CFTR; and *4*) determine the effects of these PDEis on stimulated Cl^- secretion.

Compartmentalized regulation of CFTR. In polarized epithelial cells, ion channels and transport proteins are often distributed selectively to the apical or basolateral compartments by mechanisms that are not well understood. The consequences of vectorial sorting might impinge upon the specific function of the membrane-associated protein.

In human airway (Calu-3) and colonic (T84) epithelial cell lines, the preferential activation of CFTR-dependent Cl^- secretion by membrane-associated PKA isoforms (PKAII) has been demonstrated (114, 118). PKAII is thought to be membrane associated through interactions between its regulatory (RII) subunit AKAPs (30).

Compartmentalization of regulatory proteins through scaffolding protein interactions facilitates coordinated signaling within microdomains, localizing enzymes in close proximity to both the substrate and the effectors, presumably for efficient signal transduction. The last three amino acids threonine, arginine, and leucine on the C-terminal end of CFTR have been shown to be the critical region for binding to proteins with PDZ binding domains such as ezrin-binding protein (EBP50), Na^+/H^+-exchanger type 3 kinase A regulatory protein (E3KARP), or CFTR-associated protein-70 (CAP70) (85, 128, 133). These accessory proteins, in turn, bind to AKAPs such as ezrin (84, 113, 127), and ezrin binds to the cortical actin cytoskeleton, serving as a means for anchoring membrane-targeted proteins. However, additional AKAPs that may participate in compartmentalization, coupled with recent studies demonstrating that specific proteins such as PKC, phosphatases, and phosphodiesterases have been shown to be anchored to the cell membranes through AKAP interactions, strongly suggest a role for compartmentalization in local ion channel regulation (64).

Other membrane proteins have also been found to regulate CFTR. The third predicted helical domain of syntaxin 1A, a target membrane soluble *N*-ethyl-maleimide sensitive fusion protein receptor (t-SNARE) and a component of the membrane trafficking machinery, has been shown to have a direct physical interaction with CFTR by binding to the N-terminal region (86, 87). This interaction decreases channel activity, and Munc-18,

a syntaxin 1A binding protein, blocks the interaction between CFTR and syntaxin 1A, restoring channel activity (19).

A_2 ADENOSINE RECEPTOR SIGNALING AND CHLORIDE SECRETION

Purinergic receptors and CFTR. CFTR is believed to be regulated in vivo through G-protein coupled receptors (GPCRs) which signal through cAMP. Among these, the best described are the β_2 adrenergic receptors, which couple to adenylate cyclase, increase cAMP, and activate CFTR-dependent Cl^- secretion across the human airway epithelium through PKA (65, 110, 132). Purinergic receptors of the P_1 class represent another family of GPCRs known to activate CFTR through cAMP.

Purinergic receptors are classified into two groups, P_1 (Ado-selective) and P_2 (ATP-selective) purinoreceptors, both of which are implicated in coordinating Cl^- secretion in human airway and colonic epithelium (27, 71, 99, 124, 126). ATP and analogues can activate CFTR-independent Cl^- secretion across the mammalian airway by stimulating P_{2Y2} receptors (formally $P_{2\mu}$ receptors). These pathways (P_2 purinergic receptors) are well characterized and functional in both CF and normal airway epithelia.

P_1 purinergic receptors are subdivided into four distinct classes: A_1, A_{2A}, A_{2B}, and A_3. A_1 and A_3 receptors couple to G_i, inhibiting cAMP production, while A_{2A} and A_{2B} receptors couple to G_s and stimulate cAMP production. A_{2A} receptors are thought to couple strictly to G_s, whereas A_{2B} receptors are more promiscuous and have been shown to couple with PLC, G_q, and phospholipase A_2 (PLA_2). The molecular biology of P_1 purinergic receptors is extensively reviewed in the first published section of this dissertation.

Ado receptor agonists are potent Cl^- secretagogues. Accumulating evidence suggests that adenosine (Ado) potently activates Cl^- secretion in a variety of model systems through P_1 purinergic receptors, including mammalian airway cells, canine and gerbil airway cells, and T84 colonic epithelial cell monolayers (7, 40, 71, 77, 116). In colonic epithelial cells, Ado regulates ion transport by activating A_{2B} receptors (122). CFTR regulation in the gastrointestinal tract is thought to be important for maintaining proper fluid and electrolyte balance. Activation of luminal Cl^- secretion in vivo serves to hydrate the mucosal surfaces, which is a process that is uncontrolled in secretory diarrhea (63). It has been hypothesized that Cl^- secretion in the intestine may be part of an inflammatory response to flush out luminal pathogens. In vitro studies have shown that activated PMNs and eosinophils release 5'-AMP, which is converted into Ado by ecto-5'-nucleotidases that reside on the apical surface of enterocytes (77, 100). Ado released from PMNs has been shown to activate Cl^- secretion in T84 cells (a colonic epithelial cell line that expresses a high amount of CFTR) by stimulating the A_{2B} receptor pathway (121). Ado, therefore, may serve as an endogenous, potent Cl^- secretagogue in response to states of intestinal inflammation.

Ado might also be an important endogenous regulator of CFTR in the airways. Ado concentrations are determined by the activity of transporters, as well as biosynthetic and catabolic pathways. Ado can be transepithelially absorbed or released by Na^+-dependent nucleoside transporters. Released ATP can contribute to extracellular Ado concentrations in the lumen of the airways, which is determined to a large extent by membrane bound 5' ecto-nucleotidases. These enzymes can degrade ATP, ADP, AMP, and Ado into inosine and hypoxanthine. It is not clear whether P_1 purinergic receptor

signaling in vivo results from ecto-5'-nucleotidase-dependent selective breakdown of ATP (released into the extracellular milieu), ADP, or AMP or if Ado accumulates extracellularly due to Ado released through transporters. Regardless, low micromolar concentrations of Ado have been shown to activate Cl^- secretion in vitro in airway cells. Since these concentrations reflect the binding affinity for the receptor, it is feasible that Ado might endogenously regulate CFTR in vivo.

Interest in Ado as a native regulator of CFTR is increasing. Boucher and colleagues have recently postulated that Ado may have a regulatory role in maintaining airway-surface liquid height by controlling ion transport and (indirectly) water movement across the epithelium (130). Airway surface liquid volume and content have been widely debated topics, limited by studies that vary in technique due to a relatively inaccessible, large surface area with a height estimated to be approximately 20 μm and a volume of only 2 $\mu L/cm^2$. However, Boucher and colleagues were able to measure airway-surface liquid height in tracheo-bronchial cultures by digital XY confocal deconvolution and show that spontaneous Ado accumulation regulates airway-surface liquid height by activating CFTR. This is significant since it demonstrates a possible endogenous regulatory role for nucleotides in the management of airway-surface liquid height and content, at concentrations that are likely to occur in vivo (indirectly). Furthermore, stimulation of A_2 receptor-dependent CFTR activity may be the predominate mechanism by which airyway surface liquid volume is regulated, as demonstrated by the absence of a compensatory mechanism to restore airway-surface liquid height when A_2 receptors are antagonized.

A_{2B} receptors and compartmentalized regulation of CFTR. A_{2B} receptors are thought to be involved in compartmentalized regulation of CFTR, although this process is not well understood. Interestingly, β_2 adrenergic receptors but not A_2 receptors have a PDZ binding sequence (D-S-L-L) at the C-terminal tail that allows association with the first PDZ domain of the EBP50 (43). Both receptors can be used to activate CFTR, but the signal transduction pathways may vary in light of differences in sequence and known protein-protein interactions. In apical membrane excised inside-out patches from polarized Calu-3 cells, a P-site inhibitor of adenylate cyclase completely blocked externally administered Ado activation of CFTR (49), suggesting that adenylate cyclase is present within the apical patch and couples Ado to CFTR activation. An interaction between CFTR and PDZ proteins has also been shown to be required for Ado-coupled activation of CFTR in Calu-3 cells (49), suggesting that AKAP interactions with scaffolding proteins may be important for tight coupling of A_{2B} receptors to CFTR. These data suggest that the major components that makeup this complex signaling mechanism, including PKA, adenylate cyclase, CFTR, and A_{2B} receptors, may reside predominantly in a localized microdomain at the apical surface of the cell membrane. Contained in this microdomain are all the necessary components to activate CFTR.

ADDITIONAL SIGNALING PATHWAYS: $cPLA_2$ AND ARACHIDONIC ACID

A_{2B} receptors have been shown to couple to G_s and activate adenylate cyclase in airway cells. Additional coupling to other signal transduction pathways has also been demonstrated. A_{2B} receptors have been shown to couple to mitogen associated protein (MAP) kinase pathways and phosphatidylinositol-specific PLC by stimulating G-

proteins such as $G_{q/11}$ (3, 35, 36, 73, 79, 80). This suggests that A_2 Ado receptors may couple to cAMP-independent signal transduction pathways in the airways. One goal of this dissertation is to determine the cell signaling pathways that A_2 Ado receptors utilize to activate mutant CFTR and is discussed in detail in the first manuscript.

A_2 Ado Receptors and AA signaling pathways. Previous studies have shown significant dose-dependent differences between direct activation of adenylate cyclase with forskolin and Ado stimulation of CFTR in living cells (25). Ado has been shown to activate CFTR at a 10-fold lower concentration than forskolin, despite extremely small effects on cAMP concentration compared to forskolin. While tight coupling between A_2 Ado receptors and CFTR likely accounts for some of this discrepancy, studies in other model systems suggest that Ado may activate CFTR-dependent Cl^- secretion by additional, cAMP-independent pathways.

In T84 cell monolayers, Ado activates Cl^- secretion (7). In addition to CFTR activation, it was found that Ado stimulated AA release, and exogenous AA augmented Cl^- secretion. Inhibition of cytosolic phospholipase A_2 ($cPLA_2$), an enzyme that releases AA from cell membrane phospholipids, attenuated Ado-stimulated Cl^- secretion, suggesting a possible role for $cPLA_2$ in this pathway. The nature of this effect, however, has not yet been well defined. These studies support the hypothesis that A_2 receptor regulation of CFTR might involve AA metabolism.

AA signaling and effects on ion channels. Fatty acids related to AA have been shown to regulate a variety of ion channels, including, K^+, Na^+, Ca^{2+}, and Cl^- channels

(91). However, the mechanisms by which AA stimulates CFTR-dependent transepithelial Cl^- transport are not well defined (6, 7, 37, 50, 83, 120). PLA_2 cleaves phospholipids in cell membranes, liberating free fatty acids such as AA. In addition to phospholipid digestion and metabolism, PLA_2 plays crucial roles in other cellular responses such as host defense and signal transduction. PLA_2 provides the cells with the powerful signaling molecule AA, which can be broken down into the potent class of inflammatory mediators called eicosanoids, which include prostaglandins and leukotrienes. Several structurally diverse forms of PLA_2 can exist together within the same cell. A 14-kDa PLA_2 enzyme, the secreted form ($sPLA_2$), shows no fatty acid specificity. $cPLA_2$, an 85-kDa protein, is ubiquitously expressed and is the only known enzyme that has specificity for arachidonate at the *sn*-2 position of membrane lipids. Both $sPLA_2$ and $cPLA_2$ can be regulated by an increase in intracellular Ca^{2+}, which induces translocation of the enzyme to the plasma membrane, the endoplasmic reticulum, and/or the nuclear envelope (26). A third form of $cPLA_2$ is characterized by Ca^{2+}-independent ($iPLA_2$) regulation and does not exhibit arachidonate selectivity.

Released AA is rapidly metabolized in most tissues or is reincorporated into phospholipid membranes and serves to remodel lipid-rich membranes. Quantitative analysis of AA release by cells is a useful method for measuring the mobilization of AA by various stimuli. Free AA can be metabolized by cyclooxygenase 1 and 2 (COX 1 and COX 2) and 5 lipoxygenase (5-LO) pathways (108). Both COX enzymes convert AA into prostaglandins, and, although both enzymes have similar structures, enzymatic properties, and intracellular distribution, the ways in which these enzymes are regulated differs (129). COX 1 is constitutively expressed in most cell types and produces prosta-

glandins for routine physiological maintenance, whereas COX 2 is generally induced in response to growth factors, tumor promoters, hormones, or cytokines and produces prostaglandins as part of inflammatory responsiveness.

Recent studies in CF mice describe abnormal lipid profiles, including elevated AA and reduced docohexanoic (DHA) levels in CF-affected tissues, such as the pancreas and gastrointestinal tract (37). These lipid profiles and the associated CF histopathology were normalized by supplementing the diet of CF mice with DHA. The mechanism of this protective effect is unclear. As part of this dissertation, the relationship between Ado signaling and AA liberation in airway cell monolayers was investigated in order to determine the role for $cPLA_2$ and AA release in Ado activation of CFTR. Failure to induce Cl^- secretion by this pathway as part of normal homeostatic signaling could plausibly contribute to a relative increase in AA production, with potentially pro-inflammatory effects. This hypothesis is further considered in the Discussion section.

PHARMACOLOGICAL STRATEGIES TO ACTIVATE MUTANT CFTR

Aminoglycosides and class I CFTR mutations. In addition to characterizing a novel signaling pathway to activate CFTR and Cl^- transport, the third aim of this dissertation research was to determine whether stimulation of native signaling pathways could improve mutant CFTR function. Stimulation of endogenous cell signaling pathways may complement pharmacological strategies that increase the amount of surface-localized mutant CFTR molecules. Many studies have been published that describe novel methods to overcome biosynthetic and trafficking defects in CFTR mutants. For example, many class I mutations result in biosynthetic defects due to premature stop codons that lead to

truncated and unstable mRNA transcripts that fail to translate into protein. Previous studies have demonstrated that select aminoglycosides can induce readthrough of premature stop codons, leading to full length CFTR production (13, 24, 46, 47, 137, 138, 141). This has been extended to human studies, with positive effects on Cl^- transport shown in CF patients with these types of mutations. Identifying methods to maximize surface CFTR activity may thus complement strategies that improve biosynthetic defects.

Bypassing processing defects in class II CFTR mutations. Increased transcription, expression, and surface localization of CFTR would also be a desirable outcome in the case of class II mutations. Approximately 70% of CF individuals are homozygous for ΔF508-CFTR mutation [90% of all mutant CFTR alleles, (136)]. ΔF508 CFTR is a class II mutation characterized by defective processing resulting in failure to be exported from the endoplasmic reticulum. The majority of mutant protein is targeted to the proteosome for degradation. Studies have shown, however, that ΔF508 CFTR can function as a cAMP-regulated Cl^- channel. Activity of surface localized ΔF508 CFTR, however, may be significantly reduced below that of wt CFTR. Surface localizing conditions such as growth at low temperatures or treatment with osmolytes such as glycerol and DMSO can increase the amount of mature ΔF508 CFTR at the plasma membrane. Additionally, surface ΔF508 CFTR expression can be upregulated by butyrate, and clinical trials have demonstrated that treatment with 4-phenyl butyrate partially restores ΔF508 CFTR activity in the nasal epithelia of CF patients (104). Other mechanism such as trimethylamine-*N*-oxide and antichaperone compounds (deoxyspergualin and geldanamycin) are under investigation to improve ΔF508 processing (39, 56). Few studies, however, have investi-

gated whether endogenous signaling pathways can be used to improve the activity of ΔF508 CFTR. One goal of this dissertation research was to determine whether Ado signaling is capable of improving the activity of ΔF508 CFTR at the cell membrane.

Activating mutant CFTR molecules by promoting phosphorylation. In vitro studies using transient expression systems indicate that many surface localized mutant CFTR proteins (including ΔF508 CFTR under surface localized conditions, regulation defective CFTRs such as G551D, or conduction defective CFTRs such as R117H) have a reduced ability to transport Cl^-. Such mutant CFTR molecules can be partially activated by non-physiological stimuli that promote phosphorylation (i.e., direct adenylate cyclase stimulation with forskolin, use of membrane permeant cAMP analogues, and phosphatase inhibitors). Other studies indicate that mutant CFTR molecules from classes III, IV, and V can be partially activated by agents that pharmacologically promote phosphorylation using PDE inhibitors such as 3-isobutyl-1-methylxanthine (IBMX), amrinone, and milrinone (ΔF508 and G551D CFTR) (60, 62, 117) or by phosphatase inhibition using levamisole (ΔF508 CFTR) (12). While the concentrations used in these studies were high and therefore unlikely to be useful in vivo, cumulatively, they point to a general strategy that involves improving CFTR activity by promoting PKA dependent phosphorylation.

Compounds that directly activate CFTR. Direct activators of CFTR may be useful in augmenting mutant CFTR activity from all five classes, particularly when combined with complimentary strategies to address low level of expression characteristic of class I and II mutants. Previous studies provide evidence that several compounds such as

8-cyclopentyl-1,3-dipropylxantine (CPX) and genistein are capable of activating both wt and mutant CFTR by direct interactions (2). Both compounds are currently being evaluated in clinical trials. However, the efficacy of CPX is controversial as reported in a study that showed that CPX was unable to stimulate Cl^- conductances in *Xenopus* oocytes and native epithelial cultures (70). Similarly, the tyrosine kinase inhibitor genistein, which activates ΔF508 CFTR in a protein kinase-independent manner (51, 53), has been reported to have no effect on the activation of Cl^- transport in CF tissues (78). Other compounds that might directly activate CFTR include a new class of benzoquinolizinium compounds that show promising results in vitro (11), a fluoresceine derivative phloxine B that directly activates CFTR by reducing deactivation of the channel (4), and a mucolytic compound *N*-acetyl-L-cysteine that activates CFTR Cl^- channels by an unknown mechanism (68). Strategies that fail to promote phosphorylation of mutant CFTR, therefore, may potentially be complemented by stimulation of cellular PKA-activating pathways.

Augmenting mutant CFTR activity may have clinical benefits. Achieving wt levels of CFTR function may not be necessary to normalize or improve CFTR-dependent ion transport. It has been suggested that low level CFTR function may be sufficient to ameliorate or at least reduce CF-related symptomatology. In vitro studies have shown that as little as 1 mRNA molecule per airway cell, ~ 5% of wt CFTR mRNA levels, or correction of ~10% of airway cells in a CF tissue monolayer may provide sufficient CFTR activity to improve ion transport across CF tissues (33, 65, 123). These studies provide a benchmark that is used to approximate the minimal amount of CFTR activity that may impart an improved prognosis for CF patients. Studies in this document involve

investigating the capacity of endogenous signaling mechanisms to maximize wt and mutant CFTR activity. Characterization and use of these physiologic triggers may be of benefit to pharmacological strategies aimed at augmenting the activity of mutant CFTR molecules, including mutant CFTR proteins in all five classes of mutations.

Improving the activity of mutant CFTR molecules is a reasonable approach to improve defective CFTR-dependent ion transport. However, little is known about the endogenous signaling pathways, which normally regulate CFTR in vivo. This project was designed to characterize a novel pathway used by cells and tissues to regulate CFTR activity through Ado and stimulation of Ado receptors. The results of these studies have relevance to our understanding of CFTR regulation and ion transport physiology. They also have relevance to strategies under investigation to overcome defects in CFTR biosynthesis, trafficking, and activation. These results may, therefore, point toward general strategies to improve mutant CFTR function and may thus be of significance to many areas of research in CF.

SPECFIC AIMS

Surface receptors regulate CFTR-dependent Cl^- secretion across the human airway epithelium. Recent studies suggest that Ado, through A_2 receptors, may be a potent mechanism to activate CFTR. These mechanisms by which Ado activates CFTR in the airways are not well understood but are thought to involve cAMP signaling. To better understand these mechanisms and how they relate to wt and mutant CFTR, three distinct specific aims were formed. The specific aims of this dissertation were to *1*) characterize A_2 receptor activation of CFTR-dependent Cl^- transport in living cells, *2*) examine the

mechanisms underlying A_2 receptor coupled CFTR activation, and *3*) evaluate the ability of A_2 receptors agonists, alone and in combination with other signaling molecules (including β_2 adrenergic receptor agonists, PDEis, and AA) to activate wt and mutant CFTR.

A_2 ADENOSINE RECPETORS REGULATE CFTR THROUGH PKA AND PLA_2

by

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ABSTRACT

We investigated adenosine (Ado) activation of the cystic fibrosis transmembrane conductance regulator (CFTR) in vitro and in vivo. A_{2B} Ado receptors were identified in Calu-3, IB-3-1, COS-7, and primary human airway cells. Ado elevated cAMP in Calu-3, IB-3-1, and COS-7 cells and activated protein kinase A-dependent halide efflux in Calu-3 cells. Ado promoted arachidonic acid release from Calu-3 cells, and phospholipase A_2 (PLA_2) inhibition blocked Ado-activated halide efflux in Calu-3 and COS-7 cells expressing CFTR. Forskolin- and β_2-adrenergic receptor-stimulated efflux were not affected by the same treatment. Cytoplasmic PLA_2 ($cPLA_2$) was identified in Calu-3, IB-3-1, and COS-7 cells, but $cPLA_2$ inhibition did not affect Ado-stimulated cAMP concentrations. In *cftr*(+) and *cftr*(-/-) mice, Ado stimulated nasal Cl^- secretion that was CFTR dependent and sensitive to A_2 receptor and PLA_2 blockade. In COS-7 cells transiently expressing ΔF508 CFTR, Ado activated halide efflux. Ado also activated G551D CFTR-dependent halide efflux when combined with arachidonic acid and phosphodiesterase inhibition. In conclusion, PLA_2 and protein kinase A both contribute to A_2 receptor activation of CFTR, and components of this signaling pathway can augment wild-type and mutant CFTR activity.

INTRODUCTION

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a cAMP-regulated Cl^- channel and is responsible for regulating ion transport in many tissues where it is expressed, including the airways and airway submucosal glands, sweat glands, gastrointestinal tract, pancreatic and bile ducts, and the reproductive tract (58, 74). Defective

CFTR function disrupts ion transport, which is believed to contribute to the clinical sequelae characteristic of cystic fibrosis (CF) (49, 58, 67, 74). CFTR regulation in vivo is accomplished through ATP binding and hydrolysis at nucleotide binding domain-1 and 2 and regulation of the phosphorylation status of the regulatory domain (10, 11, 36). This process is complex and depends on cAMP production through surface receptors that couple to adenylyl cyclase, cAMP degradation by phosphodiesterase (PDE) activity (40, 41), domain-domain interactions within the CFTR (55), protein-protein interactions between the CFTR and associated regulatory proteins (31, 32, 54, 64), and direct phosphorylation and dephosphorylation of the regulatory domain by protein kinase A (PKA) and phosphatases (6, 11), respectively. Receptor-based pathways that signal through cAMP are therefore relatively accessible candidates for promoting activation of the CFTR in vivo. This has been established for β_2-adrenergic receptors, which are commonly stimulated in assays to detect CFTR-dependent Cl^- secretion as measured by changes in the nasal potential difference (PD) in mice and in humans (44).

Adenosine (Ado) is a Cl^- secretagogue that signals through P_1 purinergic receptors, and recent studies have demonstrated that A_{2B} Ado receptors (ARs) can tightly couple to CFTR through PKA. In Calu-3 cells, part of this coupling is due to compartmentalized signaling of CFTR with adenylyl cyclase and PKAII through A kinase anchoring protein interactions (34, 71). T84 cells also express A_{2B}-ARs, where they have been shown to mediate neutrophil-stimulated Cl^- secretion (48, 70). Studies (2-4) have also suggested a role for phospholipase A_2 (PLA_2) in Ado-activated Cl^- secretion in T84 cells. The nature of this role and whether similar signaling contributes to CFTR activation in other CFTR-expressing cells and tissues, however, are unknown.

In this report, we describe CFTR regulation by A_2-ARs in vitro and in vivo and evaluate the contribution of cytoplasmic PLA_2 ($cPLA_2$) to A_2 receptor signaling in airway cells and the murine airway. Our findings suggest that A_2 receptors maximize CFTR activation by signaling through both adenylyl cyclase-PKA and $cPLA_2$ and that components of these receptor signaling pathways can activate common disease-causing CFTR mutations.

METHODS

Cell culture. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). COS-7 cells were grown in DMEM plus 10% fetal bovine serum (FBS) and 1% penicillin plus streptomycin. IB-3-1 cells were grown in LHC medium plus 3% FBS and 1% penicillin plus streptomycin. Calu-3 cells were grown in MEM plus 10% FBS and 1% penicillin plus streptomycin supplemented with nonessential amino acids. To study polarized Calu-3 cells at an air-liquid interface, polyester Transwell-Clear Costar filters [0.4-μm pore diameter; 6-mm insert diameter for arachidonic acid (AA) release and Ussing chamber experiments and 24-mm insert diameter for immunocytochemistry experiments; Fisher Scientific, Pittsburgh, PA] were coated with human placental collagen matrix (Becton Dickinson, Franklin Lakes, NJ) at a concentration of 5 $\mu g/cm^2$ overnight and then seeded at ~1 x 10^6 cells/cm^2. Once the filters were confluent (~1 wk), the medium was removed from the apical surface and the cells were fed only on the basolateral surface. After 48 h, resistance was checked (~1,000-2,000 $\Omega \cdot$ cm for the 6-mm filters and ~400-600 $\Omega \cdot$ cm for the 24-mm filters), and at 72-96 h the cells were studied as described in *Transepithelial short-circuit currents*. The primary human nasal

cells were isolated and grown as explant cultures on Vectabond-treated glass coverslips as previously described (15) and were studied <1 wk after being seeded.

Transient CFTR expression. CFTR was transiently expressed in COS-7 cells with a vaccinia-based expression system as previously described (16, 17). Cells grown on Vectabond-treated glass coverslips were infected with vaccinia containing the T7 polymerase (generous gift of Dr. B. Moss, National Institutes of Health, Bethesda, MD) at a multiplicity of infection of 10 for 30 min. Wild-type (WT), ΔF508, or G551D CFTR under control of the T7 promoter in the pTM-1 vector was then introduced into the cells in complex with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTAP)-propane-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; 20 μg DOTAP-DOPE and 5 μg pTM-1 CFTR/5 × 10^5 cells) for 4 h. These CFTR plasmids in the pTM-1 vector were the generous gift of Dr. S. Cheng (Genzyme, Cambridge, MA). Cells were then washed in PBS, returned to DMEM plus 10% FBS, and studied 18-24 h postinfection (for WT CFTR and G551D CFTR) or after being grown at 29°C for 48 h (ΔF508 CFTR).

Fluorescence-based halide efflux measurements. To study CFTR activation, we measured halide efflux in COS-7 cells transiently expressing WT, ΔF508, or G551D CFTR or in Calu-3 cells with the halide-quenched dye 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (SPQ; Molecular Probes, Eugene, OR) as previously described (16, 17). Briefly, COS-7 cells were seeded on Vectabond-treated glass coverslips, and Calu-3 cells were seeded on Vectabond-treated coverslips coated with 5 μg/cm^2 of human placental extracellular matrix. Cells were grown until ~80% confluent. Immediately before study,

the cells were hypotonically loaded with 10 mM SPQ for 10 min and placed in a quenching NaI buffer. The cells were then placed in a specially designed perfusion chamber and studied at 23°C. The fluorescence of individual cells was measured with a Zeiss inverted microscope (excitation 340 nm, emission >410 nm), a PTI imaging system, and a Hamamatsu camera. Baseline fluorescence was measured in isotonic NaI buffer (16, 17), and cells were then perfused with isotonic dequench buffer ($NaNO_3$ replaced NaI) at the indicated time point (generally 200 s). The perfusate was then switched to dequench buffer plus agonist and requenched at the end of the experiment. Fluorescence was normalized for each cell to its baseline value, and increases are shown as percent increase in fluorescence above basal (quenched) fluorescence. Unless otherwise specified, the means ± SE of all cells were included in the curves shown.

Protein detection. To detect the A_{2B}-AR, cells were lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.05% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl, and 10 mg/ml of phenylmethylsulfonyl fluoride, pH 8.0). For the COS-7 cells, lysates were immunoprecipitated with an isoform-specific polyclonal rabbit anti-A_{2B}-AR-antibody (Alpha Diagnostics, San Antonio, TX) raised against a 16-amino acid sequence corresponding to the third extracellular domain of human brain A_{2B}-AR cDNA as previously described (17). Briefly, anti-A_{2B}-AR antibody was linked to protein A agarose beads and incubated with cell lysates for 2 h. Precleared cell lysates (beads without primary antibody) were used as the negative control. The beads were then washed three times with PBS plus 0.1% Tween, and the immunoprecipitates were released with sample buffer incubated at 37°C for 10 min. For the Calu-3 and IB-3-1 cells,

lysates were Western blotted without immunoprecipitation. Proteins were separated by SDS-PAGE with precast 12% gels (Novex gels; Invitrogen, Carlsbad, CA) and electrophoretically blotted onto polyvinylidene difluoride membranes. The membranes were then blocked with 1% BSA in PBS for 30 min, washed three times with PBS plus 0.1% Tween, and probed with anti-A_{2B}-AR antibody (1:1,000 dilution) for 2 h. Negative control membranes were blotted with a fivefold excess (by weight) of A_{2B}-AR-specific neutralizing peptide that was added with the primary antibody (Alpha Diagnostics). The membranes were washed three times and incubated with secondary antibody (1:1,000 dilution of goat anti-rabbit antibody conjugated to alkaline phosphatase; Southern Biotechnology Associates, Birmingham, AL) for 2 h. Membranes were washed three times and developed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride in carbonate buffer (pH 9.8). Similar techniques were used to identify $cPLA_2$ in cell lysates using an affinity-purified mouse monoclonal IgG2b antibody raised against the amino-terminal domain of the human $cPLA_2$ cDNA (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody was used to identify $cPLA_2$ protein in cell lysate immunoprecipitates (Calu-3 and IB-3-1 cells; anti-$cPLA_2$ linked to precleared protein G beads; Roche Diagnostics, Indianapolis, IN) or cell lysates alone (COS-7 cells). Proteins were separated by SDS-PAGE with 8% precast gels, and after being blotted onto polyvinylidene difluoride membranes, the membranes were blocked and washed as above and probed with primary antibody (1:1,000). The secondary antibody was a goat anti-mouse IgG alkaline phosphatase conjugate (1:1,000 dilution; Southern Biotechnology Associates).

Immunofluorescence studies. Nasal explant cultures from human surgical specimens were grown on Vectabond-treated coverslips coated with 5 $\mu g/cm^2$ of human placental extracellular matrix as previously described (15). At 5 days of age, cells were fixed in 4% formaldehyde in PBS (20 min; pH 7.4), washed twice with PBS, and then permeabilized with 0.05% surfactant Triton X-100 (Pierce Endogen, Rockford, IL). The slides were then pretreated with 1% BSA in PBS to block nonspecific protein-binding sites. A_{2B}-AR antigen was detected with the polyclonal rabbit anti-A_{2B}-AR antibody (1:25 dilution) in 1% BSA for 1 h. Cells were then washed three times with PBS over 15 min and incubated with secondary antibody (goat anti-rabbit FITC conjugate, 1:100 dilution in 1% BSA) for 1 h. The cells were mounted with 4',6-diamidino-2-phenylindole Vectashield mounting fluid (Vector Laboratories). For the studies of A_{2B}-AR localization in polarized Calu-3 cells, the A_{2B}-AR antigen was identified with the filter fold technique as previously described (7). Briefly, high-resistance monolayers were fixed with 3% transmission electron microscopy-grade formaldehyde (Touismis, Rockville, MD) for 45 min at room temperature and stained without detergent permeabilization. The fixed monolayers were washed three times with PBS over 15 min, blocked with nonimmune goat serum for 30 min (1:25 dilution; Vector Laboratories), and incubated with primary rabbit anti-A_{2B}-AR antibody (1:100 dilution in nonimmune goat serum) for 1 h at room temperature. Neutralizing peptide at five times (by weight) the concentration of primary antibody was used for negative control filters. Goat anti-rabbit Alexa fluorochromes (1:80 dilution; Molecular Probes) were used to identify the primary antibody. Nuclei were identified with Hoechst 33258 staining (20 $\mu g/ml$ for 4 min). The filters were folded with the apical side exposed and mounted in 0.1% *p*-phenylenediamine (Sigma, St. Louis, MO) in PBS-

glycerol (1:9 dilution). Digital confocal images were captured and analyzed with an Olympus IX70 inverted reflective fluorescent light microscope at 623 nm excitation with UplanAPO x100 or U-APO/340 x40 objectives, a Photometric Sensys digital camera, and IPLab Spectrum software supplemented with Power Microtome extension software (Signalytics, Fairfax, VA).

Transepithelial short-circuit currents. Calu-3 cells grown as monolayers at an air-liquid interface were mounted in modified Ussing chambers (Jim's Instruments, Iowa City, IA) and initially bathed on both sides with identical Ringer solutions containing (in mM) 115 NaCl, 25 $NaHCO_3$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 D-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 5% CO_2. Solutions and chambers were maintained at 37°C. Short-circuit current (I_{sc}) measurements were obtained with an epithelial voltage clamp (University of Iowa Bioengineering, Iowa City, IA). A 3-mV pulse of 1 s duration was imposed every 100 s to monitor resistance, which was calculated with Ohm's law. To measure stimulated I_{sc}, the mucosal bathing solution was changed to a low Cl^- solution containing (in mM) 1.2 NaCl, 115 sodium gluconate, and all other components as above plus 100 μM amiloride. Increasing concentrations of Ado were added to the mucosal or serosal bathing solutions (8 min of observation at each Ado concentration). After cells were stimulated with 100 μM Ado, 200 μM glibenclamide was added to the mucosal bathing solution, effectively blocking the stimulated I_{sc} (>90%).

Murine nasal PD measurements. *Cftr*(+) and *cftr*(-/-) mice (CFTR^{unc} mice, C57BL6J genetic background) were studied by a conventional nasal PD protocol (29, 69). The *cftr*(-/-) mice carried two copies of the human *cftr* cDNA, which contains a stop codon at position 489 (S489X). *Cftr*(+) mice included *cftr*(+/+) and *cftr*(+/-) mice, which have been shown to have similar nasal ion transport characteristics (42). Genotypes were verified by PCR and dental enamel characteristics. Both male and female mice ~16-40 wk of age were studied. Mice were anesthetized with a cocktail consisting of ketamine (100 mg/ml, 82.5 µl), acepromazine (10 mg/ml, 30 µl), and xylazine (100 mg/ml, 15 µl) administered by intraperitoneal injection (0.1 ml/g body wt). The mouse tail was gently abraded, placed in lactated Ringer solution, and connected through a calomel cell to a high-impedance voltage follower (VF-1; World Precision Instruments, Sarasota, FL). An exploring bridge was established by connecting a Ag-AgCl electrode (wire) bridge to a syringe that pumped solutions at a rate of 180 µl/h. After ~5 min, mice were appropriately somnolent to permit cannulation of the nostril with a PE-10 cannula pulled to a tip diameter of ~0.15 mm. The solutions perfused included Ringer lactate plus amiloride (100 µM; *solution 1*); a low Cl^- concentration ($[Cl^-]$) solution containing 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 115 mM sodium gluconate, 25 mM $NaHCO_3$, 1.24 mM calcium gluconate, and 100 µM amiloride (*solution 2*); and *solution 3* (*solution 2* plus agonist as described in text). Each superperfused condition was studied for 6 min (total of ~18 min per protocol per mouse).

AA release. Calu-3 cells, grown to confluence on 35-mm culture dishes coated with 5 µg/ml of human placental collagen or as high-resistance monolayers at an air-

liquid interface were washed in PBS and loaded with 1 μCi/ml of [^{3}H]AA overnight (Moravek Biochemical, La Brea, CA). The plates and filters were then washed five times with PBS and placed in MEM plus 10% FBS with and without 100 μM Ado (750-μl volume for the 35-mm plates; 150-μl apical volume and 300-μl basolateral volume for the cells on filters). AA release was quantified for 20 min (cells on plates) and for 20 and 40 min (cells on filters). The cells were lysed (1 N NaOH), and the effluxed AA from each plate was quantified by scintillation counting and normalized to the percent of total number of counts in a manner similar to that previously described (13).

cAMP measurements. Cellular cAMP was measured with an ELISA-based detection kit as previously described (17) (Cayman Chemical, Ann Arbor, MI). Briefly, cells grown on 35-mm dishes (~7 × 10^6 cells/dish) were stimulated with agonist for 10 min, and the cellular cAMP was extracted with ice-cold ethanol. The supernatants were vacuum dried and resuspended in phosphate buffer, and the cAMP levels were quantified per the manufacturer's directions. For all experiments, papaverine (100 μM; nonspecific nonxanthine PDE inhibitor) was included to improve cAMP detection. Xanthine-based inhibitors were avoided because these commonly interact with Ado receptors (39).

Materials. Ado hemisodium salt, chlorpromazine (CPZ), H-89 Cl, forskolin, zaprinast, and AA were purchased from Calbiochem (San Diego, CA); albuterol (Alb; salbutamol), theophylline, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-

phenyltheophylline, rolipram, and milrinone were purchased from Sigma; papaverine HCl was purchased from Research Biochemicals International (Natick, MA).

Statistics. Descriptive statistics (means ± SE) and tests of statistical significance were performed with SigmaStat software (Jandel, CA). Paired and unpaired *t*-tests were used for samples with continuous data (cAMP levels, AA levels, stimulated PD measurements, and I_{sc}), and χ^2 analysis was used to compare the number of ΔF508 CFTR-expressing cells responding to different agonists (Ado, forskolin, DPCPX, and control). An α-level of 0.05 was considered statistically significant.

RESULTS

Human airway cells express A_{2B}-ARs. With a polyclonal anti-A_{2B}-AR antibody, Calu-3 and IB-3-1 cell lysates were probed for A_{2B} receptor expression through Western blotting. As a positive control, COS-7 cells, which our laboratory previously demonstrated to endogenously express A_{2B}-ARs (17), were also evaluated. Figure 1 shows that in all three cell types, a specific ~40-kDa band was identified. Using the same anti-A_{2B}-AR antibody, we investigated A_{2B} receptor localization in primary human nasal airway cells (Fig. 2*A*) and polarized Calu-3 cells (an airway cell line with a serous phenotype and high levels of CFTR expression; Fig. 2*B*) (37, 53). In both cell types, the antibody detected plasma membrane-localized A_{2B}-AR. Staining was predominantly along the apical surface in Calu-3 cells. Functional studies in Ussing chambers demonstrated that Ado added to either the apical or basolateral surface briskly activated I_{sc} (Fig. 2*C*, *left*). Ado was a more potent stimulus when added to the apical membrane than to the basolateral membrane, and I_{sc} stimulated from either membrane was sensitive to apical glibenclamide

blockade (~90% inhibition of stimulated I_{sc} produced by 100 μM Ado; $P < 0.05$). Although the range of concentrations capable of activating I_{sc} was most consistent with A_2 receptor stimulation, these experiments do not exclude the possibility that additional AR subtypes may contribute to Ado-stimulated I_{sc}. Because HCO_3^- transport, in addition to Cl^- transport, may contribute to I_{sc} in Calu-3 monolayers (22, 66), further functional studies of CFTR activity were performed with an SPQ-based halide efflux assay (15-17).

Ado stimulates PLA_2, adenylyl cyclase, and CFTR activation. In previous studies of CFTR regulation by A_{2B} receptors in COS-7 cells, our laboratory (17) showed that A_{2B} receptor signaling was a potent stimulus, accomplishing strong activation of CFTR despite only modest effects on cellular [cAMP] compared with forskolin. These results were similar to those reported by Barrett and colleagues (3, 4) in T84 cells. In subsequent studies by Barrett and Bigby (2), Ado-activated I_{sc} in T84 monolayers was found to be associated with AA mobilization and sensitivity to PLA_2 inhibition. To determine whether PLA_2 signaling might contribute to A_2 receptor activation of CFTR in airway and COS-7 cells, we evaluated cells for $cPLA_2$ expression and $cPLA_2$ activity after Ado stimulation. Figure 3 shows that in Calu-3, IB-3-1, and COS-7 cell lysates, a monoclonal anti-$cPLA_2$ antibody detected an ~110-kDa protein consistent with $cPLA_2$.

Activated PLA_2 releases AA from the *sn*-2 position of membrane phospholipids. To determine whether Ado activated PLA_2 and stimulated the release of AA in airway cells that express both A_{2B} receptors and CFTR, Calu-3 cells were grown on plastic and on permeable supports at an air-liquid interface, loaded with [^{3}H]AA, and exposed to Ado (100 μM; Fig. 4). Ado stimulated AA release from Calu-3 cells grown in either condition.

AA release is expressed as percent of counts released (basolateral or apical) over total counts; $P \leq 0.025$ (Fig. 4, *A* and *B*). AA was released preferentially from the apical compartment, but a detectable amount (approximately [1/10] of apical release) was released from the basolateral surface (Fig. 4*C*). Studies of the effects of $cPLA_2$ inhibition (with CPZ) or PKA inhibition (with H-89 Cl) on Ado-stimulated AA release were complicated by a mild (approximately twofold) increase in nonspecific AA release from Calu-3 cells after treatment with either inhibitor. Cell viability after treatment with either compound was preserved, however, based on cytotoxicity assay studies (data not shown), SPQ retention and response (Fig. 5), and cAMP production (Fig. 6).

In addition to investigating CFTR activity with I_{sc} measurements, studies were performed with SPQ, an established assay of CFTR activity and a specific assay of halide transport. SPQ-based studies also allowed comparison of A_2 receptor-stimulated halide transport in Calu-3 cells with that in COS-7 cells (which fail to polarize). These cells represent a simple cell type used to study A_{2B} receptor regulation of WT and mutant CFTRs because they express A_{2B} receptors but not other purinergic receptors commonly found in airway cells and other complex epithelial cells (17).

Ado-stimulated halide efflux in COS-7 cells expressing CFTR is shown in Fig. 5, *A* and *B*. Ado alone activated halide efflux at 2 μM but not at 0.2 μM, consistent with signaling through lower-affinity A_2 receptors rather than A_1 receptors (Fig. 5*A*). Treatment of cells with the PLA_2 inhibitor CPZ (50 μM) blocked Ado-activated efflux. In contrast, addition of the PLA_2 product AA (25 μM) augmented the Ado stimulus, shifting activation of halide efflux to 0.2 μM. AA alone (25 and 100 μM), however, failed to activate halide efflux in CFTR-expressing COS-7 cells. These results suggest that $cPLA_2$ sig-

naling is part of the pathway by which A_2 receptors activate CFTR in this model system. The $cPLA_2$ product AA when used alone, however, was insufficient and unable to substitute for Ado. The results therefore suggest that for A_2 receptors to activate CFTR, additional signaling pathways need to be stimulated. In control experiments (Fig. 5*B*), forskolin-activated halide efflux in COS-7 cells (+ CFTR) was not inhibited by CPZ.

To test whether these observations applied to airway cells that express both CFTR and A_{2B} receptors, halide efflux experiments were performed in Calu-3 cells. Ado-activated halide efflux was blocked by CPZ (50 μM), whereas AA alone failed to activate efflux. High concentrations of Ado (100 μM) were able to partially overcome the CPZ blockade (Fig. 5*E*). The Ado response was also blocked by the PKA inhibitor H-89 Cl (5 μM), indicating that Ado also utilizes adenylyl cyclase and cAMP to activate halide efflux. In control experiments, β_2-receptor activation of halide efflux with Alb was sensitive to H-89 Cl^- but not to $cPLA_2$ inhibition with CPZ (Fig. 5*D*). These results are consistent with those in the COS-7 cells and indicate that distinct differences exist in the mechanisms by which A_2 receptors and β_2-receptors activate CFTR and halide transport in these in vitro systems.

A_2-ARs traditionally couple to adenylyl cyclase through G_s and elevate cellular cAMP, and previously, our laboratory (17) has shown that A_{2B} receptor stimulation in COS-7 cells increases cAMP in a dose-dependent manner (17). Whether A_2 receptor stimulation of cAMP involves PLA_2 signaling, however, is not known. Figure 6 shows that in COS-7, IB-3-1, and Calu-3 cells, Ado (10 μM) increased cAMP. Treatment of cells with the PLA_2 inhibitor CPZ (50 μM) had no effect on Ado-stimulated cAMP production in all three cell lines, yet completely abolished Ado-activated halide efflux in both

COS-7 and Calu-3 cells (Fig. 5). Production of cAMP by lower doses of Ado (0.1 and 1 μM) was also not inhibited by CPZ treatment (Calu-3 cells; Fig. 6*D*). These results confirm that A_2 receptors do couple to adenylyl cyclase and elevate cAMP but also show that the inhibitory effect that CPZ exerts on Ado stimulation of CFTR is independent of cAMP. Rather, the results shown in Figs. 4-6 suggest that Ado stimulation leads to both $cPLA_2$ and PKA activation, with each pathway required for the stimulation of CFTR.

AA alone (100 μM) had variable effects on [cAMP], stimulating some cAMP production in airway cells ($P < 0.001$ for the Calu-3 cells compared with control conditions) but not in COS-7 cells. The cAMP produced by high-dose AA appeared to contribute little to CFTR activation. AA alone was insufficient to acutely activate halide efflux in either Calu-3 cells or COS-7 cells expressing CFTR (Fig. 5), and AA had no additive effect on [cAMP] when combined with Ado stimulation in any of the three cell types. This was in contrast to the functional results, such as those shown in Fig. 5*A*, in which the addition of AA to Ado stimulation appeared to augment CFTR-dependent halide efflux.

Ado activation of CFTR-dependent Cl^- transport in vivo. The results given in *Ado stimulates PLA_2, adenylyl cyclase, and CFTR activation* provide a framework in which to investigate Ado-activated Cl^- transport in vivo. For these studies, we investigated ion transport across the murine nasal mucosa using the nasal PD, a well-established bioelectric assay of CFTR activity in vivo. Figure 7 shows examples of nasal PD tracings from a *cftr*(+) (Fig. 7*A*) and a *cftr*(-/-) (Fig. 7*B*) mouse. In the *cftr*(+) mouse, Ado stimulated further hyperpolarization (Fig. 7*A*, arrow #3), consistent with Cl^- conductance. In the *cftr*(-/-) mouse, depolarization continued during perfusion with a low [Cl^-] solution and a

low [Cl^-] solution plus Ado (500 μM). Figure 8*A* summarizes a comparison of Ado (100 μM)-, Alb (100 μM)-, isoproterenol (Iso; 100 μM)-, and forskolin (10 μM)-stimulated Cl^- secretion in *cftr*(+) mice. Ado was a potent Cl^- secretagogue, producing further polarization in 10 of 12 mice studied ($P < 0.005$ compared with the no-agonist or Iso control mice). Alb was also a strong agonist (similar to forskolin), producing further polarization in six of eight mice studied ($P < 0.05$ for Alb and forskolin compared with Iso and control animals). In contrast, Iso was less predictable, producing further hyperpolarization in only 5 of 14 mice. Both AR stimulation with Ado (500 μM) and β_2-receptor stimulation with the more specific β_2-receptor agonist Alb (500 μM) failed to activate Cl^- conductance in *cftr*(-/-) mice (Fig. 8*B*). These studies confirm that both receptors stimulate CFTR-dependent Cl^- transport. Ado-stimulated Cl^- conductance in *cftr*(+) mice was sensitive to A_2 receptor blockade with 8-phenyltheophylline ($P < 0.02$), indicating that Ado activates Cl^- conductance through A_2 receptor signaling (Fig. 8*C*).

We next investigated the contribution of PLA_2 to Cl^- secretion across the murine nasal mucosa. Figure 9 shows the effect of the PLA_2 inhibitor CPZ (100 μM) on Ado- and Alb-stimulated Cl^- conductance. Ado (500 μM) strongly stimulated Cl^- secretion that was blocked by CPZ treatment ($P < 0.02$). In contrast, Alb (500 μM)-stimulated Cl^- secretion was not affected by CPZ. Costimulation with Ado and Alb (500 μM each) failed to produce additive effects on Cl^- conductance, indicating that the two signaling pathways converge on CFTR-dependent and not other (CFTR-independent) Cl^- transport pathways (data not shown). These results also confirm our in vitro observations, which demonstrated that A_2 receptor activation of CFTR in vivo depends on PLA_2 activity.

A_{2B}-ARs activate ΔF508 CFTR and G551D CFTR. The results discussed in *Ado activation of CFTR-dependent Cl^- transport in vivo* show that Ado and A_2 receptor signaling potently activate CFTR-dependent Cl^- transport in vitro and in vivo. To determine whether A_{2B} receptor signaling could be used to improve the activity of common disease-causing CFTR mutations, we transiently expressed ΔF508 CFTR (class II mutation) and G551D CFTR (class III mutation) in COS-7 cells. Figure 10 shows that after localization to the cell membrane (growth at 29°C for 48 h), ΔF508 CFTR was activated by A_{2B} receptor stimulation (10 μM Ado) in a fashion similar to direct adenylyl cyclase activation with forskolin (20 μM). Halide efflux was stronger than that produced by stimulation with DPCPX, an agent that can acutely stimulate ΔF508 CFTR-dependent halide efflux in ΔF508 CFTR-expressing cells [and is currently in clinical trials as an activator of ΔF508 CFTR in CF patients (24, 30); $P < 0.001$ comparing the proportion of responding cells stimulated by Ado with DPCPX]. We did not test the ability of A_{2B} receptor stimulation to activate ΔF508 CFTR after prolonged treatment with DPCPX. Activation of ΔF508 CFTR was accomplished despite only modest effects of Ado on [cAMP] compared with forskolin (17).

In Fig. 11, COS-7 cells expressing G551D CFTR were stimulated with forskolin, Ado, or AA. A series of PDE inhibitors was also evaluated because previous reports (62, 63, 77) suggested that surface-localized mutant CFTRs might be partially responsive to stimulation with certain PDE inhibitors. Isotype-specific inhibitors (rolipram, PDE-4 specific; milrinone, PDE-3 specific; and zaprinast, PDE-5 and -6 specific) were used at concentrations ~20-fold above the inhibitor constant of their respective PDEs (72). Two non-

specific PDE inhibitors (papaverine and theophylline) were studied at 200 μM. PDE inhibitors such as milrinone have been evaluated in clinical trials for their ability to activate Cl^- conductance in normal subjects and in CF patients carrying the G551D CFTR mutation (68). At the concentrations used, none of the PDE inhibitors alone consistently elevated cAMP in COS-7 cells (10-min exposure; data not shown), and none of the stimuli (including Ado) activated G551D CFTR-specific halide efflux (Fig. 11*A*). WT CFTR-expressing cells stimulated with forskolin are shown for comparison. In Fig. 11*B*, G551D CFTR-expressing COS-7 cells were exposed to combinations of Ado (200 μM), AA (100 μM), and PDE inhibitors. In contrast to the results in Fig. 11*A*, A_{2B} receptor stimulation, when combined with AA and PDE inhibition, strongly activated halide efflux in a fashion similar to that produced in WT CFTR-expressing cells. Together, the results shown in Figs. 10 and 11 indicate that the activity of the two most common disease-associated CFTR mutations can be increased dramatically with A_{2B}-AR signaling.

DISCUSSION

In this report, we investigated the cellular pathways utilized by Ado to activate CFTR-dependent ion transport. Using a series of experimental systems, including single cells, polarized cell monolayers, and in vivo measurements in *cftr*(+) and *cftr*(-/-) mice, we demonstrated that Ado, through A_2-ARs, activates CFTR-dependent halide transport. These receptors are expressed in human airway cells and signal in part through cAMP and PKA. We also demonstrated the dependence of this signaling pathway on PLA_2 activity in vitro and in vivo. Finally, we showed that the two most common disease-associated CFTR mutations can be activated by A_{2B} receptors alone (ΔF508 CFTR) or by

using messenger pathways stimulated by A_{2B} receptors (G551D CFTR). These results help to establish the physiological role of Ado-stimulated Cl^- secretion in vivo and may identify new targets for improving the function of mutant CFTR molecules.

Ado is a ubiquitous signaling molecule, serving protean functions ranging from regulation of neurotransmission to cardiovascular tone and inflammation in many organ systems. Our results, together with those of other investigators, indicate an important role for Ado as a regulator of CFTR. Ado and A_2 receptors are potent activators of Cl^- secretion in primary human airway cell monolayers, in addition to canine and gerbil airway cells and T84 intestinal cells (28, 46, 59, 70). In each of these systems, Cl^- secretion has been accomplished in the setting of low cAMP levels. Part of this high efficiency is due to compartmentalization of A_{2B} receptors, transduction proteins, and CFTR (34, 7). Our results suggest that in addition to spatial compartmentalization, efficient transduction between A_2 receptors and CFTR involves $cPLA_2$ activation and AA signaling. A_2 receptors, including A_{2A} and A_{2B} subtypes, classically signal through stimulation of adenylyl cyclase, cAMP, and, ultimately, PKA (56). Although A_{2A} receptors appear to hold more strictly to this observation, A_{2B} receptors have been shown to stimulate additional signaling pathways such as phospholipase C, direct regulation of calcium channels, and, in our experience, PLA_2 (26). These observations are of particular interest because recent studies (27) have demonstrated abnormal lipid profiles, including elevated AA levels, in murine CF tissues. Our findings demonstrate that lipid signaling may be important to the regulation of CFTR activity by Ado in vitro and in vivo. The present results therefore set the stage for future studies designed to test the relationship between membrane lipid composition, A_2 receptors, and CFTR activity.

There are two general classes of PLA_2 enzymes: $cPLA_2$ (three subtypes) and secretory PLA_2 (seven subtypes) (33). Both are expressed in many tissues including the lung and leukocytes. $cPLA_2$ translocates to cell membranes after activation and primarily releases AA from membrane lipids. $cPLA_2$ has been shown to play an important immunologic role in human airway epithelial cells, governing AA release from epithelia after immunostimulation (76). Studies (50) have also observed dysregulation of PLA_2 in the CF airway, with increased AA release seen after bradykinin exposure in ΔF508 CFTR-expressing airway cells compared with normal control cells. AA is the parent molecule of two important inflammatory signaling cascades including *1*) the cyclooxygenase (COX) pathway, which leads to the production of prostanoids and thromboxanes and *2*) the lipoxygenase pathway in which AA is metabolized to 5-hydroperoxyeicosatetraenoic acid, 5-hydroxyeicosatetraenoic acid, and the leukotrienes A_4-E_4. Clinical studies suggest that products of AA metabolism, including proinflammatory species, have direct relevance to CF-related inflammation. The COX-1 inhibitor ibuprofen is routinely used in the pediatric CF population to reduce lung inflammation and slow the progression of CF lung disease (45). Glucocorticoids, which suppress COX-2 expression, have also been studied in CF clinical trials, attenuating the decline in pulmonary function in pediatric CF patients (25). The relevance of AA signaling pathways to CFTR activity and ion transport, however, has not been previously demonstrated. Our results suggest that a buildup in AA may have beneficial effects on CFTR activity in the context of its activation by surface receptors.

The mechanism by which A_2 receptors activate CFTR-dependent Cl^- transport is complex. CFTR activation by A_2 receptors could be blocked by either PKA or $cPLA_2$ in-

hibition (Fig. 5). Production of cAMP was not reduced by PLA_2 inhibition in any of the three cell lines (Fig. 6). Although 10 min of high-dose (100 μM) AA exposure could stimulate some cAMP production in the two airway cell lines (primarily Calu-3 cells, likely through the actions of distal metabolites such as PGI, PGE_2, $PGF_{2\alpha}$ or PGD) (73), it failed to augment cAMP production by Ado or acutely activate CFTR when used alone (COS-7 or Calu-3 cells; Fig. 5). These results suggest that the additional effects of AA on CFTR-dependent halide transport were not through influences on total cellular cAMP. Rather, A_2 receptors appear to activate both adenylyl cyclase and $cPLA_2$ in parallel, with each signaling pathway contributing to the maximization of CFTR activity. PLA_2 signaling was not necessary to activate CFTR through receptors, however, because β_2-adrenergic stimulation activated CFTR-dependent Cl^- transport in vitro and in vivo despite $cPLA_2$ inhibition (Figs. 5 and 9).

Figure 12 is a summary model that provides three possible mechanisms by which $cPLA_2$ and AA could contribute to A_2 receptor activation of CFTR. It is possible that Ado and/or AA exerts its effects in part by activating K^+ channels and increasing the driving force for halide efflux. AA has been shown to have both stimulatory and inhibitory effects on epithelial K^+ channels, and modulation of basolateral K^+ channel activity can strongly influence transepithelial Cl^- transport (20, 21). In related experiments, Ado-activated halide efflux in CFTR-expressing COS-7 cells was not reduced by two K^+ channel blockers, including barium and tetraethylammonium (10 mM each) (18, 19). These results suggest that the stimulatory effects of Ado on CFTR seen in our studies were independent of K^+ channel activation. The observation that AA augmented the stimulation of CFTR by Ado and forskolin (Figs. 5 and 11), but failed to stimulate CFTR

alone in vitro (Fig. 5), suggests that AA may exert a permissive effect on CFTR activation. Membrane-derived fatty acids, including AA and its metabolites, have been demonstrated to interact with ion channels and influence their activation by other signaling molecules, including nucleotide sensitivity of ATP-activated K^+ channels (5, 65), light-sensitive transient receptor potential, and transient receptor potential-like channels (14), small Cl^- channels in rabbit parietal cells (61), the nicotinic acetylcholine receptor (9), and calcium channels as part of oscillating calcium currents (51). AA does interact with CFTR and alter its Cl^- channel activity. Cytoplasmic AA produces a flickery block of CFTR in inside-out membrane patches after heterologous expression (1, 35, 47). Membrane lipids (including AA) can have differential effects on the function of other channels, stimulating channel activity on one surface and inhibiting channel activity on the other (52). We speculate that AA or one of its metabolites may interact with an external portion of the channel or with an unidentified regulatory factor that secondarily influences CFTR Cl^- channel function. Alternatively, products of AA metabolism could accelerate local cAMP production and, in this way, increase CFTR channel activity. This process would be difficult to detect with total cellular assays to quantitate [cAMP]. The effect would need to be quite dramatic, however, because G551D CFTR, which has previously been shown to be poorly responsive to powerful phosphorylating stimuli, appears to be activated by costimulation with AA (Fig. 11*B*, Refs. 38, 58, and 77). This occurs despite no detectable change in [cAMP] when combined with Ado (Fig. 6*A*) but is most pronounced when combined with PDE inhibition. Single-channel-based studies will be necessary to help clarify the mechanism by which $cPLA_2$ and AA promote CFTR activity. Finally, our results do not exclude the possibility that additional processes may be in-

volved because CPZ has been shown to have effects on other cell signaling pathways in addition to PLA_2 (8, 12, 23, 43, 57).

The results shown in Figs. 7-9 indicate that Ado is a potent Cl^- secretagogue in vivo, activating CFTR-dependent Cl^- transport across the murine nasal mucosa more predictably than Iso (when studied at 100 μM). This is of relevance to human nasal PD protocols, which typically use β_2-receptor stimulation with Iso to aid in the detection of CFTR-dependent Cl^- transport. Our results suggest that comparisons of these two receptor pathways in human subjects may improve the sensitivity of the nasal PD, which could have implications for clinical studies that use this assay to measure low-level CFTR function. The importance of PLA_2 signaling to Ado activation of Cl^- transport was also demonstrated in vivo because Ado- but not Alb-stimulated Cl^- conductance was sensitive to PLA_2 inhibition.

A_2 receptor signaling may also be useful in improving the activity of disease-related CFTR mutations. Using an in vitro system, we have demonstrated that CFTR mutations from three subclasses (classes II, III, and IV) can be activated by A_{2B} receptors (17) (Figs. 10 and 11). Although the expression levels of the mutant CFTRs were far higher than those found in vivo, the results shown in Figs. 10 and 11 support the notion that mutant CFTRs, when localized to the cell membrane, can be activated by endogenous receptor-based signaling pathways. Current approaches to increase mutant CFTR activity, including improvement of CFTR biosynthesis [e.g., suppression of premature stop mutations with aminoglycoside antibiotics (7, 15, 75)], increase in trafficking of ΔF508 CFTR to the cell membrane [e.g., treatment with butyrate compounds (60)], or treatment of surface mutant CFTRs with activating compounds [e.g., genistein or PDE inhibitors (38,

68)] may be complemented by the use of A_2 receptor signaling pathways. Our findings also suggest that common mutant CFTRs should be at least partially responsive to this signaling pathway when available at the cell membrane.

In summary, our studies demonstrate that A_{2B}-ARs are expressed in CF and normal airway cells, localizing predominantly to the apical membrane of polarized Calu-3 cells. A_2 receptors mediate activation of CFTR-dependent Cl^- transport in vitro and in vivo, utilizing $cPLA_2$ and AA in addition to cAMP and PKA. Ado and A_2-ARs compare favorably with other agents as activators of CFTR-dependent Cl^- conductance, stimulating Cl^- secretion better than the β_2-adrenergic receptor agonist Iso in mice and activating ΔF508 CFTR similar to forskolin in vitro. Our studies therefore provide a rationale for the investigation of the effects of Ado and A_2 receptor signaling on measured Cl^- secretion in humans.

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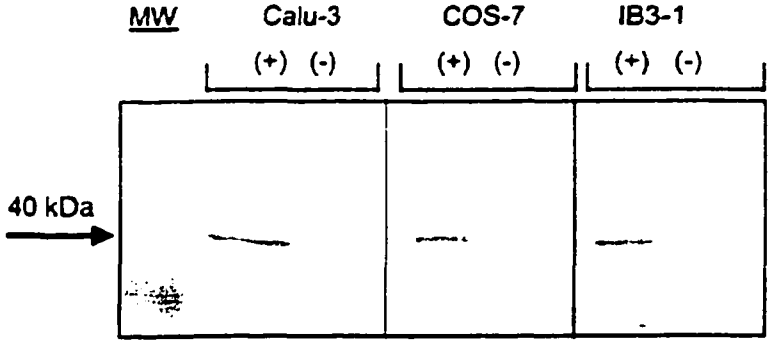

Fig. 1. Identification of A_{2B} adenosine (Ado) receptors (ARs). Characteristic 40-kDa band was identified (+) in 3 cell types. MW, molecular mass. Negative controls (-) were precleared beads with cell lysates (COS-7 cell immunoprecipitates, no primary antibody conjugated to beads) or with addition of neutralizing peptide during Western blotting (Calu-3 and IB-3-1 cells, 5-fold excess of neutralizing peptide added during incubation with primary antibody).

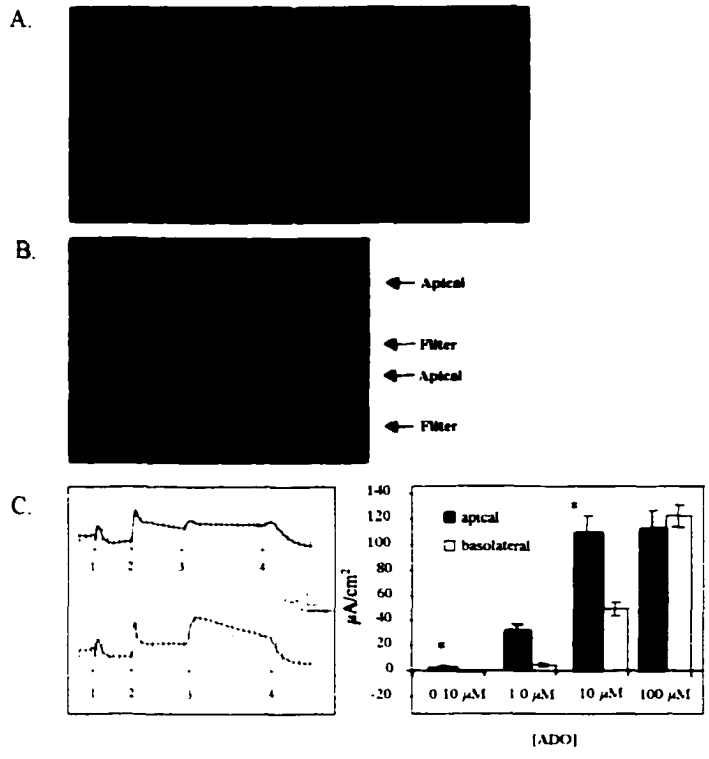

Fig. 2. Immunofluorescence of primary human bronchial epithelial cells and Calu-3 cell monolayers studied with immunofluorescence (*A* and *B*, respectively) and in Ussing chambers (*C*). Cells were prepared and studied as described in METHODS. *A*, *left*: A_{2B}-AR antigen is identified with a membrane-staining pattern (green). *A*, *right*: negative control cultures (fivefold excess of neutralizing peptide during incubation with primary antibody) eliminated membrane staining. *B*, *top*: A_{2B}-AR antigen in Calu-3 monolayers (green) shows predominantly apical membrane staining. *B*, *bottom*: addition of neutralizing peptide (as in *A*) to negative control cultures eliminated membrane staining. *C*: effects of apical and basolateral addition of Ado on short-circuit current (I_{sc}) in Calu-3 cells. Cells were grown at an air-liquid interface and studied in Ussing chambers. *C*, *left*: representative I_{sc} tracings. *Top*: Calu-3 cells were initially cultured in lactated Ringer solution followed by (from *left* to *right*) *1*) mucosal low-Cl^- concentration ($[Cl^-]$) buffer + amiloride (100 μM), *2*) addition of mucosal 1 μM Ado, *3*) addition of basolateral 1 μM Ado, and 4) blockade with mucosal 200 μM glibenclamide. *Bottom*: same experiment as above, except basolateral Ado (1 μM; *2*) was added before mucosal Ado (1 μM; *3*). *Right*: Ado was a strong stimulus when added to either membrane. Apical: $P = 0.002$ for 0.1 μM compared with 1.0 μM and 1.0 μM compared with 10 μM. Basolateral: $P < 0.03$ for 0.10 μM compared with 1.0 μM; $P < 0.001$ for 1.0 μM compared with 10 μM; and $P < 0.001$ for 10 μM compared with 100 μM. Apical-stimulated I_{sc} was greater than basolateral-stimulated I_{sc} for 0.1 and 10 μM (*$P < 0.02$) and 1.0 μM ($P < 0.001$). Approximately 90% of the stimulated I_{sc} (100 μM, either membrane) was blocked by glibenclamide (200 μM added to the mucosal compartment; data not shown).

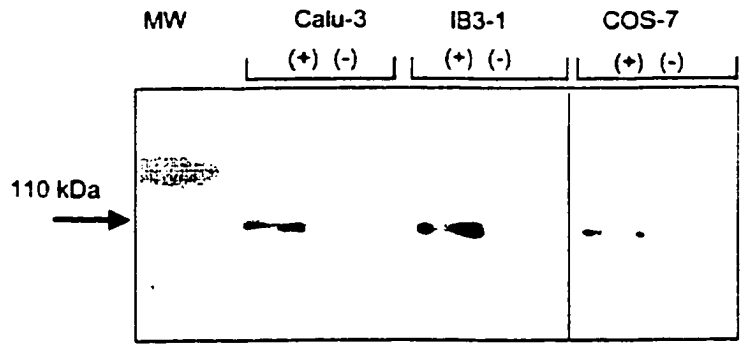

Fig. 3. Identification of cytoplasmic phospholipase A_2 (cPLA$_2$) in 3 cell types. Cell lysates were immunoprecipitated with anti-cPLA$_2$ antibody (Calu-3 and IB-3-1 cells) and separated by SDS-PAGE. cPLA$_2$ antigen (~110 kDa) was detected with monoclonal anti-cPLA$_2$ antibody (arrow). Control conditions were precleared beads from cell lysates without primary antibody (Calu-3 and IB3-1 cells). COS-7 cell lysates were separated by SDS-PAGE and Western blotted with anti-cPLA$_2$ antibody. Controls received no primary antibody during blotting.

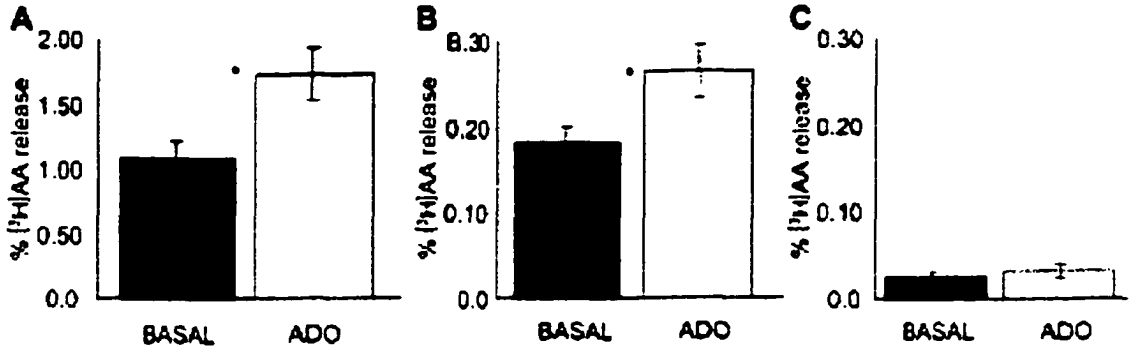

Fig. 4. Ado stimulates arachidonic acid (AA) release from Calu-3 cells. Cells were loaded with [^{3}H]AA overnight, washed 5 times, and then studied. Values are means ± SE. *A*: AA release from cells grown on 35-mm plastic dishes stimulated with Ado (100 μM) for 20 min compared with cells in unstimulated dishes (n = 10 dishes/condition). *P < 0.025. *B*: apical release of AA from Calu-3 cells grown on permeable supports at an air-liquid interface and stimulated with 100 μM Ado compared with cells on unstimulated filters (n = 6 filters/condition). Cells were stimulated for two 20-min time points. Release over the second 20 min is shown. For the entire 40-min period, Ado stimulated AA release by ~20% over the unstimulated condition (P = 0.06). *P = 0.025. *C*: basolateral release of AA for same 20-min time point. Total no. of counts (released to medium and retained in cells) for the stimulated and unstimulated conditions was not different for cells grown on dishes (*A*) or on filters (*B* and *C*). P = 0.08 for Ado-stimulated compared with control cells. P = 0.06 for entire 40-min time period.

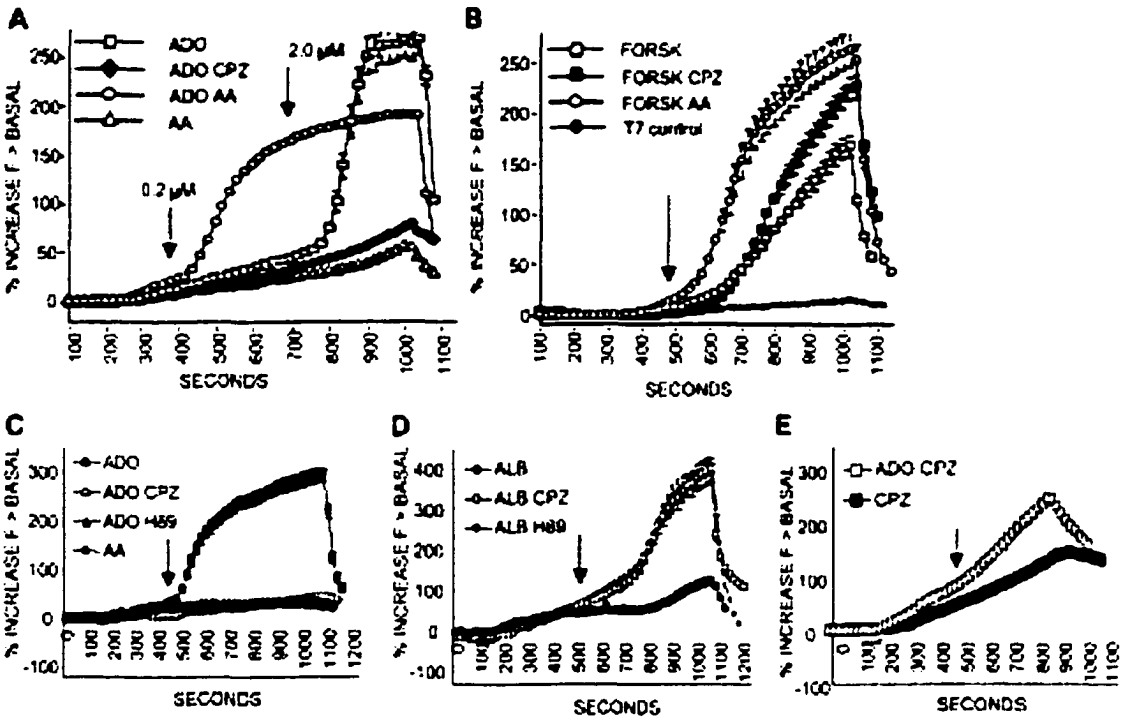

Fig. 5. Ado activation of halide efflux in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing COS-7 cells (*A* and *B*) and Calu-3 cells (*C-E*). Values are means ± SE in % increase in fluorescence over basal (NaI-quenched fluorescence); $n >$ 40 cells/condition. *A*: COS-7 cells expressing CFTR were stimulated with Ado (arrows), with activation seen at the higher concentration. Same experiment was performed in the presence of 25 μM AA, from 200 s, with shift of CFTR activation to 0.2 μM Ado. Treatment of cells with chlorpromazine (CPZ; 50 μM) from 200 s blocked CFTR activation by 2.0 μM Ado. AA at 25 and 100 μM (arrows) failed to activate halide efflux. *B*: COS-7 cells expressing CFTR were stimulated with forskolin (Forsk) alone (20 μM; arrow) or with addition of 25 μM AA (200 s) to forskolin stimulation of CFTR (20 μM). Treatment with CPZ (50 μM) had no effect on forskolin activation. T7 controls (no CFTR) stimulated with AA (25 μM at 200 s) and forskolin (20 μM, arrow) failed to activate halide efflux. *C*: Calu-3 cells stimulated with Ado (2 μM). Treatment with CPZ (50 μM from 200 s) or H-89 (5 μM for 4 h) blocked Ado-activated halide efflux. AA alone (100 μM; arrow) failed to activate halide efflux. *D*: Calu-3 cells stimulated with albuterol (Alb; 0.2 μM) activated halide efflux. Treatment with CPZ (50 μM from 200 s) had no effect on Alb activation, whereas treatment with H-89 Cl- (5 μM for 4 h) blocked activation of halide efflux. *E*: Calu-3 cells treated with CPZ (50 μM) from 200 s. High-concentration Ado (100 μM) partially overcame CPZ blockade.

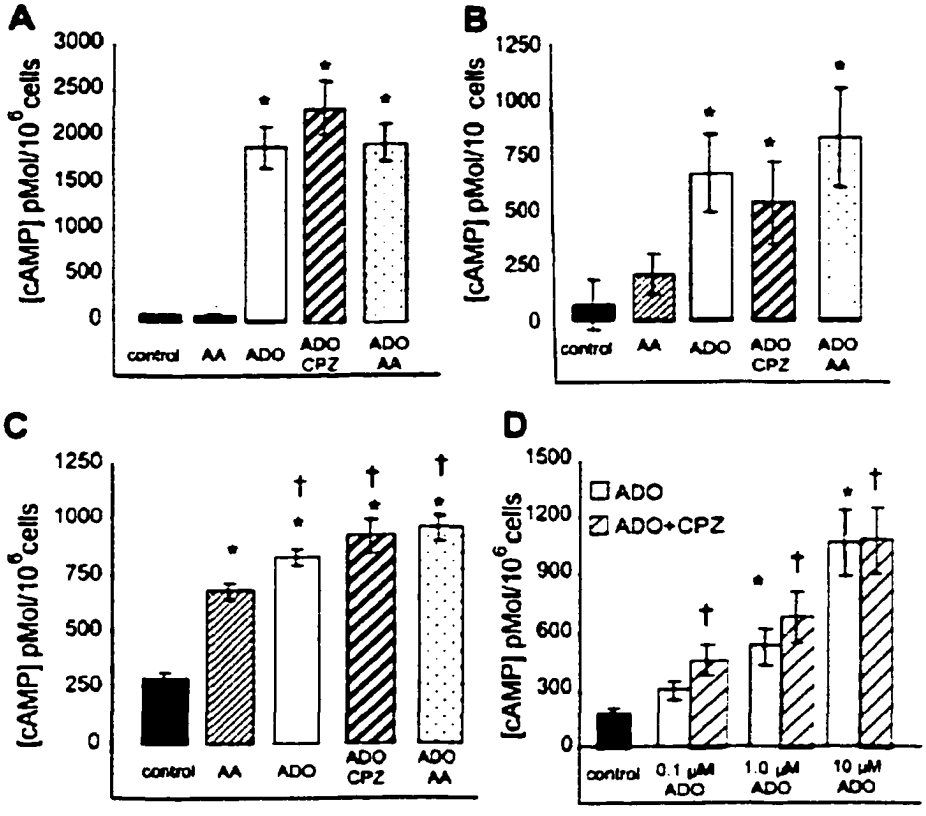

Fig. 6. Ado stimulates cAMP production in COS-7, IB3-1, and Calu-3 cells. cAMP levels were measured by ELISA as described (see METHODS). Cells were stimulated for 10 min before extraction, n = 4-12 cultures/condition. Concentrations were 10 μM Ado, 50 μM CPZ, and 100 μM AA. Papaverine (100 μM) was included in all conditions to enhance cAMP detection as previously described (17). Values are means ± SE for each condition. *A*: COS-7 cells. $^*P < 0.001$ compared with control (100 μM papaverine). *B*: IB3-1 cells. $^*P < 0.02$ compared with control. *C*: Calu-3 cells. $^*P < 0.001$ compared with control. $P < 0.02$ compared with AA alone. *D*: Calu-3 cells cAMP dose-response curve. Ado (0.1, 1.0, and 10 μM) stimulation of cAMP production was not inhibited by treatment with 100 μM CPZ. $^*P < 0.02$ for Ado alone compared with papaverine control (100 μM). $P < 0.01$ for Ado + CPZ compared with papaverine control.

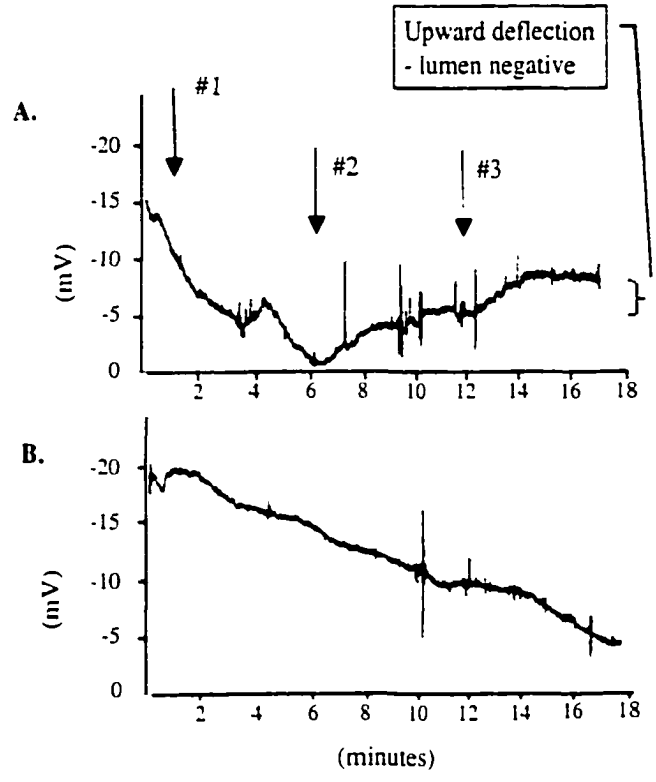

Fig. 7. Examples of nasal potential differences (PDs) in *cftr*(+) and *cftr*(-/-) mice. Mice underwent a standard nasal PD protocol as described in METHODS. *Solution 1*, lactated Ringer + 100 μM amiloride; *solution 2*, low [Cl-] solution + 100 μM amiloride; *solution 3*, *solution 2* + agonist. Upward deflections are hyperpolarization (lumen negative), conventionally taken to represent Cl^- ^{secr}etion. *A*: *cftr*(+) mouse with 100 μM Ado included in *solution 3*. *B*: *cftr* (-/-) mouse with 500 μM Ado included in *solution 3*. A depolarizing phenotype throughout the entire protocol is seen.

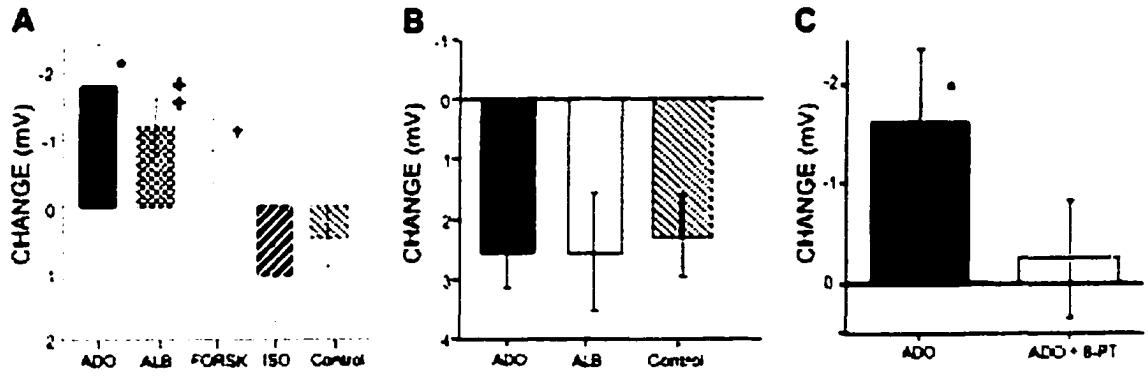

Fig. 8. Stimulated Cl- transport in *cftr*(+) (*A* and *C*) and *cftr*(-/-) (*B*) mice. Change in PD after the switch to *solution 3* is shown. *A*: *cftr*(+) mice stimulated with Ado (100 μM; n = 12), Alb (100 μM; n = 8), Forsk (10 μM; n = 10), isoproterenol (Iso; 100 μM; n = 14), and low [Cl-] control (no agonist; n = 18) included in *solution 3* are shown. $^*P < 0.005$ for Ado compared with ISO or control. $P < 0.025$ for FORSK compared with ISO or control; $P < 0.05$ for Alb compared with ISO or control. *B*: *cftr* (-/-) mice stimulated with Ado (500 μM; n = 10) or Alb (500 μM; n = 10). A depolarizing phenotype is seen with both agonists that is similar to that of *cftr*(-/-) control mice (low-[Cl-] alone in *solution 3*; n = 14). *C*: *cftr* (+) mice stimulated with 50 μM Ado in *solution 3* (n = 8) and 50 μM Ado (*solution 3*) + 100 μM 8-phenyltheophylline (8-PT) in *solutions 2* and *3* (n = 10). $^*P <$ 0.02 for change in PD after stimulation with *solution 3* (+ Ado) compared with *solution 2* (no agonist).

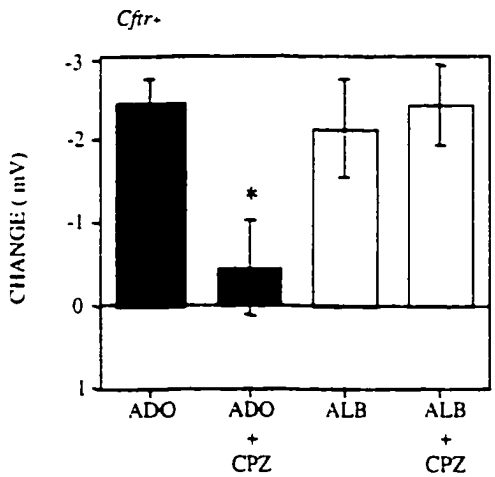

Fig. 9. $cPLA_2$ inhibition blocks Ado-stimulated Cl^- transport. Nasal PDs were performed in *cftr*(+) mice as described in Fig. 7 (n = 10-12 mice/condition). Mice were stimulated with Ado (500 μM) or Alb (500 μM) in *solution 3* in the presence and absence of CPZ (100 μM, included in *solutions 2* and *3*). *$P < 0.02$ for Ado + CPZ compared with Ado alone, Alb alone, or Alb + CPZ.

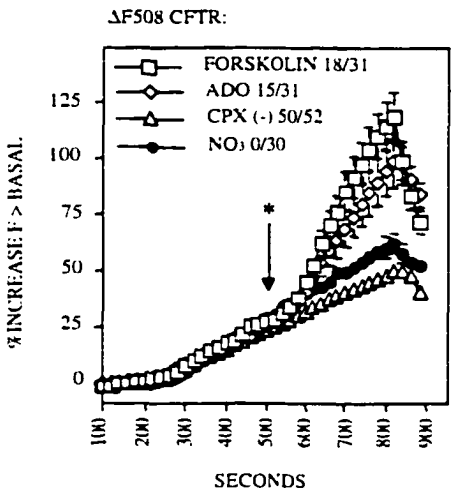

Fig. 10. A_{2B} receptor activation of halide efflux in ΔF508 CFTR-expressing cells. COS-7 cells expressing ΔF508 CFTR were studied after growth at 29°C for 48 h. Cells were perfused with NaI quenching buffer from 0 to 200 s, NO_3 dequenching buffer from 200 to 500 s, and NO_3 buffer + agonist from 500 s (arrow). Nos. in parentheses, total no. of responding cells/total no. of screened cells per condition (as described in METHODS). No. of responding cells in each condition was compared by the χ^2 test. Values are means ± SE of responding cells in each stimulated condition for Forsk (20 μM) and Ado (10 μM) or means ± SE of nonresponding cells for the 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 30 nM)-stimulated cells and NO_3 control cells. Concentration of DPCPX used has previously been shown to maximally induce acute $^{36}Cl^-$ release from ΔF508 CFTR-expressing cells (24, 30). $^*P < 0.001$ for Ado or Forsk compared with DPCPX or NO_3 conditions.

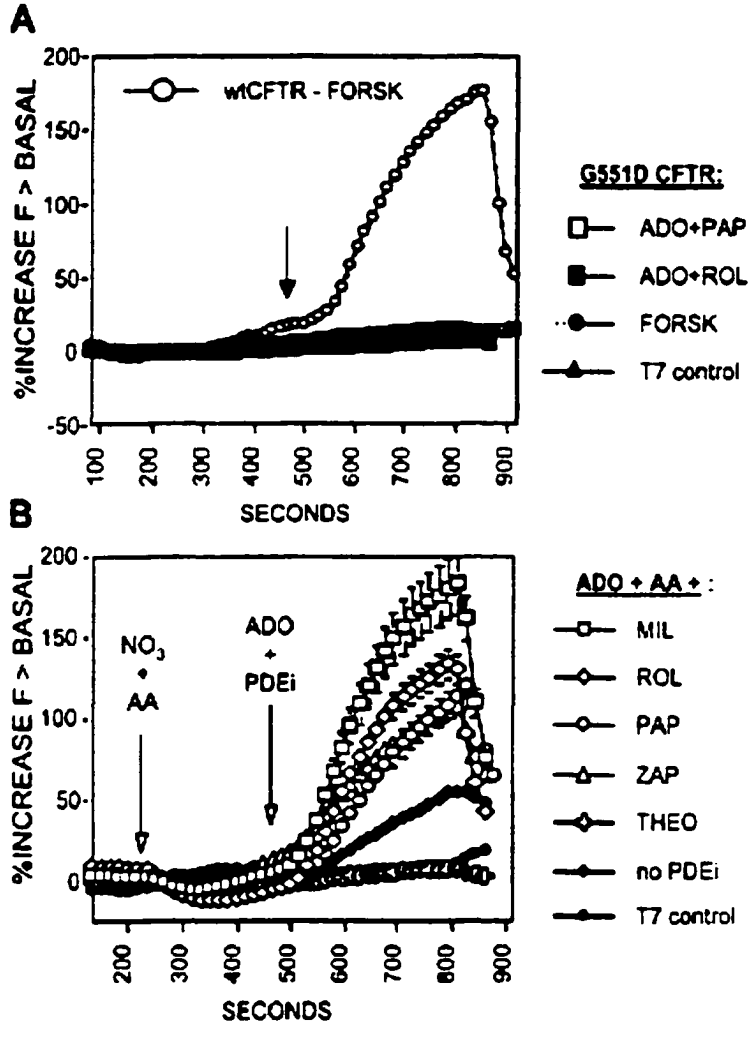

Fig. 11. Activation of halide efflux in G551D CFTR-expressing cells. *A*: G551D CFTR-expressing cells compared with wild-type (WT) CFTR-expressing cells. COS-7 cells transiently expressing WT or G551D CFTR were studied with 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) as described in METHODS; $n = 40$ cells/condition. G551D CFTR-expressing cells failed to activate halide efflux when stimulated with Ado (200 μM) + papaverine (PAP; 200 μM), Ado + rolipram (ROL; 20 μM) in combination, or Forsk (20 μM; arrow). WT CFTR-expressing cells stimulated with Forsk (CFTR + Forsk; 20 μM) are shown for comparison. *B*: G551D CFTR-expressing COS-7 cells stimulated with Ado (200 μM) and AA (100 μM) combined with a series of phosphodiesterase inhibitors (PDEis). Cells were perfused with NO_3 buffer + AA from 200 s and Ado + PDEi was added (arrows). Isotype-specific PDEis were studied at concentrations ~20-fold over the inhibition constant of their respective PDEs. Cells were also studied with Ado + AA alone (i.e., no PDEi). Values are means ± SE of all cells studied in each condition ($n > 40$). Concentrations of PDEis were (in μM) 20 milrinone (MIL), 20 ROL, 200 PAP, 5 ZAP, and 200 theophylline (THEO).

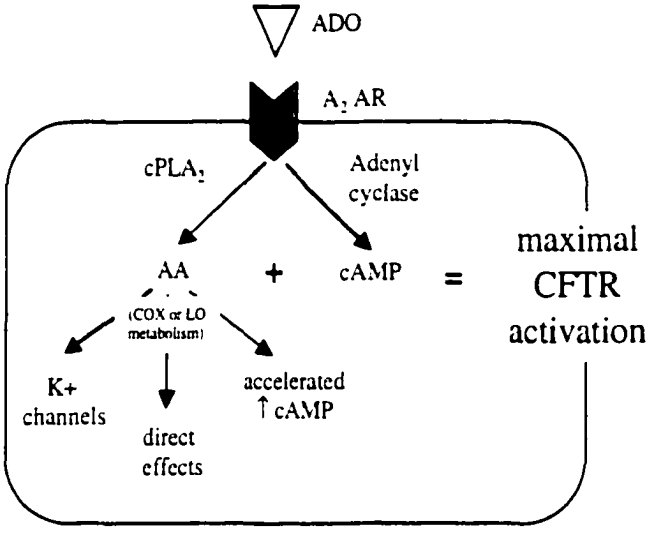

Fig. 12. Three potential mechanisms by which A_2 receptors signal CFTR activation. Ado stimulates A_2 adenosine receptors (A_2-AR), which activate $cPLA_2$ and adenylyl cyclase. AA or products of AA metabolism, when combined with cAMP, helped maximize CFTR transport of Cl^-. The mechanism could involve effects on the electrochemical driving force for ion transport through CFTR (i.e., activation of K^+ channels) through direct effects of lipid species with CFTR or associated factors (direct effects) or possibly by increasing the rate of cAMP production in the vicinity of CFTR (accelerated cAMP production). COX, cyclooxygenase; LO, lipoxygenase.

ACTIVATION OF CHLORIDE SECRETION IN CALU-3 MONOLAYERS BY CLINICAL PHOSPHODIESTERASE INHIBITORS

by

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ABSTRACT

We compared short circuit current (I_{sc}) and cAMP production in response to isotype-specific and nonspecific phosphodiesterase inhibitors (PDEi) in human airway cell lines. The concentrations used approached therapeutic serum levels for many of the PDEis investigated. Milrinone and cilostazol (Pletal, PDE3-specific), papaverine (nonspecific), rolipram (PDE4-specific), and sildenafil (Viagra, PDE5-specific) stimulated I_{sc} 20-85% of that produced by forskolin while theophylline failed to activate I_{sc}. PDEi-stimulated cAMP levels were generally ≤10% of that produced by 20 μM forskolin (Calu-3 and IB-3-1 cells). Cilostazol, papaverine, and sildenafil activated R117H cystic fibrosis transmembrane conductance reguatlor (CFTR) expressed in IB-3-1 cells. Stimulation was similar to that produced by A_2 adenosine (Ado) receptor stimulation and direct adenylate cyclase stimulation with forskolin. In Calu-3 cell monolayers, papaverine, cilostazol, and rolipram augmented both the magnitude of Ado (1 μM)-stimulated I_{sc} and the duration of I_{sc} following very low dose stimulation with Ado (0.1 μM, $P = 0.01$). Our results provide functional evidence that many PDEis activate CFTR-dependent I_{sc} in the setting of low cAMP concentrations and suggest that PDEis can augment endogenous CFTR-activating pathways.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel

that is activated through protein kinase A (PKA)-dependent phosphorylation of its regulatory domain, coupled with ATP binding and hydrolysis at nucleotide binding domains 1 and 2 (2-4, 36). Activation in vivo is believed to be accomplished through surface receptors that stimulate adenylate cyclase and raise cellular cAMP. A_{2B} Ado receptors, for example, spatially compartmentalize with CFTR through scaffolding protein interactions with A-kinase anchoring proteins (AKAPs), which modulate adenylate cyclase and PKAII (14). CFTR is also regulated by phosphodiesterases, which catalyze the conversion of cAMP to 5' AMP. Previous studies have indicated that certain phosphodiesterase inhibitors (PDEis) can activate CFTR-dependent Cl^- transport in nonpolarized cells [3-isobutyl-1-methylxanthine (IBMX), milrinone] and in wildtype (WT) and ΔF508 CFTR transgenic mice (milrinone) (11, 18-20, 44). No studies, however, have comprehensively evaluated the dose-response relationships of nonspecific and isotype specific PDEis on CFTR activity, cAMP levels, and Cl^- transport, particularly in polarized airway cell model systems.

In this report, we investigated several clinically approved isotype specific and nonspecific PDEis and tested their effects on Cl^- transport and cAMP levels in Calu-3 and IB-3-1 cells. The majority of PDEis had much stronger effects on CFTR-dependent short circuit current (I_{sc}) in Calu-3 cells than would be predicted based solely on stimulated levels of cAMP. A similar relationship was established between cAMP concentration and halide efflux in IB-3-1 cells expressing R117H CFTR in response to PDEis. Our results provide functional evidence that PDE3 isotypes contribute to CFTR regulation in polarized airway cells and that PDE inhibition can augment A_{2B} Ado receptor activation of CFTR-dependent Cl^- transport. Collectively, these studies provide a rationale to fur-

ther evaluate the effects of PDEis on receptor-based pathways that activate CFTR in mice and humans.

MATERIALS AND METHODS

Cell culture. All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). IB-3-1 cells (53) were grown in LHC-8 + 3% FBS and 1% penicillin + streptomycin. Calu-3 cells were grown in MEM media (ATCC) + 10% FBS and 1% penicillin + streptomycin. To study polarized Calu-3 cells at an air-liquid interface, polyester Transwell-Clear Costar filters (Fisher Scientific, Pittsburgh, PA; 0.4 μm pore diameter, 6 mm insert diameter for Ussing chamber experiments) were coated with human placental collagen extracellular matrix (Becton Dickinson, Franklin Lakes, NJ) at a concentration of 5 $\mu gm/cm^2$ overnight and then seeded at approximately 1×10^6 cells/cm^2. Once the filters were confluent, the media were removed from the apical surface, and cells were fed only on the basolateral surface. Resistance was monitored, and the cells were studied when resistance was $600 - 800\ \Omega \cdot cm^2$ (generally between 72-96 h after establishing an air-liquid interface).

Transient CFTR expression. CFTR was transiently expressed in IB-3-1 cells using a vaccinia-based expression system as previously described (5, 6). Cells grown on vectabond-treated glass coverslips were infected with vaccinia containing the T7 polymerase (generous gift of Dr. B. Moss, National Institutes of Health, Bethesda, MD) at a multiplicity of infection of 10 for 30 min. R117H CFTR under control of the T7 promoter in the pTM-1 vector was then introduced into cells in complex with *N*-[1-(2,3-

dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTAP)-propane-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; 20 μg DOTAP-DOPE and 5 μg pTM-1 CFTR/5 × 10^5 cells) for 4 h (6). These CFTR plasmids in the pTM-1 vector were the generous gift of Dr. S. Cheng (Genzyme Corporation, Cambridge, MA). Cells were then washed in PBS, returned to DMEM + 10% FBS, and studied 18-24 h post-infection.

Fluorescence-based halide efflux measurements. To study CFTR activation, we measured halide efflux in IB-3-1 cells (transiently expressing R117H CFTR) using the halide-quenched dye 6-methoxy-*N*-(3-sulfopropyl-quinolinium) (SPQ; Molecular Probes, Eugene, OR) as previously described (5, 6). Briefly, IB-3-1 cells were seeded onto Vectabond-treated glass coverslips, and cells were grown until ~80% confluent. Immediately prior to study, cells were hypotonically loaded with 10 mM SPQ for 10 min and then placed in a quenching NaI buffer. The cells were then placed in a specially designed perfusion chamber and studied at 23°C. Fluorescence of individual cells was measured using a Zeiss inverted microscope (excitation at 340 nm, emission >410 nm), a PTI imaging system, and a Hamamatsu camera. Baseline fluorescence was measured in isotonic NaI buffer, and then cells were perfused with isotonic dequench buffer ($NaNO_3$ replaced NaI) at the indicated time point (200 s). The perfusate was then switched to dequench buffer plus agonist and requenched at the end of the experiment. Fluorescence was normalized for each cell to its baseline value, and increases are shown as percent increase in fluorescence above basal (quenched) fluorescence. The mean percent increase in fluorescence over basal of all cells were included in the curves shown (± SE).

Transepithelial short-circuit currents. Calu-3 cells grown as monolayers at an air-liquid interface were mounted in modified Ussing chambers (Jim's Instruments, Iowa City, IA) and initially bathed on both sides with identical Ringer solutions containing (in mM) 115 NaCl, 25 $NaHCO_3$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 D-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 95% O_2 and 5% CO_2. Solutions and chambers were maintained at 37°C. I_{sc} measurements were obtained by using an epithelial voltage clamp (University of Iowa Bioengineering, Iowa City, IA). A 3 mV pulse of 1 s duration was imposed every 100 s to monitor resistance, which was calculated using Ohm's law. To measure stimulated I_{sc}, the mucosal bathing solution was changed to a low Cl^- solution containing (in mM) 1.2 NaCl, 115 sodium gluconate, and all other components as above. Increasing concentrations of PDEis were added to the mucosal and serosal bathing solutions as noted in the text. At the end of most experiments, 200 μM glybenclamide was added to the mucosal bathing solution, effectively blocking the majority of stimulated I_{sc} (~90%). Studies investigating multiple conditions were carefully paired and completed on cells of similar age and passage (10-20).

cAMP measurements. Cellular cAMP was measured using an ELISA-based detection kit as previously described (6) (Cayman Chemical, Ann Arbor, MI) . Briefly, cells grown on 35-mm dishes (~7 × 10^6 cells/dish) were stimulated with agonist for 10 min, and the cellular cAMP was extracted with ice cold ethanol. The supernatants were vacuum dried and resuspended in phosphate buffer, and the cAMP levels were quantified per manufacturer's directions.

Statistics. Descriptive statistics (s and SE) and tests of statistical significance were performed using SigmaStat software (Jandel, CA). Paired and unpaired *t*-tests were used for studies with continuous, parametric data (cAMP levels, I_{sc}; Figs. 1 and 4) and the Mann Whitney U test was used for studies with continuous non parametric data (Fig. 5).

Materials. Ado hemisodium salt, forskolin, dipyridamole, and rolipram were purchased from Calbiochem (San Diego, CA). Theophylline and milrinone were purchased from Sigma Chemicals (St. Louis, MO); papaverine HCl was purchased from Research Biochemicals International (Natick, MA). Cilostazol and sildenafil were obtained from the University of Alabama at Birmingham pharmacy.

RESULTS

PDEis activate Cl⁻ secretion in Calu-3 cell monolayers. We investigated the ability of various isotype-specific and nonspecific PDEis to activate I_{sc} across Calu-3 cell monolayers grown at an air-liquid interface. Calu-3 cells have an airway serous gland phenotype, polarize, and express high levels of CFTR as well as receptors that couple to CFTR, including A_{2B} Ado receptors (7, 13-16, 26, 40, 51). PDEis were tested at 2, 20, and 200 μg/mL (n = 4-8 filters studied in each condition). This dose range was chosen because it includes clinically used serum levels for the well-described PDEi theophylline, which has been used for decades to treat a variety of pulmonary disorders (therapeutic range 2-20 μg/mL) (1). Table 1 summarizes therapeutic serum levels and IC_{50} values of PDEis for each PDE subtype. Theophylline (nonspecific PDEi), cilostazol (PDE3-specific), and sildenafil (PDE5-specific) are all orally bioavailable and routinely used in

Table 1. *Characteristics of isotype specific and nonspecific PDEis.*

PDEi	Isotype	[μM] at 2 μg/mL	IC_{50}	[Peak Serum]
Cilostazol	PDE3	29.9 μM	200 nM	1.2 μg/mL*
Milrinone	PDE3	9.47 μM	300 nM	100-300 ng/mL
Rolipram	PDE4	3.63 μM	800 nM	N/A
Sildenafil	PDE5	54.1 nM	3 nM	500 ng/mL
Papaverine	Nonspecific	5.32 μM	10-50 μM	1 μg/mL†
Theophylline	Nonspecific	11.1 μM	120-300 μM	~ 2-20 μg/mL

Information concerning peak serum concentrations and IC_{50} values (1), * (17), †(21).

clinical medicine to treat non-cystic fibrosis (CF) pulmonary diseases and other disorders. We also studied two vasoactive agents that are dosed intravenously (papaverine, a non-specific PDEi, and milrinone, a PDE3-specific PDEi) and rolipram, a PDE4-specific PDEi. Although PDE4-specific PDEis are not currently approved for human use in the United States, rolipram has been used as part of treatment for depression in Europe and Japan (43). Stimulated I_{sc} was normalized to the percentage of the response produced by forskolin (20 μM), a direct activator of adenylate cyclase that produces a global increase in cellular cAMP. At the end of experiments, glybenclamide (a CFTR channel blocker, 200 μM) was added to the apical surface of polarized monolayers (examples shown in Fig. 1*B*).

Monolayers were exposed to increasing concentrations of agonists, and the I_{sc} 10 min following stimulation were compared. In the presence of a serosal to mucosal [Cl^-] gradient (mucosal [Cl^-] = 6 mM, basolateral Ringer's [Cl^-] = 130 mM), papaverine, sildenafil, cilostazol, rolipram, and milrinone (but not theophylline) were able to activate Cl^-

secretion ranging from ~20-85% of the forskolin response at 200 μg/mL (Fig. 1*A*). Activation was generally immediate, suggesting that resting PDE activity provided tonic negative regulation of CFTR (examples of I_{sc} tracings are provided in Fig. 1*B* and 4*B*). Nonspecific PDE inhibition with papaverine (2 μg/mL) stimulated Cl^- secretion most efficiently, producing ~ 35% of the forskolin response at the lowest concentration tested (Fig. 1, $P < 0.02$ compared with all other agonists). Theophylline, a xanthine-based nonspecific PDEi, failed to activate Cl^- secretion at any dose studied. In general, all isotype specific PDEis produced a dose-dependent activation of Cl^- secretion, with milrinone achieving >50% of the forskolin response at 20 μg/mL and close to 85% at the highest dose tested. These concentrations, however, are well above those used clinically (300-500 ng/mL) (1). Sildenafil (PDE5-specific) activated I_{sc} at higher concentrations (20 and 200 μg/mL); however, these concentrations were also well above peak clinical serum levels (500 ng/mL) (1). Cilostazol (PDE3-specific) displayed a clear dose-dependent effect on I_{sc}, achieving 14.92% (±1.61 SE), 27.35% (±5.91), and 39.07% (±5.25) of the forskolin response at 2, 20, and 200 μg/mL, respectively. Dipyridamole, a PDEi with activity against PDE7, PDE8, and PDE11, failed to activate I_{sc} at all concentrations tested (2, 20, and 200 μg/mL, data not shown). Milrinone and cilostazol had the strongest effects on I_{sc} of the isotype-specific inhibitors investigated, suggesting that the PDE3 isotype may be particularly important in regulating CFTR in serous gland cells. While the majority of stimulated I_{sc} produced by the different PDEis was blocked by glybenclamide (Fig. 1*B*), Cl^- secretion produced by high dose milrinone was only partially glybencla-

mide sensitive ($\simeq$ 50%). Due to these potentially nonspecific effects on I_{sc}, further functional studies of PDE3 inhibition (Figs. 3, 4, and 5) were limited to cilostazol.

PDEi effects on cAMP in Calu-3 and IB-3-1 cells. In an attempt to correlate PDEi-stimulated I_{sc} with cAMP production, we measured cell cAMP in Calu-3 and IB-3-1 cells following PDEi exposure for 20 min at concentrations of 2, 20, and 200 μg/mL (Fig. 2). cAMP production was again normalized to forskolin-stimulated cAMP production (20 μM). We found that all PDEis produced dose-dependent increases in cAMP concentration in both cell types. The effects on cAMP, however, were less than what might have been expected based on I_{sc} measurements. In Calu-3 cells, all PDEis tested (except for sildenafil) increased cAMP concentration at 200 μg/mL, ranging from ~8-15% of that produced by forskolin. These differences between stimulated I_{sc} and cAMP production were more pronounced at lower concentrations. For example, low dose papaverine (2 μg/mL) produced less than 5% of the cAMP produced by forskolin yet stimulated Cl^- secretion I_{ac} close to 40% of the forskolin response (Fig. 1). Milrinone and rolipram produced comparatively similar increases in cAMP concentration at 20 and 200 μg/mL. Cilostazol (20 μg/mL), also a PDE3 inhibitor, activated Cl^- secretion to a lesser extent than milrinone (Fig. 1), but this was accomplished at cAMP concentrations that were below the sensitivity of our assay. Stimulation with sildenafil (PDE5, cGMP-dependent) had negligable effects on cAMP concentrations but was nonetheless able to activate Cl^- secretion at 20 μg/mL. IB-3-1 cells stimulated with the highest doses (200 μg/mL) of theophylline and papaverine had the greatest increases in cAMP concentration (19.9% and 29.9% of cAMP produced by 20 μM forskolin, respectively). Stimulation of

IB-3-1 cells with sildenafil (PDE5-specific), rolipram (PDE4-specific), milrinone (PDE3-specific), and cilostazol (PDE3-specific) at 200 μg/mL produced cAMP responses similar to those seen in Calu-3 cells, ranging from ~5-10% of forskolin-stimulated cAMP.

In general, the amount of cAMP detected in both airway cell types stimulated with PDEis was dose dependent and produced concentrations that were <10% of the forskolin response. Coupled with the functional data in Fig. 1, the results suggest that PDE isotypes police discrete regions within the cells and confer a local regulation of cAMP levels and CFTR activity.

PDEis activate R117H CFTR expressed in IB-3-1 cells. Previous in vivo studies have shown that milrinone alone or in combination with isoproterenol (a β_2 adrenergic receptor agonist) can activate CFTR-dependent Cl^- transport in normal subject, but fails to activate Cl^- secretion across the nasal mucosa of CF patients homozygous for ΔF508 CFTR or patients heterozygous for ΔF508/G551D CFTR (44). However, partial CFTR function was restored in the nasal mucosa of transgenic mice homozygous for the ΔF508 CFTR mutation following perfusion of milrinone combined with forskolin (20). No studies, however, have systematically investigated whether PDEis, when used alone, can activate partial function, disease-causing CFTR mutations that can localize to the cell membrane in a fashion similar to WT CFTR. To perform these studies, we transiently expressed R117H CFTR (class IV mutation with reduced single channel conductance) in IB-3-1 cells and measured ion transport. We measured R117H CFTR activation using SPQ, a halide sensitive dye that is a well-established and specific assay of CFTR activity (5, 6). Experiments were performed using the clinically approved PDEis at 200 μg/mL.

In Fig. 3, R117H CFTR-expressing cells stimulated with Ado (25 μM) or forskolin (20 μM) are shown for comparison. The mean rate of dequench for each condition (ΔRFU/20 s) from 560-720 s is shown. Mock transfected cells stimulated with both Ado and forskolin did not activate halide efflux, demonstrating that stimulated halide efflux required CFTR expression. Similar to the stimulated I_{sc} in Calu-3 cells (Fig. 1), cilostazol, papaverine, and, to a lesser extent, sildenafil activated halide efflux in R117H CFTR-expressing IB-3-1 cells. Theophylline had the smallest effects on halide efflux in this system. These studies indicate that isotype specific and nonspecific PDEis are capable of activating a partial function mutant CFTR molecule in airway cells in a fashion similar to receptor stimulation or direct adenylate cyclase stimulation with forskolin. Activation was observed despite only small effects on cell cAMP concentrations (Fig. 2*B*).

Papaverine, cilostazol, and rolipram augment Ado-activated Cl^- secretion in Calu-3 cell monolayers. Previously, it has been shown by our laboratory and others that CFTR is efficiently activated by A_{2B} Ado receptors in Calu-3 cells, in part due to compartmentalized signaling that links the receptor, adenylate cyclase, and PKAII to CFTR (14, 49). These observations provide a rationale to investigate the additive effects of PDE inhibition on Ado-activated Cl^- transport. For these studies, we stimulated Calu-3 cell monolayers ($n = 4$ filters in each condition) with each PDEi at a low dose (2 μg/mL, mucosal and serosal) for 5 min prior to stimulation with Ado at concentrations that modestly activate I_{sc} (1 μM mucosal, Fig. 4*A* and 4*B*). Stimulated I_{sc} 5 min following PDEi addition (black bars) and 5 min following Ado addition (gray bars) is shown (Fig. 4*A*). Papaverine had a strong additive effect on Ado-stimulated Cl^- secretion ($P < 0.005$ com-

pared with Ado only), while cilostazol and, to a lesser extent, rolipram produced modest additive effects with Ado ($P < 0.05$ compared to Ado only). These three PDEis alone were also able to acutely simulate I_{sc} at 2 μg/mL (while sildenafil and theophylline did not) with minimal effects on cAMP production (Fig. 4*B* and see Fig. 2*A*). These results suggest that these PDEis can be combined with Ado stimulation to increase the magnitude of A_2 Ado receptor-stimulated Cl^- secretion in Calu-3 cell monolayers.

To further characterize localized signaling through Ado, A_{2B} Ado receptors, PDEs, and CFTR, we investigated the effects of PDEi pretreatment on the duration of Ado-stimulated Cl^- secretion. Calu-3 cell monolayers were again prestimulated with 2 μg/mL of cilostazol, papaverine, and rolipram followed by very low dose Ado (0.1 μM, all agonists added to mucosal surface only, Fig. 5, $n = 6$ filters in each condition). The time courses of stimulated I_{sc} produced by the PDEis alone and 0.1 μM Ado are compared in Fig. 5*A*. All PDEis produced prolonged activation of I_{sc} (>1,000 s) compared with 0.1 μM Ado alone, which produced only a transient stimulation of I_{sc}. Pretreatment with cilostazol and, to a lesser extent, papaverine and rolipram augmented the duration of Ado-stimulated I_{sc}, producing a sustained response above the pre-Ado baseline that persisted for 1,000 s, 500 s, and 300 s long, respectively (Fig. 5*B*). These results suggest that PDEs limit the duration of CFTR activation and Cl^- secretion produced by stimulation of A_{2B} Ado receptors. Furthermore, low concentrations of PDEis may be capable of "priming" CFTR, enabling prolonged activation following stimulation by Ado as part of in vivo signaling.

DISCUSSION

In this report, we investigated and compared the ability of several clinically relevant PDEis to activate halide transport. Using Calu-3 cells grown as monolayers, we demonstrated that both a nonspecific PDEi and several isotype specific PDEis could activate CFTR-dependent Cl^- secretion in a dose-dependent manner. PDEi-stimulated I_{sc} did not correlate well with changes in total cellular cAMP in Calu-3 cells, and a similar relationship was observed between stimulated halide efflux and cAMP in IB-3-1 cells expressing R117H CFTR. We also found that specific PDEis could have additive effects on the magnitude (papaverine) and the duration (cilostazol) of Ado-stimulated I_{sc}. These results help delineate the physiologic role of PDEs as regulators of CFTR and support further investigations of PDEis as agents to improve the function of surface localized CFTR molecules.

Many PDE subtypes, including members of the PDE2, PDE3, PDE4, PDE5, PDE6, PDE7, PDE8, PDE9, and PDE10 families, have been shown to be expressed in mammalian airways and/or lungs by either Northern blot analysis or RT-PCR (10, 22-25, 27, 28, 35, 37, 45, 50, 52). Of those isotypes, PDE3 has most consistently been demonstrated to regulate CFTR activity. Previous studies have shown that inhibition of PDE3 activity stimulates halide efflux in human airway cells (in the setting of low cAMP), human airway tissues, and the murine intestine (19, 44, 47). Our studies, however, are among the first to evaluate a battery of PDEis (including several that were studied close to clinically achievable serum concentrations) to activate Cl^- secretion in airway cell monolayers and to test the ability of PDEis (other than milrinone) to activate a partial

function, surface localized, mutant CFTR molecule. The results from Fig. 1 are consistent with the previously published reports demonstrating PDE3 regulation of CFTR but also indicate that several other clinically useful PDEis can augment CFTR activity. The results from Fig. 3 extend previous studies of mutant CFTR activation through PDEis and indicate that R117H CFTR can be stimulated by PDEis in a fashion similar to endogenous receptor signaling.

One goal of our investigations was to determine whether clinically available PDEis could influence CFTR activity and Cl^- secretion in airway cell monolayers at relatively low concentrations. Although the effects of milrinone on I_{sc} were stronger than cilostazol (Fig. 1), the concentrations necessary to achieve activation were quite high relative to clinically established serum levels (Table 1). Cilostazol, in contrast, achieved moderate activation of I_{sc} at concentrations near serum levels achieved as part of oral therapy for claudication (2 μg/mL, Fig. 1 and Table I). Furthermore, cilostazol strongly activated halide efflux in IB-3-1 cells expressing R117H CFTR (Fig. 3), produced prolonged activation of I_{sc} at 2 μg/mL (Fig. 5*A*), and augmented Ado-activated I_{sc} in terms of magnitude (Fig. 4) and duration (Fig. 5*B*). These effects on Ado-stimulated I_{sc} were demonstrated at relatively low Ado concentrations (1 and 0.1 μM), suggesting that cilostazol may be able to influence CFTR activation as part of normal in vivo signaling.

To date, 26 PDE genes within 11 isotype families have been identified. It is possible that other PDEs, in addition to those inhibited by the isotype-specific PDEis investigated in this study, play a role in regulating CFTR in airway cells. Papaverine was particularly interesting in this regard. This nonspecific PDEi *1*) potently activated Cl^- secretion in Calu-3 cells (Fig. 1), *2*) stimulated R117H CFTR in IB-3-1 cells (Fig. 3), *3*) pro-

duced prolonged Cl^- secretion at low doses (2 μg/mL, Fig. 5*A*), and *4*) augmented low dose Ado-stimulated I_{sc} (Figs. 4 and 5). This is a clinically approved vasoactive PDEi for the treatment of male impotence, with a peak effective concentration following direct administration in the corpus cavernosa of approximately 1 μg/mL (Table 1). In our model system, papaverine was capable of activating sustained I_{sc} twofold and almost 10-fold above a PDE3-specific and a PDE4-specific PDEi, respectively (Fig. 4). While our studies do not exclude combined effects of papaverine on multiple PDEi isotypes, they do support future studies to investigate CFTR regulation by additional PDEs.

Sildenafil, a PDE5 inhibitor (cGMP-specific), activated I_{sc} at relatively high concentrations (Fig. 1) with negligible effects on cAMP (Fig. 2*A*). While this could represent cGMP-dependent regulation of I_{sc}, it should also be noted that cGMP exerts a negative feedback on PDE3 activity (48). Therefore, this type of PDE "cross-talk" could explain how, at higher concentrations, cGMP might downregulate endogenous PDE3 activity, leading to larger local cAMP concentrations produced from in vivo signaling mechanisms that are capable of activating CFTR. Finally, theophylline was a very poor stimulus of WT CFTR-dependent I_{sc} in Calu-3 cells (Fig. 1) and R117H CFTR activity in IB-3-1 cells (Fig. 3), despite relatively strong effects on cAMP (Fig. 2). The reasons for this are uncertain but may indicate that this nonspecific PDEi primarily influences PDE activity physically removed from CFTR. Additionally, theophylline is a xanthine-based PDEi with known antagonism of P_1 purinergic receptors. It is possible that this sort of receptor antagonism influences CFTR activation in Calu-3 monolayers. Regardless of the primary effect of theophylline, our results indicate that it is unlikely that this agent

would have beneficial effects on CFTR activity and Cl^- secretion at concentrations tolerated clinically (<20 μg/mL).

The results from Figs. 1 and 2 demonstrate that many PDEis can activate Cl^- secretion in the setting of very low (and often undetectable) cAMP levels. While this could suggest that cAMP-independent mechanisms contribute to PDEi activated I_{sc}, we favor the interpretation that the PDEis exert compartmentalized regulation of cAMP pools and CFTR. Several studies have led to the conclusion that CFTR interacts with scaffolding proteins of the cytoskeleton and that these interactions can influence CFTR regulation by surface receptors and PKA (12, 29-32, 42). Our results provide functional evidence that PDEs may be part of this organized signaling cascade. First, several PDEis alone and at low concentrations were able to acutely activate Cl^- secretion in Calu-3 cell monolayers (*Fig. 4B*). These results suggest that PDEis provide tonic negative regulation to CFTR through reduction of local cAMP and that a threshhold, localized elevation of cAMP (through PDE inhibition) is adequate to activate CFTR. This same pattern was observed with most of the PDEis tested and over a variable range of concentrations. Activation was seen despite structural differences between the compounds, arguing against idiosyncratic affects that produce CFTR activation independent from PDEi activity. Moreover, a number of recent studies in other model systems suggest that PDEs may participate in compartmentalized protein regulation. For example, PDE3A and 3B have six predicted transmembrane helices, a requirement for binding to the membrane fraction of the endoplasmic reticulum (39). Additionally, certain PDE4 subtypes contain proline-rich Src homology 3 (SH3)-binding motifs in their amino terminal tails, which could allow interactions with anchoring proteins possessing SH3 domains (34, 38). Finally, PDE6A, B,

and C (expressed predominantly in the retina), like CFTR, have a putative post-synaptic density zona occludin (PDZ) binding domain that may be important for targeting to specific intercellular regions (46). Future studies that investigate the nature of PDE:CFTR compartmentalization and regulation may be of particular interest to our understanding of CF. Our results suggest that removal of tonic negative regulation imparted by PDEs can acutely increase resting CFTR activity (including WT and a mutant CFTR) in the absence of specific exogenous stimuli.

PDEis may be useful in improving the activity of partial function, disease-related CFTR mutations. We investigated R117H CFTR, a class IV CFTR mutation that matures appropriately, localizes to the cell surface, and is characterized by defective single channel Cl^- conduction (41). The amount of R117H CFTR localization to the cell membrane in vivo is also dependent on R117H CFTR mRNA transcript levels (that are reduced depending on the thymidine tract length at the end of *cftr* intron 8) (8, 9, 33). Patients carrying the R117H CFTR allele typically suffer from the respiratory manifestations characteristic of CF but are in general pancreatic sufficient. We used an in vitro airway cell expression system to demonstrate that R117H CFTR can be activated by cilostazol, papaverine, and to a lesser extent sildenafil in CF bronchial airway cells. Activation by these PDEis was similar to that produced by Ado (25 μM) or forskolin (20 μM). Although our CFTR expression levels are much higher than those found in vivo, the results from Fig. 3 provide support for the notion that PDEis may be capable of contributing to activation of mutant, partial function CFTRs. Furthermore, the additive effects of cilostazol or papaverine, when combined with an A_2 Ado receptor stimulus (Figs. 4 and

5), support the notion that PDEs which regulate CFTR can influence endogenous, receptor-based signaling pathways.

In summary, our data demonstrate that PDEis alone can activate CFTR-dependent Cl^- secretion across airway cell monolayers in vitro in the setting of low cAMP concentrations. Inhibition of PDE activity can also have additive effects on I_{sc} when combined with A_2 Ado receptor stimulation, increasing the duration and magnitude of receptor-based activation of CFTR and Cl^- secretion. These studies support a strategy utilizing endogenous cell signaling pathways to augment surface-localized CFTR activity and provide a rationale for further studies of novel PDEis in CF mice and humans.

ACKNOWLEDGEMENTS

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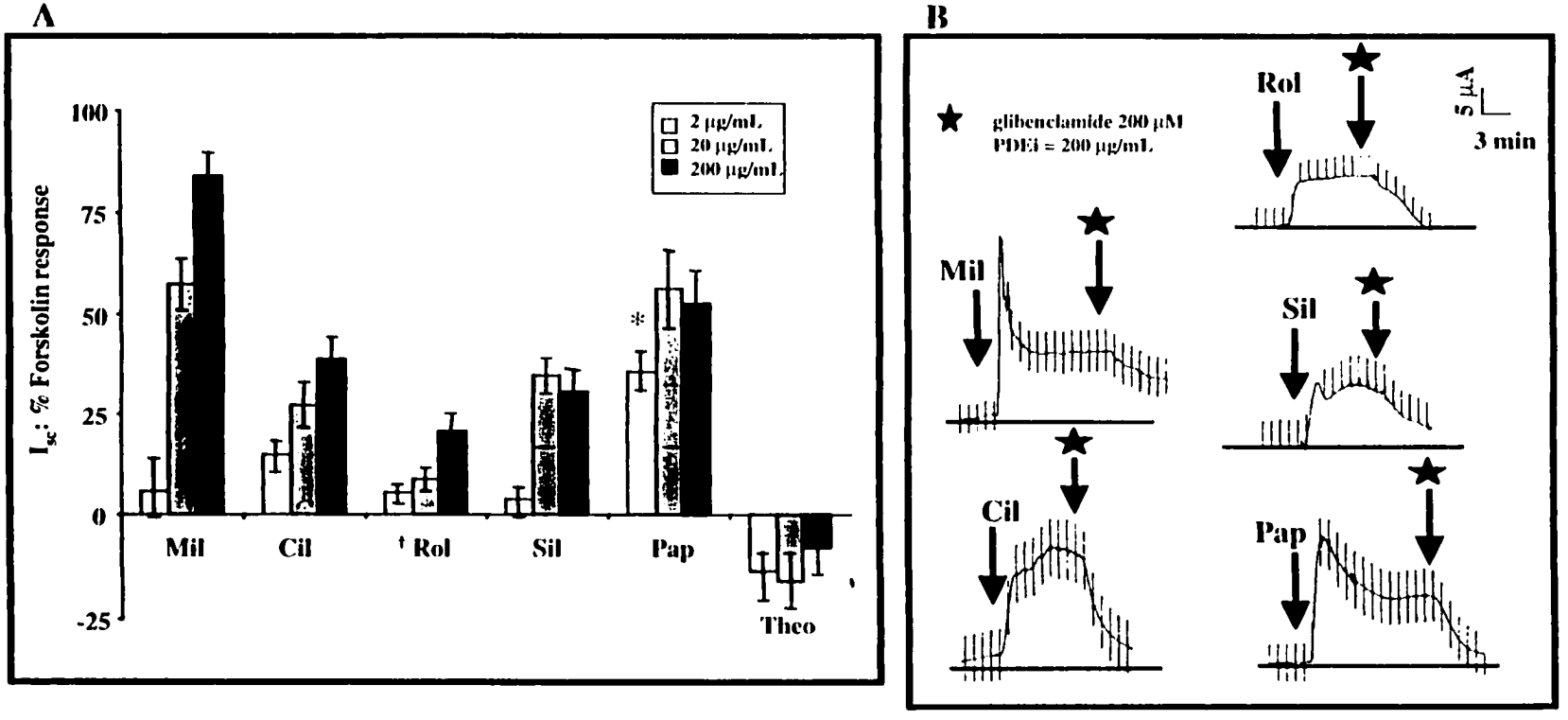

Fig. 1. PDEis activate Cl^- secretion in Calu-3 cell monolayers. Cells were grown at an air-liquid interface and studied in Ussing chambers as described in METHODS. *A*: All PDEis, except for theophylline, activated I_{sc} in a dose-dependent manner ranging from 10%- 85% of the forskolin response (20 μM). Values represent the percent of forskolin (20 μM) response ± SE for each condition (n = 4-8 filters/condition). Papaverine was the most potent agent to activate I_{sc} at low concentrations (2 μg/mL, $^*P < 0.02$). Due to an absence of data concerning therapeutic serum concentrations for rolipram, this agent was studied at 1, 10, and 100 μM †. *B*: examples of PDEi-activated I_{sc} (200 μg/mL – arrows). Each pulse = 100 s. ★ = addition of glybenclamide (200 μM, mucosal). Mil = milrinone, Cil = cilostazol, Rol = rolipram, Sil = sildenafil, Pap = papaverine, Theo = theophylline.

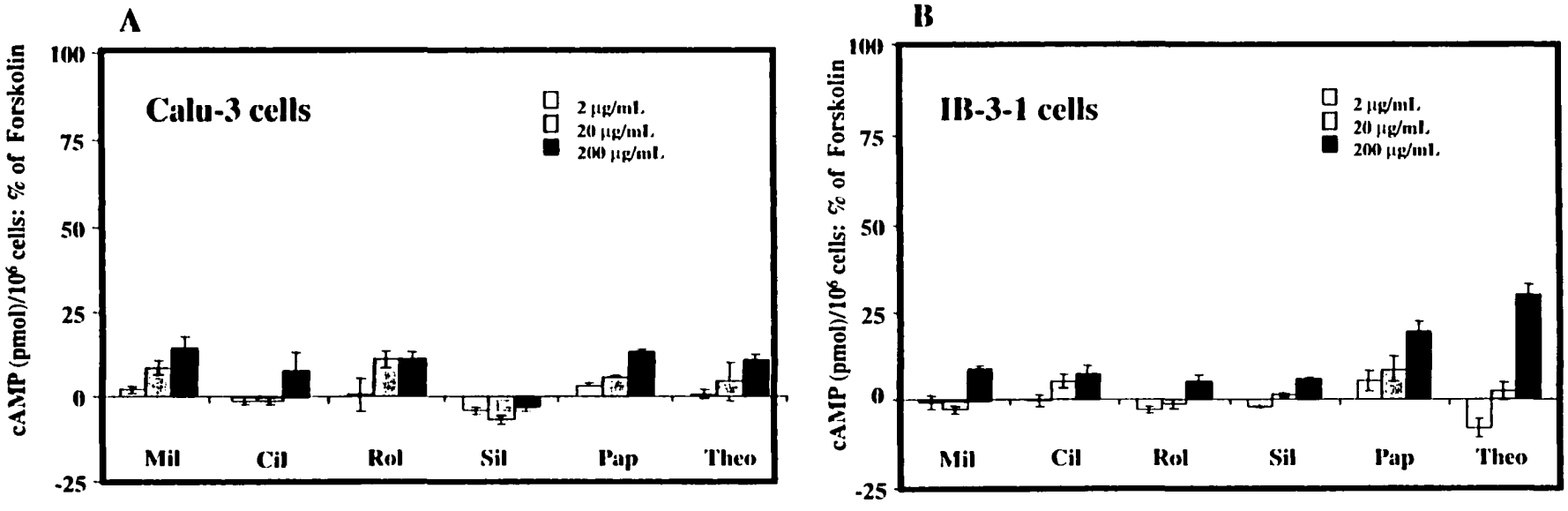

Fig. 2. PDEis modestly stimulate cAMP production in (*A*) Calu-3 and (*B*) IB-3-1 cells. cAMP levels were measured by ELISA as described (see METHODS). Cells were stimulated for 20 min before extraction (n = 4 dishes/condition). Values represent the percent of forskolin (20 μM) response $\pm$SE for each condition. *A*: Calu-3 cells. *B*: IB-3-1 cells.

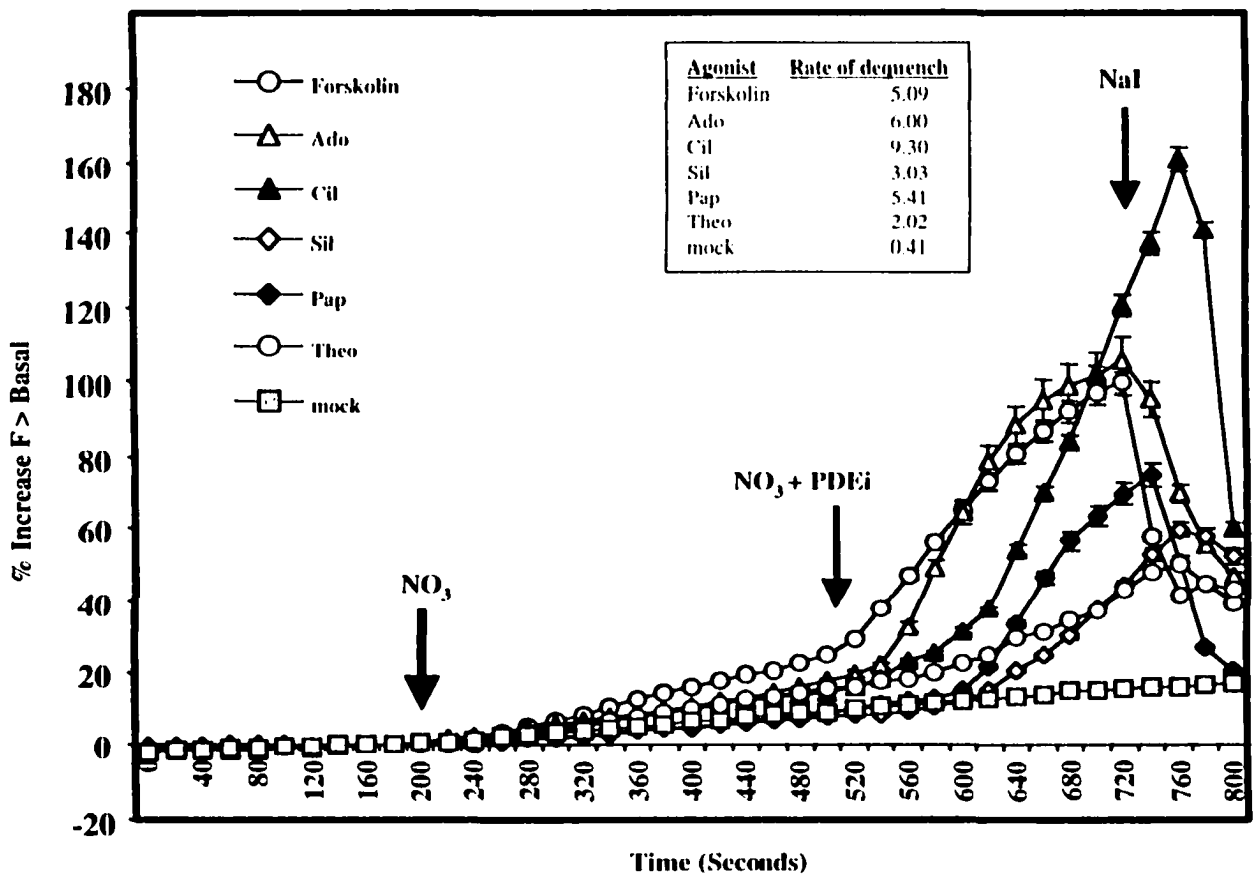

Fig. 3. PDEis activate halide efflux in R117H CFTR-expressing IB-3-1 cells. IB-3-1 cells transiently transfected with R117H CFTR were studied using SPQ, as described in the METHODS. Values are means ± SE of all cells in each stimulated condition for Forskolin (20 μM), Ado (25 μM), and each PDEi at 200 μg/mL (n = 40-50 cells/condition). Mock cells represent IB-3-1 cells transfected in the absence of R117H CFTR plasmid. Numbers in inset are the mean dequench rate (RFU/20 s) from 560-720 s for all conditions. The lag between agonist addition and dequench represents the time for solution change.

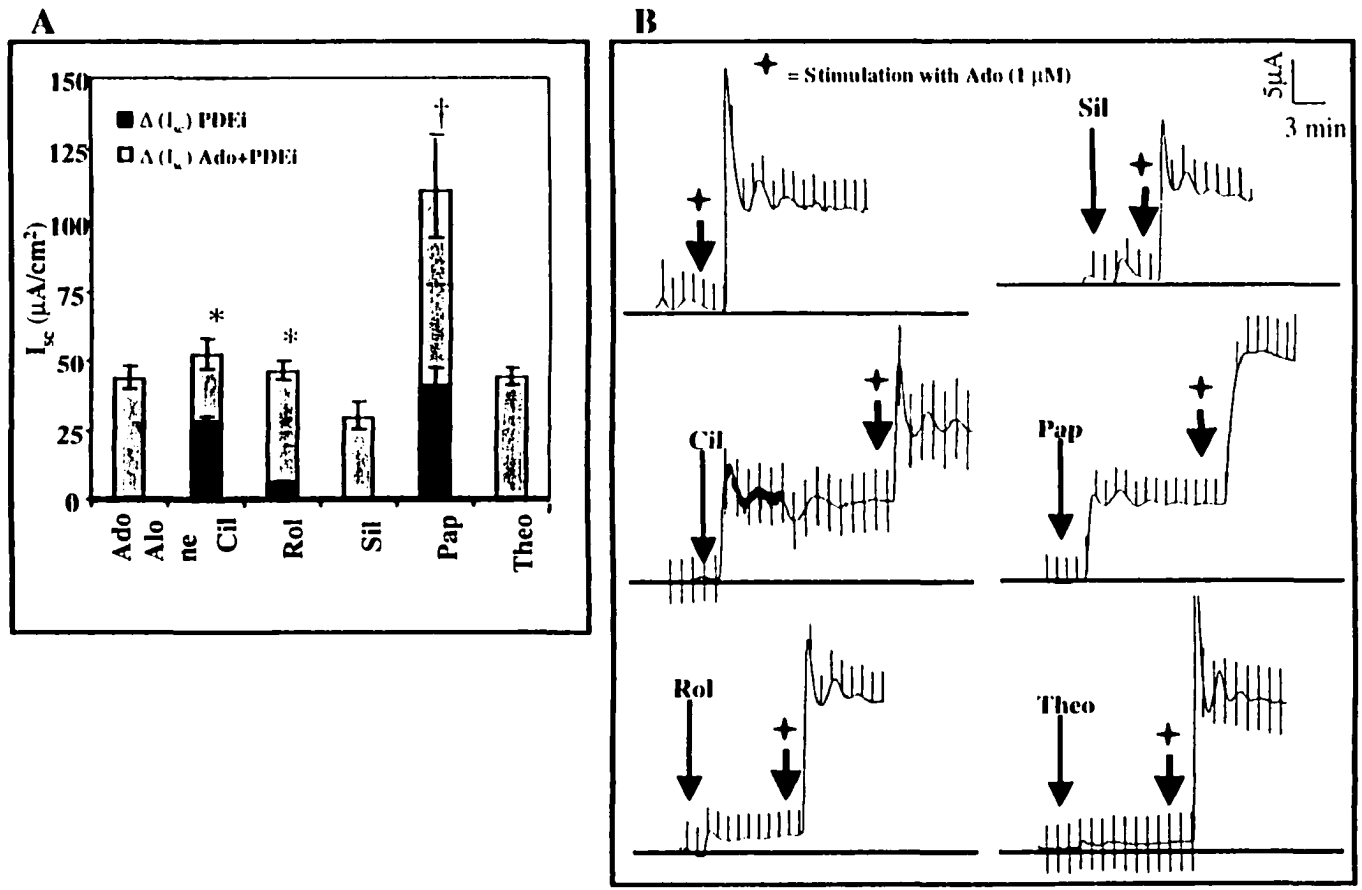

Fig. 4. Additive effects of PDE inhibition and Ado-stimulated I_{sc}. Calu-3 cell monolayers were exposed to PDEis (2 μg/mL, mucosal and serosal) followed by Ado stimulation (1 μM, mucosal, $n = 4$ for each PDEi). *A*: *Black bars*, change in I_{sc} following 5 min exposure to PDEi. *Gray bars*, change in I_{sc} following 5 min exposure to Ado. Values are means ± SE of stimulated I_{sc}. *P* values represent Ado stimulation alone compared to Ado with PDEi pretreatment (*$P < 0.05$, †$P < 0.005$.) *B*: examples of PDEi- and Ado-stimulated I_{sc} tracings. Each pulse = 100 s.

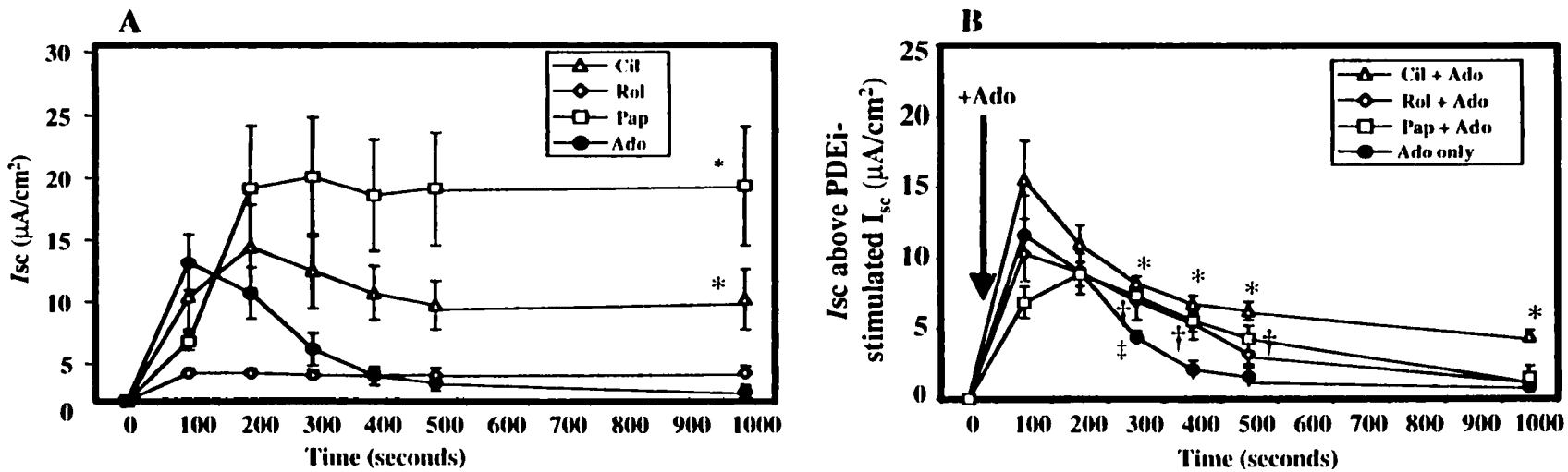

Fig. 5: I_{sc} measurements in Calu-3 cell monolayers prestimulated with PDEis (2 μg/mL, mucosal) followed by stimulation with Ado (0.1 μM, mucosal). *A:* Time course of stimulated I_{sc} produced by PDEis compared with Ado. All PDEis tested produced a prolonged (>1,000 s) response compared with 0.1 μM Ado alone. Papaverine and cilostazol-stimulated I_{sc} were greater than Ado at 1,000 s ($^{*}P = 0.02$). *B*: time course of Ado-stimulated I_{sc} alone compared with Ado-stimulated I_{sc} of monolayers pretreated with PDEis. Pretreatment with cilostazol produced a sustained I_{sc} response to Ado stimulation ($^{*}P < 0.02$) compared with Ado alone at 300 - 1,000 s. Pretreatment with papaverine modestly prolonged the Ado response ($^{\dagger}P < 0.05$) at 400 and 500 s compared with Ado alone. Pretreatment with rolipram slightly prolonged the Ado response ($^{\ddagger}P < 0.05$) compared with Ado alone at 300 s ($n = 6$ filters for each condition, values are mean ± SE).

MOLECULAR BIOLOGY OF ADENOSINE RECEPTORS

by

BRYAN COBB AND JOHN PAUL CLANCY

In preparation for *Academic Press*

Format adapted for dissertation

ABSTRACT

Two classes of purinergic receptors, P_1 and P_2, have been identified which are selective for either adenosine (Ado) or ATP and related compounds, respectively. P_1 purinergic or Ado receptors are G protein coupled receptors that are classified into A_1, A_{2A}, A_{2B}, and A_3 subtypes based on structural and functional characteristics, agonist selectivity series, and effector coupling systems. The intracellular signaling pathways that result from stimulation of each receptor subtype are dependent on the specific subtype expressed, the level of expression, the concentration of agonist, and the effector systems available. Agonist interaction with a specific receptor subtype imparts downstream physiological consequences such as alterations in ion channel activity, neurotransmission, or regulation of gene expression. Having both stimulatory and inhibitory effects, P_1 purinergic receptors serve as homeostatic regulators that function in the fine tuning of various modulatory pathways. Due to the ubiquitous expression of Ado receptors, dysregulation due to events such as cellular injury or aberrant Ado metabolism might contribute to pathological processes involved in cardiovascular, immunological, neurological, and epithelial disorders. Ado receptors and the agonists or antagonists that interact with them may, therefore, be appropriate targets for therapeutic drug development to treat a variety of diseases. This chapter reviews these recent and well-studied aspects of the molecular and cell biology of Ado receptors.

INTRODUCTION

The purine nucleoside adenosine (Ado) has been of interest to researchers and clinicians for nearly 70 years due to its physiological role in cell signaling as well as its'

potential and observed therapeutic applications (45). The therapeutic potential was first considered when Ado, unlike adenine, was found to be nontoxic and rapidly metabolized ($t_{1/2}$ = 3-6 s) when administered intravenously to humans. As early as 1929, the physiologic effects of Ado were beginning to be defined, primarily due to its effects on the cardiovascular system, including coronary vasodilation and systemic hypotension (45). In modern medicine, Ado is the treatment of choice for certain supraventricular tachyarrhythmias and can be useful to aid in the diagnosis of other tachycardias due to its profound, but short lived, effects on atrioventricular nodal conduction (11). Ado exerts its effects through receptor signaling, and the development of Ado receptor agonists is currently an important line of research that may have relevance to many physiologic processes and medical conditions. This includes cardiovascular functions, degenerative central nervous system diseases, schizophrenia, memory and behavior, rheumatic and other inflammatory disorders, and epithelial ion transport.

Ado is a unique signaling molecule in that it utilizes both extracellular and intracellular signaling pathways to produce widespread effects in many different cells and organ systems (12, 138, 169). Found in virtually all living cells, Ado produces effects by activating specific cell surface P_1 purinergic receptors in a concentration-dependent, tissue and subtype specific manner (12, 103, 160). Ado receptors are members of the G-protein-coupled receptor (GPCR) superfamily with seven characteristic membrane-spanning domains, extracellular tertiary structure that determines agonist selectivity, and intracellular structure which contributes to coupling with many G proteins (18, 103, 160). The purpose of this manuscript was to discuss specific aspects of P_1 purinergic receptor

molecular and cell biology, including pharmacological and structural characteristics, functional significance, and the modulatory role these receptors can have on signaling as part of both homeostatic and pathophysiologic processes.

MOLECULAR PHARMACOLGY

Identification and characterization of subtypes. Ado primarily interacts with receptors that are distinct from those that interact with other nucleotides, such as ATP. Using biochemical, physiological, and pharmacological methods, two classes of purinergic receptors, P_1 (Ado selective) and P_2 (ATP selective), have been identified based on the following findings: *1*) methylxanthines competitively antagonize Ado-mediated cell processes but not those mediated by ATP, *2*) Ado but not ATP frequently increases intracellular cAMP levels, and *3*) ATP, ADP, AMP, and Ado have different relative potencies at Ado-selective receptors compared with ATP-selective receptors. P_1 purinergic receptors are characterized by their Ado-selectivity over its phosphorylated nucleotides, while P_2 purinergic receptors are selective for ATP and related compounds over Ado (20).

With the help of molecular cloning techniques, human A_1, A_{2A}, A_{2B}, and A_3 P_1 purinergic receptors have been identified with ~45% sequence homology exhibited between the four receptor subtypes (35, 97). With this information, it has become possible to begin characterizing each subtype and developing selective agonists and antagonists. With the aid of molecular cloning technology, researchers have also begun to better understand receptor activation and desensitization, as well as other regulatory processes, including cell signaling, and the role that these receptors play in health and disease. (For more extensive detail on structure/function relationships between P_1 purinergic receptors

and ligands, the reader is directed toward several excellent, focused reviews; (85, 124, 126, 127, 130, 131)

A_1 Ado receptors. The A_1 receptor subtype is a 326 amino acid protein of approximately 36 KDa that has been cloned from a wide variety of species (Table 1). Heterologous mammalian expression systems have shown that agonists bind to A_1 receptors with an order of potency of (-)-RG 14719 > 2-chloro-N^6-cyclo-pentylAdo (CCPA) > N^6 –cyclopentylAdo > (-)-N^6-(R-phenylisopropyl)Ado (R-PIA) > 5'-*N*-ethylcarboxamidoAdo (NECA) > (+)-N^6-phenylisopropylAdo (S-PIA) > NECA > (2-[[4-(2-carboxyethyl)phenethyl]-amino]Ado-5'-*N*-ethlcarboxamide (CGS-21680) that *1*) is distinct from other P_1 purinergic receptors and *2*) possesses a higher (up to10-fold) affinity for both agonists and antagonists than other (A_2 or A_3) P_1 receptors (Table 2) (87, 98, 105, 123). For example, the K_D-value for [^{3}H]CCPA in chinese hamster ovary cells transiently expressing human A_1 receptors is extremely low (0.6 nM). In general, N^6-substituted Ado analogs are selective for A_1 receptors, with CCPA being the most selective identified thus far. (-)-RG 14718 is considered the most potent A_1 receptor agonist, with high selectivity (2,000-fold) for A_1 over A_{2A} receptors. NECA is a relatively non-selective and potent agonist for both A_1 and A_2 receptors.

Many of the A_1 receptor antagonists are xanthine based, including commonly known drugs such as caffeine and theophylline. 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, K_D value = 3.9 nM) and the non-xanthine, nonselective compound CGS 15943 (9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine, Ki value = 3.5 nM) appear to be the most potent A_1 receptor antagonists in radioligand competitive binding studies

Table 1. *Characterization of P_1 Purinergic Receptors*

	size	species (cloned)	chromosome location
A_1	326 aa, 36 KDa	human, bovine, dog, rabbit, chick, guinea pig, rat, mous	1q32.1
A_{2A}	412 aa, 44 KDa	human, dog, guinea pig, rat, mouse	22q11.2
A_{2B}	332 aa, 36 KDa	human, chicken, rat, mouse	17p11.2-12
A_3	318 aa, 36 KDa	human, sheep, dog, rabbit, rat, mouse	1p13.3

aa = amino acids

Table 2. *Prominent P_1 Purinergic Receptor Agonists/Antagonists and Relative Affinities*

	agonist selectivity	antagonists	affinity (for adenosine)
A_1	CCPA>CPA>R-PIA>ADO>NECA>S-PIA>CGS-21680	DPCPX, CGS 15493, CPX	high affinity (15-100 nM) *
A_{2A}	CGS-21680>ADO>NECA>R-PIA~S-PIA	DPMDX, KF17837	highest affinity for CGS-21680 (~ 150 nM)*
A_{2B}	NECA>ADO≥R-PIA=IBMECA>>CGS-21680	Enprofylline, XAC	low affinity (0.5- 20 μM) *
A_3	2Cl-IB-MECA>DBXRM>>ADO	MRE300F20, MRS1220	low affinity (1- 10 μM) *

MRS1220: 9-chloro-2-(2-furyl)-5-phenylacetylamino[1,2,4]triazolo[1,5-c]quinazoline

CGS15943: 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine (Williams, et al. 1987; Dunwiddie, et al. 2001)

KF17837: (E) -1,3-Dipropyl-8- 3,4-dimethoxy-styryl -7-methylxanthine

CPX: 8-cyclopentyl-1,3- dipropylxanthine

DBXRM, 1,3-dibutylxanthine-7-riboside-5'- N-methyl-carboxamide

MRE3008F20: 5N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo [4,3-e] -1,2,4- triazolo[1,5-c]pyrimidine

XAC: 8-[4-[[[(2- aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine

(79, 87). A_1 receptors are structurally distinct from other P_1 purinergic receptors as well as other GPCRs, possessing several unique characteristics that are presently of uncertain functional significance. For example, the third intracellular loop has a short sequence of 34 amino acids which is roughly one sixth the size of other GPCRs (such as muscarinic and adrenergic receptors) capable of inhibiting adenylate cyclase (123). There is also a potential fatty acid binding site on the carboxy-terminal tail, in addition to relatively few (n = 5) consensus sequences for phosphorylation (see Fig. 1).

A_{2A} receptors. A_{2A} receptors are ~45 KDa proteins composed of 410-412 amino acids and have been cloned from multiple mammalian species, including human, mouse, dog, guinea pig, rat, and canine (60, 128). The carboxy-terminal tail of the A_{2A} receptors is comparatively larger than the A_1 subtype, yet the functional significance of this extended sequence is unknown. A_{2A} receptors are pharmacologically distinct from other subtypes primarily because of their high affinity binding to [^{3}H] CGS 21680 and [^{125}I]-2-[4-[2-[2- phenylmethlycarbonylamino] ethylaminocarbonyl]ethyl]phenyl]ethylamino-5'-*N*-ethyl-carboxamidoAdo ([^{125}I]), both of which are much more selective for A_{2A} receptors than [^{3}H]-NECA and the PIA stereoisomers. Typically, A_2 subtype selectivity is accomplished by modifying the 2-position of Ado. Recent synthetic ligands generated using comparative molecular field analysis have enhanced selectivity for A_{2A} receptors (143). This technology uses physiochemical modeling to design agonists with reduced A_1 receptor and enhanced A_{2A} receptor selectivity.

Several potential A_2 receptor antagonists with 8-styryl modification of 1,2,7-alkylxanthines have been synthesized. 1,3-Dipropyl-7-methyl-8-(3,5-dimethoxystyryl)

xanthine (DPMDX) was found to be a potent (K_I = 24 nM) Ado antagonist that is 110-fold more selective for A_2 receptors over A_1 receptors (82).

A_{2B} receptors. A_{2B} receptors, first evaluated in human fibroblasts (17), have ~75 fewer amino acids than A_{2A} receptors (332 amino acids total), with a molecular weight of approximately 35 KDa. These receptors have also been cloned from many different species and tissue types, including humans, and are expressed throughout the brain (Table 3) (37, 60). A_{2B} receptor characterization has required unique approaches to distinguish it from other P_1 receptors. A_1 and A_{2A} receptors have historically been characterized in terms of agonist selectivity by performing radioligand binding studies using receptor clones heterologously expressed in eukaryotic cells. To characterize A_{2B} receptors, however, demonstration of direct adenylate cyclase activity has been required in order to determine isotype-specific activity. This is due to a lack of known selective agonists that distinguish A_{2B} receptors from other P_1 receptors. NECA nonselectively binds A_{2B} receptors (EC_{50} = 2 μM), while activating other subtypes (A_1 and A_{2A}) with greater (in nM) affinity. The A_{2B} receptor is characterized by a potency profile of NECA > R-PIA = N^6-(3-iodobenzyl)-Ado-5'-*N*-methyl-uronamide (IB-MECA) > CGS 21680, but the receptor cannot be distinguished from other P_1 purinergic receptor subtypes based on this potency series alone. Interestingly, the A_{2B} subtype has a relatively low affinity for [^{3}H]NECA, while the A_{2A} subtype binds [^{3}H]NECA with much higher affinity (18). CGS 21680 is a relatively ineffective agonist for A_{2B} receptors (but very selective for A_{2A} receptors), while R-PIA and IB-MECA in micromolar concentrations are nonselective for A_{2B} versus A_{2A} receptors (2, 15, 50, 84). Identification of A_{2B} receptors, therefore, has involved

Table 3. *Expression of P_1 Purinergic Receptor Subtypes in Various Tissues*

A_1		A_{2A}		A_{2B}		A_3	
Brain, widespread	(1-5)	Heart	(6)	Caecum, colon	(6, 7)	Neurons various	(8)
Spinal cord	(3)	Kidney	(6)	Bladder	(6, 7)	Brain	(2)
Smooth muscle	(9)	Platelets	(10)	Brain, widespread	(1, 2)	sperm, widespread	(6)
Adipocytes	(11)	Brain, Striatum	(12)	Neurons, various	(6)	Mast cells	(6)
Heart	(13)	Smooth muscle	(9)	Mast cells	(14)	Lung (abundant)	(6, 15)
Kidney	(16)	Neutrophils	(17)	Airway cells	(18, 19)	Kidney	(6)
Thyroid	(20)	olfactory tubercule	(21)	Neutrophils	(17)	Heart	(6)
Neutrophils	(22, 23)	Neurons, various	(6)	Fibroblasts	(24)	Neutrophils	(25)
Macrophages	(22, 23)	Lung	(6)	Macrophages	(26)	Macrophages	(27)
Neurons, various	(6)	Liver	(6)			Testes, widespread	(28)
Retina	(29)					Lung, abundant	(15)
Bladder	(6)						
Testis	(3, 28, 30)						

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demonstration of direct adenylate cyclase activation in a xanthine-sensitive, NECA-inducible manner that is poorly responsive to CGS 21680 or PAP-APEC stimulation.

Although highly selective antagonists are preferable for subtype identification, they have yet to be developed for A_{2B} receptors. However, some xanthine derivatives are moderately potent A_2 receptor antagonists, similar to that of other P_1 purinergic receptor subtypes (15, 50). Enprofylline, for example, was found to have a dissociation constant of approximately 7 μM and appears to be a selective albeit not particularly potent A_{2B} receptor antagonist. Other more potent yet less selective antagonists have been characterized and are reviewed by Feoktistov and Biaggioni (48). More potent and specific A_{2B} receptor ligands are needed to aid in the characterization of this receptor, to distinguish it from other P_1 purinergic receptors, and to clarify its physiologic role.

A_3 receptors. Using chinese hampster ovary (CHO) cells stably expressing a unique clone with sequence similarity to the A_1 and A_{2A} receptors (isolated from a rat brain cDNA library), Zhou and colleagues discovered a P_1 purinergic receptor with a pharmacological profile distinct from A_1 and A_2 subtypes (177). Additionally, the cloned receptor was found to be relatively insensitive to antagonism by most xanthine derivatives, while NECA stimulation resulted in inhibition of forskolin-stimulated cAMP accumulation through a pertussis toxin-sensitive G-protein. This novel receptor subtype, composed of 337 amino acids, was designated A_3 and has subsequently been cloned from human, rat, dog, rabbit, and sheep (74, 99, 119, 145).

Selective activation of A_3 receptors requires modification of Ado in the N^6- and 5' positions. An example is IB-MECA, which is a potent agonist with moderate selectivity

(83). 2-chloro-N^6 -(3-iodobenzyl)-Ado-5'-*N*-methyluronamide (2Cl-IB-MECA), however, is 2500- and 1400-fold more selective for the rat A_3 receptor subtype compared with the A_1 or A_{2A} receptor, respectively (81). Additional synthetic ligands selective for the A_3 receptor subtype have been developed over the last 3 years (161-165). Differences in mRNA and protein expression among species homologs may represent distinct A_3 receptor subtypes rather than species variation of a single A_3 receptor. Additional genomic, proteomic, and pharmacological studies will be needed to better characterize the A_3 subtype.

ADO RECEPTOR-EFFECTOR COUPLING AND INTRACELLULAR SIGNALING

The best studied intracellular signaling pathway that Ado receptors influence is the adenylate cyclase system (147). A_{2A} and A_{2B} receptors are thought to couple to G_s and activate adenylate cyclase, which catalyzes the conversion of ATP to 5' cAMP. A_1 and A_3 receptors, in contrast, couple to G_i and, in general, inhibit adenylate cyclase and cAMP production. Studies over the past decade have broadened our view of the signaling pathways influenced by P_1 purinergic receptors, with many of the receptor subtypes modulating second messenger systems independent of cAMP (see Table 4). In general, the specific effector systems that couple to P_1 receptor subtypes vary in a tissue-specific manner that is dependent on both the level of receptor subtype expression and the diversity of the effector coupling systems innate to the specific tissue of interest. This view might help to explain some of the apparent discrepancies found when comparing different methodologies that use different model systems.

Table 4. *Effector Coupling Systems*

Receptor	G Protein	additional coupling systems	ion channels
A_1	$G_{i\alpha 1,2,3}$ and G_o	PKA(-), PLC, PKC, IP_3, MAP kinase, PLA_2	Ca^{2+}, K^+, Cl^- channels
A_{2A}	$G_{s\alpha}$ and G_{olf}	PKA, PKC	N-/P-type Ca^{2+} channel
A_{2B}	$G_{s\alpha}$ and G_o	MAP Kinase (via $G_{q/11}$), PLC, PKA, PLA_2	Ca^{2+}-regulated Cl^- channel
A_3	$G_{i\alpha 2,3}$ and G_o	PLC, PLD, PLA_2	Ca^{2+} (undefined) channel

A_1 receptors and their effector coupling systems. It has been demonstrated in reconstituted systems that A_1 receptors can interact with the $G_{i1,2,3}$ and G_o and inhibit adenylate cyclase (61). A_1 receptors are somewhat promiscuous, with the capacity to couple to a variety of effector systems other than adenylate cyclase, including PLC, PKC, IP_3 signaling system, and several ion channels including, Ca^{2+}, K^+, and Cl^- channels (1, 13, 40, 100, 135, 146, 166).

Characterizing A_1 receptors in terms of their signaling systems has largely been made possible by using A_1 receptor expression systems in CHO cells. Using this approach, stimulation with A_1 receptor agonists have been shown to result in the following effects: *1*) activation of the inositol phosphate signaling cascade (with increases in IP_3 and the release of stored Ca^{2+}), *2*) the activation of the mitogen-activated protein (MAP) kinase signaling pathway, and *3*) direct coupling to PLC in a $G_{3\alpha 3\beta\gamma}$-dependent manner (39-41, 120, 125, 130).

The effector systems that couple to A_1 receptors also have been studied extensively in neurons. A_1 receptor stimulation can produce excitatory neurotransmission by modulating K^+ and Ca^{2+} conductances (134). For example, Ado inhibits voltage-gated Ca^{2+} influx via A_1 receptor activation. Preventing Ca^{2+} accumulation within neurons prevents the release of neurotransmitters (mainly glutamate), which can dually inhibit both presynaptic excitatory effects and (by default) downstream postsynaptic excitatory effects.

Recent studies have also focused on the role of A_1 receptors and PKC signaling cascades. Ado release, acting through A_1 receptors, is capable of positively regulating in-

sulin and leptin in isolated rat adipocytes utilizing a PLC/PKC-dependent pathway (21). Ado stimulates insulin-dependent leptin release through PLC/PKC activity.

A_{2A} receptors and their effector coupling systems. A_{2A} receptors are thought to couple predominantly to adenylate cyclase. This has been demonstrated in a variety of tissues and cell types, including platelets, striatum, basal ganglia, vasculature, and smooth muscle cells. Unlike A_1 receptors, few alternative effector systems have been shown to link this receptor subtype to PKA-independent signaling pathways. However, A_{2A} receptors have been shown to regulate additional G-proteins that are not coupled to adenylate cyclase. In striatal cholinergic neurons, A_{2A} receptor subtype stimulation has been shown to lead to two parallel signaling pathways that utilize different G-proteins (68). One of the pathways (that activates adenylate cyclase) involves G_s coupling and PKA-dependent activation of P-type Ca^{2+} channels. The other pathway involves a cholera-toxin insensitive G-protein that activates a PKC-dependent N-type Ca^{2+} channel. Both pathways are sensitive to an A_{2A} receptor antagonist (KF17837) and are not additive, and PKA signaling is capable of inhibiting the second one. Finally, evidence suggests that A_{2A} receptors are also able to couple to G_{olf} (named for its identification in olfactory epithelium), which is a receptor subtype that is highly expressed in neurons of the striatum in the brain, in excess of G_s expression (90).

A_{2B} receptors and their effector coupling systems. A_{2B} receptors couple to G_s proteins and activate adenylate cyclase, and this has been demonstrated in various systems including, the brain, fibroblasts, and airway cells. Additionally, A_{2B} receptors cou-

ple to various intracellular signal transduction pathways outside the adenylate cyclase system. For example, A_{2B} receptors have been shown to couple to MAP kinase pathways via $G_{q/11}$ proteins (5, 49, 53). In mast cells, A_{2B} receptors also couple to $G_{q/11}$ and activate phophatidylinositol-specific PLC, resulting in the release of diacylglycerol (DAG) and IP_3 (49, 101, 106, 107). DAG, in turn, stimulates PKC, while IP_3 mobilizes Ca^{2+} from intracellular stores.

Other studies support a role for A_{2B} receptors in a PLC signaling. Activation of A_{2B} receptors expressed in Xenopus oocytes induces a Ca^{2+}-regulated Cl^- conductance, thought to be modulated by PLC (174). In human mast cells, A_{2B} receptors couple to G_q and activate β-PLC, resulting in both Ca^{2+} mobilization in a phosphotidylinositol-specific manner and the release of interleukin-8 (IL-8) (49). In these studies, A_{2B} receptor stimulation led to Ca^{2+} mobilization that was insensitive to both cholera and pertussis toxin. Additionally, IL-8 release was sensitive to the A_{2B} receptor antagonist, enprofylline.

In human erythroleukemia cells, A_{2B} receptor coupling to G_s also influences intracellular Ca^{2+} levels by potentiating Ca^{2+} influx in a cAMP-independent manner (51). Stimulation of A_{2B} receptors can also potentiate Ca^{2+} influx through a Ca^{2+} channel, activating PKA in neurons which innervate the guinea pig hippocampus (112). These studies, along with others, reinforce the role for the receptor subtype in coupling mechanisms outside of adenylate cyclase.

A_3 receptors and effector coupling systems. A_3 receptors are thought to predominantly couple to either $G_{i\alpha 2}$ or $G_{i\alpha 3}$ in a pertussis toxin-sensitive manner, producing in-

hibitory effects on adenylate cyclase and cAMP production. In schaffer collateral-CA1 synapses and in a rat mast cell line, A_3 receptors are also able to stimulate PLC (130) and PLD (3), respectively. In addition, A_3 receptors expressed in cultured airway smooth-muscle cells appear to be capable of activating phospholipase A_2 (PLA_2) and releasing arachidonic acid (AA) from cell membranes (110). In this system, ATP and 5-hydroxy-tyrptamine were shown to stimulate Ca^{2+} release, which could be enhanced by a specific A_3 receptor agonist (1B-MECA). The enhancing effects of IB-MECA were shown to be sensitive to $AACOCF_3$ (a PLA_2-specific inhibitor), suggesting a role for $cPLA_2$ and AA signaling.

Although additional signaling mechanisms are not well documented for this less promiscuous subtype, activation of A_3 receptors in human monocytes has been shown to inhibit NADPH oxidase activity independent of cAMP elevation or alterations in Ca^{2+} levels. These results suggest that A_3 receptors might utilize additional signaling path-ways not yet described (16).

Receptor desensitization. Upon prolonged agonist exposure, GPCRs are known to undergo a loss of sensitivity to agonists (66). Several studies support desensitization of P_1 receptors (22, 23, 129, 130, 133). Two phases characterize this desensitization and are based on short-term versus long-term agonist exposure. Short-term exposure results in uncoupling of P_1 receptors from G-proteins through receptor phosphorylation (of ser-ine and tyrosine residues by PKA and PKC), while long-term agonist exposure results in receptor downregulation, including receptor internalization and/or reduced receptor syn-

thesis. In CHO cells transiently expressing the A_1 or the A_3 receptor subtype, different uncoupling rates have been demonstrated following sustained agonist exposure. The differences appear to be in part due to the ability of A_3 receptors (but not A_1 receptors) to undergo rapid phosphorylation (and subsequent internalization) through the activation of G protein-coupled receptor kinases (GRK) (54). A_{2A} and A_{2B} receptors have also been shown to undergo short-term desensitization mediated by phosphorylation through a specific GRK (GRK2). For these A_2 receptors, P_1 receptor phosphorylation was dependent on both agonist concentration and the level of GRK expression (118).

A_{2B} receptor stimulation with NECA induces pulmonary vasodilation in isolated lung and pulmonary artery smooth muscle cells, followed by desensitization that requires $G_{s\alpha}$-adenylate cyclase signaling (73). A_{2B} receptors found on the apical and basolateral surfaces of polarized T84 epithelial monolayers differentially desensitize following agonist stimulation (152). Prolonged stimulation of A_{2B} receptors on the basolateral surface produces desensitization of A_{2B} receptors on the apical membrane, but not the converse. The nature of this cross-cellular desensitization, however, is not clear.

ADO RECEPTORS IN HEALTH AND DISEASE

The intracellular and extracellular concentration of Ado is regulated by many factors, including *1*) its production by both de novo and salvage pathways for purine nucleotide biosynthesis, *2*) the amount of Ado released from cells by facilitated diffusion, *3*) Ado reuptake, *4*) the activity of degradation pathways, and *5*) metabolic conversion following removal of phosphate groups from ATP and related compounds through 5'ectonucleotidase activity. The cellular effects that Ado influences are determined by

the tissue levels and the regional P_1 purinergic receptor subtypes that are expressed. Ado capacity to modulate multiple biological processes in numerous cell types, including inflammatory cells, mast cells, smooth muscle cells in bronchi and vasculature, intestinal and airway epithelial cells, neurons, and platelets, makes it a versatile, quintessential signaling molecule. Although concentrations of Ado are tightly regulated, alterations in the levels of Ado can have pathophysiological ramifications, in that Ado serves as a cardioprotector, chemoprotector, immunomodulator, ion transport regulator, and neuromodulator.

Ado receptors and protective effects in the cardiovascular system. The role of Ado in the cardiovascular system has been well studied but continues to be an active area of research. Through P_1 purinergic receptor signaling, Ado acts as a potent modulator of many cardiovascular functions, including blood pressure, vascular tone, and heart rate, and may be particularly useful in understanding the pathogenesis of myocardial infarction in animal models. During ischemic challenge, Ado is released to the interstitium, where it is believed to exert a cardioprotective effect and reduce myocardial infarct extension. In addition, ischemia followed by reperfusion can extend vascular injury, leading to contractile dysfunction, apoptosis, and cellular necrosis. Ischemia-reperfusion injury is characterized by neutrophilic invasion, alterations in ionic pump activity, edema, and generation of superoxide radicals. Ado appears to modulate many of the components that contribute to cardiac injury. Although P_1 purinergic receptors have been known to be expressed in the heart for some time, the specific mechanisms by which Ado exerts its cardioprotective effects have only been recently investigated. The nature of the cardiopro-

tective effect of Ado depends on whether it is used as pretreatment, during ischemia, or with reperfusion. Cardioprotection by Ado is thought to be mediated by A_1 receptor activation and opening of an ATP-sensitive potassium channel (K_{ATP}) when used prior to or during ischemia (10, 122). Pretreatment with Ado during ischemic preconditioning (following 5 min of ischemia prior to receiving longer, more damaging ischemia) has been shown in several animal models to reduce myocardial infarct size and augment postischemic recovery (109). Ado receptor activation during ischemia can also result in reduced infarct size, while enhanced postischemic recovery requires P_1 purinergic stimulation during ischemia and reperfusion.

Neutrophil recruitment is characteristic of ischemic vascular injury. Each receptor subtype seems to harness its defense through distinct signaling pathways. During reperfusion, A_2 receptor activation may inhibit neutrophil attachment to the endothelium and, therefore, reduce inflammation and myocardial injury. Stimulating A_2 receptors before reperfusion inhibits neutrophil function and does not appear to involve K_{ATP}-channel activation (176). Activation of A_3 receptors has also been shown to inhibit neutrophil adhesion to the endothelium, as well as attenuate postischemic contractile dysfunction through PKC-dependent activation of K_{ATP} channels (96, 158, 159). Following coronary artery occlusion in rabbits, infarct size can be reduced by activating K_{ATP} channels. Ado can accomplish this by stimulation of A_1 receptors and nitric oxide synthetase (NOS)-dependent signaling or A_3 receptors and NOS-independent signaling (157).

The cardioprotective effects of Ado are not limited to ischemia-induced vascular injury. Ado accumulates in the systemic circulation in patients with both ischemic and

nonischemic chronic heart failure (62). In a recent study, patients with chronic heart failure who were treated with either dilazep or dipyridamole (which inhibit Ado transport into the cell) had increases in plasma Ado levels, associated with improved maximal oxygen consumption and ejection fraction (86). These studies suggest that there may be therapeutic implications for the management of cardiac and noncardiac vascular disorders by influencing Ado metabolism or by using P_1 receptor ligands.

Ado receptors and cancer. Several observations have demonstrated that Ado stimulation of specific P_1 purinergic receptors modulates the growth and differentiation of both normal and cancer cells. At micromolar concentrations, Ado stimulates proliferation of bone marrow cells, thymocytes, and swiss mouse 3T3 cells. Treating HT29 cells (a colonic adenocarcinoma cell line) with Ado deaminase (ADA, which converts Ado into inosine) or with an A_1 receptor antagonist decreases cell growth rates (94). At higher concentrations (>100 μM), Ado can induce apoptosis in both normal and transformed cells in vitro. This has been demonstrated in lckNT transgenic mice that overexpress ecto-5'-nucleotidase (5' NTase) in cortical thymocytes. In these mice, 5' NTase activity was increased 100-fold without alterations in the plasma concentration of Ado, whereas ADA activity was shown to prevent accumulation of Ado and maintain it at low concentrations (140). Treatment with an ADA inhibitor (2'-deoxycoformycin) resulted in a 30-fold increase in Ado concentration, levels which are capable of inducing apoptosis.

Released Ado has been shown to abrogate muscle cell tumor growth, and, in a rat lymphoma cell line, A_3 receptor stimulation has been shown to have antiproliferative ef-

fects by inducing cell cycle arrest during G_o/G_i (58). In vivo, a combined effect of chemotherapy and A_3 receptor stimulation may theoretically enhance antiproliferative characteristics.

In hypoxic tissues, Ado modulates angiogenesis. In a recent study, antagonists of A_2 receptors were shown to mediate hypoxia-induced antiangiogenic properties in CD45 positive lymphocytes isolated from peritoneal ascitic fluid of ovarian cancer patients (7). Additionally, cell motility imperative to tumor progression was found to be enhanced in Ado-stimulated melanoma cells, which was inhibited by a P_1 receptor antagonist (172). In another study, Ado released from hypoxic regions within solid tumors (in the presence of an inhibitor of ADA) inhibited the adhesion of anti-CD3-activated killer lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells by up to 60%. This effect appeared to be through stimulation of A_3 receptors on the effector cells (104).

These studies and others highlight the role that Ado might play in cellular differentiation and tumor development. Ado has been shown to have both inhibitory and stimulatory effects that are consistent with signaling through specific P_1 purinergic receptor subtypes. A better understanding of the effects of Ado on tumor growth (particularly in regions of hypoxia), angiogenesis, and metastasis may lead to new targets for future cancer therapy.

P_1 purinergic receptors as regulators of inflammation. P_1 purinergic receptors mediate both pro- and anti-inflammatory effects in various organ systems and white blood cell types. This section will highlight examples of both pro- and anti-inflammatory actions mediated through P_1 purinoreceptors with an emphasis on mucosal inflammation.

Evidence suggests that Ado can function as a potent anti-inflammatory agent. Extracellular Ado can be produced during cell stress and following ecto-5'NTase break-down of ATP and other Ado nucleosides, with accumulation in inflammatory exudates. Ado release is promoted by neutrophil-dependent production of oxygen radicals, in addition to clinically used anti-inflammatory agents (26, 64). Methotrexate and sulfasalazine are part of the treatment of chronic inflammatory conditions such as rheumatoid arthritis and Crohn's disease. The anti-inflammatory effects of these agents appear to be mediated by enhanced Ado release with subsequent inhibition of neutrophil activity (discussed below) (32, 33, 64, 114). A_{2B} receptors may also play a protective role in arthritis. Activation of this receptor subtype in cultured synoviocytes has been shown to inhibit gene expression of matrix metalloproteinases, which are involved in the pathologic degradation of bone and cartilage seen in rheumatoid arthritis (14).

The most potent anti-inflammatory effects of Ado come from studies involving A_{2A} receptors. Activation of this receptor subtype during an inflammatory response results in inhibition of neutrophil adhesion and a reduction in the generation of superoxide radicals (19, 27, 29-31). In macrophages, A_{2A} receptor activation also inhibits superoxide anion generation, TNF-α, IL-12 secretion, and the proliferation of T-cells and promotes apoptosis of activated T-cells (65, 92, 95, 102, 136). These findings demonstrating the toxic effects of Ado on T-cells may be important to better understanding T-cell dysfunction in immune disorders, such as severe combined immune deficiency, which is a genetically inherited disorder that involves defective ADA function.

P_1 purinergic receptors not only regulate the function of inflammatory cells, but can also effect gene expression. For example, A_{2B} receptors play a crucial role in cycloxygenase-2 gene regulation and the synthesis of prostaglandin E2 in microglial cells, as well as inducing IL-6 expression (57). A_{2B} receptors, therefore, may play a role in the inflammatory responses seen in neurodegenerative diseases such as Alzheimer's disease, where IL-6 may contribute to disease pathogenesis (9, 59, 78, 171). A_3 receptor agonists have also been shown to regulate gene expression, inhibiting transcriptional activation of macrophage inflammatory protein 1α, in which a protein that promotes neutrophil chemotaxis (156).

Several studies also suggest a role for Ado in asthma pathogenesis. Evidence for this role was first recognized when it was shown that Ado and AMP inhalation produced dose-dependent bronchoconstriction in asthmatics with allergic triggers, but not in normal subjects or subjects with other airway diseases (CF, bronchectasis) (36, 75). Excessive amounts of Ado have also been found in bronchial lavage fluid of asthmatic individuals (44). It appears that A_1 receptors are involved in asthma pathogenesis since they are overexpressed in allergic rabbits and rats, and A_1 receptors are upregulated in bronchial smooth-muscle tissue exposed to human asthmatic serum (4, 44, 47, 70, 132). Ado also induces mast cell degranulation through stimulation of A_{2B} receptors, leading to histamine and IL-8 release (31, 52). A_{2B} receptor specific antagonists might, therefore, one day serve a role in asthma therapy, blocking mast cell degranulation.

Ado may also "fine tune" white blood cell activity, depending on local Ado concentrations and differential P_1 purinergic receptor affinities. For example, A_1, A_{2A}, A_{2B},

and A_3 receptors are all expressed in neutrophils. However, each receptor subtype plays a different role in regulating neutrophil-induced inflammatory responses. A_1 receptors stimulate pro-inflammatory functions such as chemotaxis, superoxide radical formation, and neutrophil adherence (27-29, 31, 88, 144). The stimulation of A_2 receptors, however, inhibits many inflammatory neutrophil functions, including superoxide formation, inflammatory mediator release, phagocytosis, and neutrophil adherence. Different concentrations of Ado, therefore, may mediate pro- or anti-inflammatory responses, supporting a model in which ambient production of Ado in tissues modulates inflammation. Peripheral human neutrophil suspensions also spontaneously release Ado, which, upon binding to A_2 receptors, inhibits important pro-inflammatory functions such as leukotriene B_4 production (89).

Ado has also been shown to modulate important inflammatory functions in peripheral macrophages, including the modulation of Ado receptor expression by various inflammatory mediators. For example, Xaus and colleagues have demonstrated that interferon upregulates A_{2B} receptor expression in murine bone marrow macrophages (173). Ado stimulation also inhibits several pro-inflammatory functions in peripheral macrophages and macrophage cell lines, including interferon-γ induced expression of major histocompatibility class II genes; NOS, lipopolysaccharide induced IL-10, TNFγ, and NO production; macrophage colony stimulating factor induced macrophage proliferation; macrophage inflammatory protiein α expression; and immunostimulated IL-12, IL-6, and NO production (71, 76, 145, 156, 173).

Effects of P_1 purinergic receptor agonists on ion channel activity in intestinal and airway epithelia. Many studies have investigated the role of P_1 purinergic receptors in both intestinal and airway epithelium. Mucosal stimulation of canine airway monolayers with Ado results in Cl^- secretion that is sensitive to P_1 receptor blockade (137). Lazarowski and colleagues extended these findings by demonstrating that human airway epithelial monolayers secrete Cl^- in response to Ado and Ado analogues with a rank order of potency supporting A_2 receptor subtype stimulation (91). More recently, Furukawa and coworkers found that Ado and its analogs stimulate Cl^- secretion in primary cultures of gerbil middle ear epithelium (63). In each of these investigations, Ado led to the production of cAMP.

In the intestinal epithelium, migration of white blood cells, including eosinophils and neutrophils, are thought to play a role in transepithelial ion transport characteristic of inflammatory disorders such as infectious diarrhea and allergic colitis. Activated neutrophils release a secretory factor thought to be an ATP metabolite that is capable of activating electrogenic Cl^- secretion across T-84 monolayers (108). Similarly, activated eosinophils also invoke Cl^- secretion across T-84 colonic cell monolayers, and the nature of this response is dependent on the conversion of 5'-Ado monophosphate to Ado by apically localized ecto-5'-nucleotidase activity (139). Later studies concluded that A_{2B} receptors were responsible for activated Cl^- secretion in these experiments, signaling predominantly through cAMP and the cystic fibrosis transmembrane conductance regulator (CFTR) (153).

Evidence for a cAMP-independent pathway involved in A_2 receptor activation of Cl^- secretion has been suggested by Barrett and Bigby (8). Their studies showed that Ado stimulates AA release from T-84 cells and that exogenous AA augmented Cl^- secretion. An inhibitor of cytosolic phospholipase A_2 ($cPLA_2$), an enzyme that releases AA from cell membrane phospholipids, was found to attenuate Ado-stimulated Cl^- secretion. This study provided evidence that additional, cAMP-independent signaling mechanisms such as AA signaling might influence Ado stimulated Cl^- secretion in intestinal epithelia.

Recent studies have also begun to more fully investigate the relationship between P_1 receptors and CFTR. Defective or absent CFTR activity leads to abnormal airway epithelial Cl^- and Na^+ transport in a variety of tissues where it is expressed, which, in turn, is felt to alter the composition of the epithelial surface liquid and/or mucus (148, 170). CFTR is a PKA-regulated Cl^- channel, which also regulates a variety of other channels (including the epithelial sodium channel (ENaC), the outwardly rectified chloride channel (ORCC), the renal potassium channel (ROMK), and possibly an ATP release pathway). Using rabbits exposed to SO_2 as a model for bronchitis, Iwase and coworkers found that injury to the rabbit airway induced the upregulation of CFTR transcription which was associated with the appearance of a new ATP-activated Cl^- current (80). This current was dependent on conversion of ATP to Ado by surface 5'-ectonucleotidase activity. More recently, Ado, in addition to its phosphorylated nucleosides (AMP, ADP, ATP), activated wildtype (WT) CFTR-dependent halide efflux which was mediated by A_{2B} receptors (24). Additionally, clinically relevant, mutant CFTR molecules available at the cell membrane could be activated using A_{2B} receptor stimulation in vitro. Collec-

tively, these studies provide a rationale for investigating A_{2B} and their role in regulating CFTR and ion transport in vivo.

New evidence suggests that Ado receptors might also be involved in signaling transducisomes that reside at the cell surface, anchored through protein-protein interactions. CFTR is thought to be preferentially regulated by membrane-bound PKAII, which is anchored at specific sites to A Kinase anchoring proteins (AKAPs) (25, 67, 72, 111, 113, 116, 117, 151, 167, 168). CFTR has been shown to bind a scaffolding protein, EBP50 (ezrin binding protein), that in turn binds to ezrin, an AKAP (43). The role of ezrin in CFTR regulation is believed to involve linking the protein to the actin cytoskeleton and possibly participating in membrane targeting. In Calu3 cells (an airway epithelial serous cell line that expresses A_2 receptors and CFTR), a synthetic peptide, Ht-31, was used to block the interaction between PKAII and ezrin in an attempt to determine how A_2 receptors regulate CFTR (77). Ht-31 blocked Ado-stimulated activation of CFTR in whole cell voltage-clamp studies, suggesting a role for this AKAP in A_2 receptor coupled activation of CFTR. Additionally, forskolin and ATP were able to activate CFTR when applied to the cytoplasmic surface of excised membrane patches. Together, these elegant studies provide evidence that A_2 receptors and CFTR may be spatially compartmentalized and allow efficient regulation of CFTR activity by Ado.

P_1 purinergic receptors and the role of Ado in the CNS: Studies from an A_{2A} receptor knockout. Accumulating evidence points toward Ado as an important homeostatic modulator and, in general, a neuroprotector of the central nervous system (CNS). With the exception of A_{2A} receptors which are expressed primarily in the striatum, olfactory

tubercle, and nucleus accumbens, P_1 receptors are widely expressed in the brain based on studies involving ligand binding, in situ hybridization, and RT-PCR (42). Manifestations of P_1 purinergic receptor activity in the CNS include regulation of behavior, sleep and arousal, locomotor activities, pathologic conditions such as CNS ischemia, and possibly influencing neurodegenerative diseases, including Parkinson disease. Throughout the CNS, Ado is felt to frequently "fine tune" the primary components of synaptic transmission, and this modulation is accomplished through P_1 purinergic receptors. For a more complete discussion of Ado and P_1 receptor activity in the CNS, the reader is directed toward several excellent recent reviews (34, 46, 85, 115, 142, 154, 155).

Similar to the heart, Ado protects against ischemia-induced neuronal injury in the CNS. Ado influences pre- and postsynaptic neurotransmission by modulating the release and actions of specific neurotransmitters and neuropeptide modulators, including vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (141, 149). Recently, a role for Ado receptors as regulators of other receptors in the nervous system such as *N*-methyl-D-aspartate receptors, metabotropic glutamate receptors, and nicotinic autofacilitary receptors, has been identified. A_1 receptor stimulation also protects against methamphetamine-induced neurotoxicity to nigrostriatal dopaminergic neurons in animal models (38). Ado activation of A_1 receptors has been suggested to play a role in glutamate-mediated neuronal injury following ischemia. During ischemic stress, Ado is released and has been shown to be associated with attenuated glutamate neurotoxicity and delayed onset of paraplegia in a New England white rabbit paraplegic model (121). Although the nature of this effect is unclear, it appears that Ado may act through A_1 receptors to reduce

the effect of excitatory amino acids (like glutamate) by altering *N*-methyl-D-aspartate receptors receptors.

Recent studies completed in A_{2A} receptor knockout mice have provided insight into the role of this receptor in the brain in vivo. Caffeine, a well established A_{2A} receptor antagonist, is known to produce both exploratory behavior as well as alertness, both of which were found to be depressed in A_{2A} receptor knockout mice (93). Caffeine also has been shown to enhance anxiety levels and act as an analgesic. Consistent with these observations, A_{2A} receptor knockouts were found be more aggressive and anxious and have notably reduced pain responses compared to WT mice. Finally, knockout mice had increased blood pressure and heart rate as well as more efficient platelet aggregation compared to WT mice. These studies suggest that the effects of Ado on vasodilation and inhibition of platelet aggregation are likely to be A_{2A} receptor-specific.

A_{2A} receptor knockout mice have also provided evidence for involvement of these receptors in the pathogenesis of Parkinson's disease, a neurodegenerative disease that results from depletion of dopaminergic neurons in the nigrostriatal pathway of the brain. Clinical manifestations of Parkinson's disease are thought to result from alterations in the dopaminergic pathways within the basal ganglia and the GABA-releasing spiny neurons of the striatum. Ado activation of A_{2A} receptors is thought to antagonize dopaminergic signaling by decreasing the binding affinity of dopamine to D_2 dopamine receptors (55, 56). Since Parkinson's disease is characterized by depletion of dopamine, A_{2A} receptor antagonists could theoretically be therapeutic targets for developing drugs to treat patients with Parkinson's disease. In a D_2 receptor transgenic knockout mouse model of Parkinson's disease (6), an A_{2A} receptor antagonist was found to rescue motor deficits

observed in the knockouts in a dopamine-independent manner. In a separate study, caffeine was found to have less locomotor effects in D_2 receptor knockout mice (versus WT mice), suggesting a functional coupling between D_2 receptors and A_{2A} receptors (175). P_1 purinergic receptor signaling might also be relevant to additional neurodegenerative disorders such as Alzheimer's disease and Huntington's disease (69, 150).

CONCLUDING REMARKS

Ado, by interacting with P_1 purinergic receptor subtypes, acts as a potent regulatory autocrine that "fine tunes" many physiological processes in different tissues and organ systems. Each receptor subtype is wired into a distinct and complex network of signaling pathways that allows Ado to have both stimulatory and inhibitory effects necessary for proper homeostatic control. Alterations in this regulatory relationship may be associated with many different pathological processes, including progressive neurological disorders, inflammatory disorders, defective epithelial ion transport, and cardiovascular disorders that involve arrhythmias and ischemia. A better understanding of the cell biology of P_1 purinergic receptors and signaling may provide novel therapeutic targets for these and other pathologic conditions.

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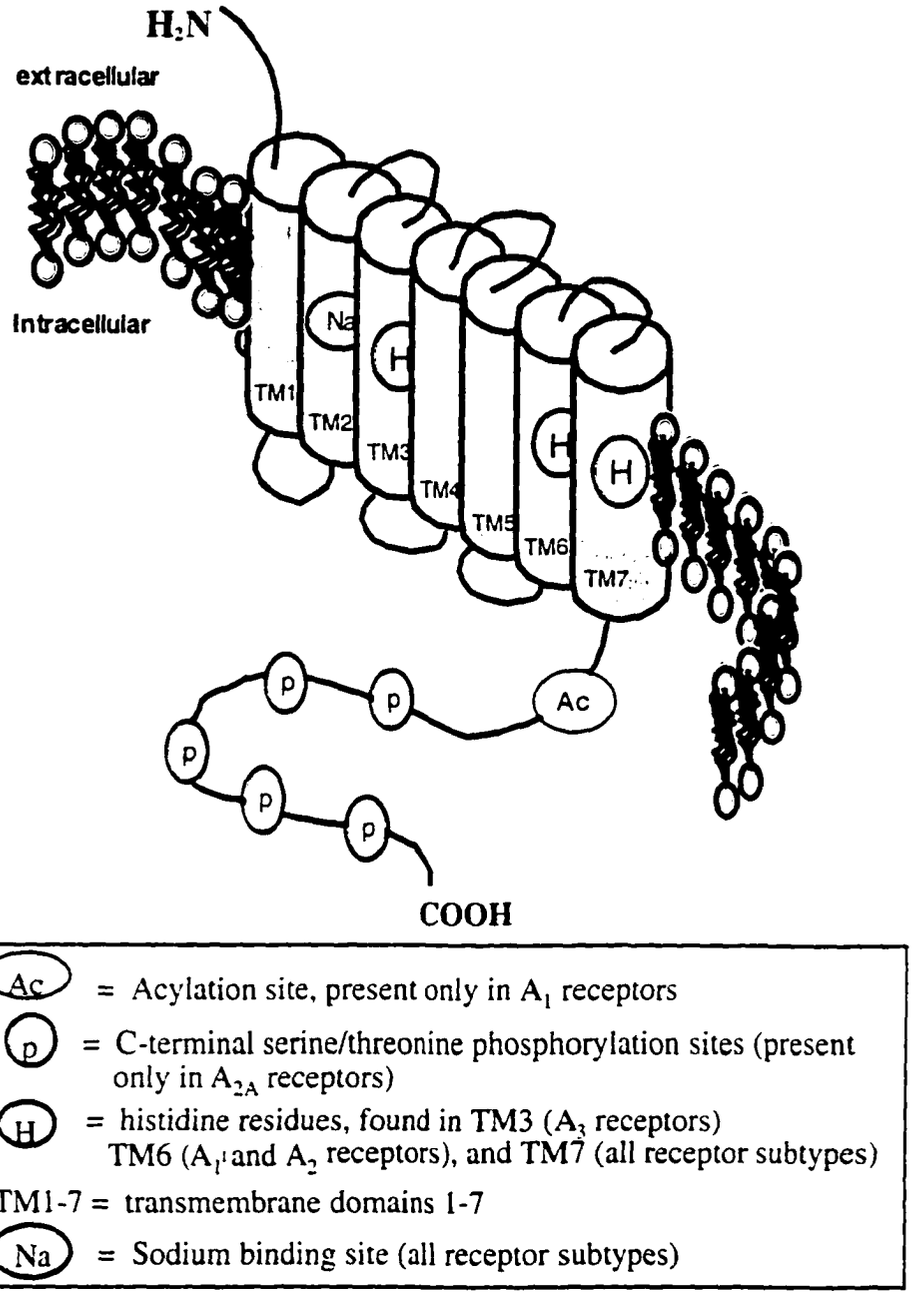

Fig. 1: Structural comparisons between P_1 purinergic receptors. All P_1 purinergic receptors have seven transmembrane domains, characteristic of G-protein coupled receptors. Consistent among subtypes are three extracellular loops and three intracellular loops that vary in size. The main differences between receptor subtypes include phosphorylation sites, fatty acylation sites, histidine residues (that may be involved in ligand binding or desensitization), and sodium binding sites.

CONCLUSIONS

IN SUMMARY

A_2 Ado receptor coupled activation of CFTR involves $cPLA_2$ signaling. Accumulating evidence suggests that Ado, through A_2 Ado receptors, is a potent mechanism to activate CFTR-dependent Cl^- secretion (7, 40, 71, 116). Activation has been shown in the setting of low cAMP production in human airway cell monolayers, and this may be in part due to compartmentalized regulation (49). A_{2B} Ado receptors couple to PKA as well as to PLC, which is important for the direct regulation of Ca^{2+} channels (72, 73). For these reasons, this study was designed to investigate additional, cAMP-independent pathways that might be involved in Ado activation of CFTR.

It has been demonstrated that, in colonic epithelial cell monolayers (T84 cells), Ado stimulates AA release, and inhibition of AA release attenuates Ado-stimulated Cl^- secretion (7). However, it is unclear whether the effects in T84 cell monolayers reflect intestinal epithelial responses to endogenously released Ado in vivo. The nature of this effect, whether it is exclusive to this cell line, and the relevance of AA signaling pathways to CFTR activity in the human airways have not been investigated.

The first report in this dissertation addressed the relationship between Ado stimulation of CFTR and AA signaling in human airways cells. In this report, it was demonstrated that in, addition to adenylate cyclase, cAMP, and PKA signaling, A_{2B} receptor activation of CFTR also included $cPLA_2$ activation. Calu-3 cells were shown to

express $cPLA_2$ and release AA in response to Ado stimulation (predominantly from theapical surface of polarized monolayers). Ado activation of CFTR was also sensitive to $cPLA_2$ inhibition in both Calu-3 cells and Cos-7 cells transiently expressing CFTR. In contrast, $cPLA_2$ blockade did not affect β_2 adrenergic receptor stimulation of CFTR-dependent halide efflux in Calu-3 cells, suggesting that AA signaling was unique to the A_2 receptor pathway. The effects of AA or $cPLA_2$ inhibition appeared to be independent from changes in total cell cAMP production, and AA combined with Ado did not augment cAMP levels over that produced by Ado alone.

An important goal of these studies was to determine the role of AA and $cPLA_2$ in Cl^- secretion in vivo, by investigating the effects of $cPLA_2$ inhibition on agonist stimulated Cl^- secretion across the murine nasal mucosa. Ado-stimulated Cl^- secretion in *cftr*$^{-/-}$ mice was sensitive to both A_2 receptor and $cPLA_2$ blockade, suggesting that A_2 receptor and PLA_2 signaling contribute to transepithelial ion transport. Consistent with the in vitro data, $cPLA_2$ receptor blockade did not affect β_2 adrenergic receptor activated of Cl^- secretion with albuterol. These studies were extended to mutant CFTR molecules, demonstrating that Ado alone activated halide efflux in Cos-7 cells transiently expressing ΔF508 CFTR (under surface localizing conditions). Ado also activated the severe, surface localizing mutant G551D CFTR. This activation required the combined effects of Ado, AA, and PDE inhibition. From these results, it can be concluded that A_2 receptors stimulate both adenylate cyclase and $cPLA_2$ activity and that each pathway is required to achieve maximal CFTR activation.

Utilization of the A_2 receptor signaling pathway may represent a useful strategy to improve the activity of mutant CFTR molecules in humans with CF. This study, com-

bined with a previous report, demonstrates that A_2 receptors can activate class II, III, and IV CFTR mutations (25). These data suggest that mutant CFTR molecules, when present at the cell membrane, should be responsive to native Ado-based signaling. This receptor signaling pathway might also complement other approaches that increase mutant CFTR activity (i.e., suppression of premature stop mutations using aminoglycosides, treatment with compounds to improve CFTR trafficking, treatment with PDEis, and treatment with phosphatase inhibitors). Finally, these studies suggest that Ado stimulates Cl^- secretion in mice more predictably than the β_2 adrenergic receptor agonist isoproterenol and may improve the sensitivity of the nasal potential difference (PD) protocol to measure low level CFTR function. These results, therefore, provide preclinical support for human studies testing the efficacy of Ado to activate CFTR and Cl^- secretion in vivo.

Clinical PDE inhibitors activate Cl^- secretion. PDEs degrade cAMP, and PDEis raise intracellular cAMP concentrations. Recent studies have investigated the use of PDEis to activate Cl^- secretion in human airway cells and across the nasal mucosa in mice and humans. Preliminary studies suggest that milrinone (PDE3-specific PDEi), two non-specific xanthine derivatives that inhibit PDEs (IBMX and X-33), and rolipram (PDE4-specific) might be useful in augmenting the activity of surface-localized, mutant CFTR molecules. These studies were performed using either the nasal PD protocol in CF humans or mice, $^{36}Cl^-$ efflux assays, or the whole cell patch clamp technique in nonpolarized airway cells. Of these studies, milrinone was the only PDEi identified that was able to augment Cl^- secretion in vivo. Combined with direct activation of adenylate cyclase with forskolin (but not alone), milrinone has been shown to activate Cl^- secretion across

the murine nasal mucosa of *cftr*$^{\Delta F508/\Delta F508}$ transgenic mice (62). However, the concentrations of milrinone used in these studies were too high to be used clinically, while cytotoxic effects of forskolin preclude its clinical use. Studies performed using the nasal PD protocol in mice and humans that have ΔF508/G551D *cftr* or ΔF508/ΔF508 *cftr* genotypes demonstrate that, even in the presence of a β_2 adrenergic agonist (isoproterenol), milrinone failed to activate Cl^- secretion in those subjects (117). However, results reported in the first manuscript of this dissertation indicate that G551D CFTR, transiently expressed in Cos-7 cells, can be activated by a combination AA and Ado that requires PDE inhibition for activation.

Despite these studies suggesting that PDEis may have the capacity to augment mutant CFTR activity, few PDEis have been investigated in this regard. As part of this dissertation, a panel of isotype-specific and nonspecific PDEis were evaluated in terms of their importance in regulating CFTR activity, total cell cAMP production, the ability to augment both Ado-activated Cl^- secretion across polarized epithelial Calu-3 cells, and halide efflux in R117H CFTR expressing CF airway cells.

To date, no published studies have evaluated the efficacy of PDEis as Cl^- secretagogues in the context of polarizing airway epithelial cell monolayers. Using Calu-3 cells, PDEi-stimulated I_{sc} and cAMP production were compared to that produced by forskolin, a direct activator of adenylate cyclase. Milrinone and cilostazol (PDE3-specific), papaverine (nonspecific PDEi), rolipram (PDE4-specific), and sildenafil (PDE5-specific) stimulated I_{sc} from 20-80% of that produced by forskolin, with a potency series at the lowest concentration (2 μg/mL) of papaverine > cilostazol > rolipram ≥ milrinone ≥ sildenafil. All PDEi-activated currents were inhibited (~90%) by glybencla-

mide (200 μM, mucosal) except for those produced by milrinone (~50% inhibition). PDEi-stimulated Cl^- secretion did not correlate well with cAMP production compared to forskolin, with levels generally ≤10% of that produced by forskolin (20 μM).

PDEis were then combined with Ado stimulation to determine whether the stimulated I_{sc} was additive. Papaverine, cilostazol, and to a lesser extent rolipram augmented both the magnitude of Ado (1 μM) stimulated I_{sc} and the duration following very low dose stimulation with Ado (0.1 μM, $P < 0.01$). These studies are the first to demonstrate functional evidence that many PDEis can activate CFTR-dependent I_{sc} in Calu-3 cell monolayers in the setting of low cAMP concentrations and suggest that low concentrations of PDEis are capable of augmenting CFTR activity that is regulated by native signaling pathways.

As part of this study, the abililty of PDEis to activate mutant CFTR molecules was also evaluated. R117H CFTR, characterized by defective ion channel conductance, was selected because it localizes to the cell surface and is partially functional. R117H CFTR is thought to be clinically significant depending on the genetic background. For example, if R117H CFTR is inherited along with a truncated thymidine tract at the end of intron 8 of *cftr*, clinical manifestations are likely to arise. A 5T allele (5 thymidine tract) leads to ineffective splicing and the loss of exon 9 in the mRNA transcript, resulting in protein maturation abnormalities. An R117H CFTR allele with the 5T truncation in the cis configuration along with a severe mutation on the other allele results in CF and the CBAVD. If the 5T allele is instead a 7T allele, the individual may have a milder (if any) CF phenotype with CABVD. Finally, a 9T allele results in a normal, carrier phenotype.

To determine if PDEis were capable of activating R117H CFTR, a pTM-1 plasmid vector containing R117H CFTR was transiently expressed in IB-3-1 cells, a CF bronchial airway epithelial cell line from a patient with a ΔF508/W1282X CFTR genotype. Papaverine, cilostazol, and to a lesser extent sildenafil activated halide efflux to levels comparable to activation with forskolin and Ado.

Cumulatively, these studies point toward PDE3 as a specific regulator of CFTR through degradation of cAMP in the region where CFTR localizes. Cilostazol, an orally available PDE3-specific inhibitor, activated CFTR at low concentrations that are near the peak serum concentration of the drug used to treat claudication in humans. Papaverine, a nonspecific PDEi, was also a very effective agonist when used alone or in combination with Ado. These findings suggest that additional PDEs may play an important regulatory role relevant to CFTR. Since multiple isotype specific PDEs have been shown to target to intracellular locations and interact with cell membranes, specific isotypes might localize close to CFTR and regulate endogenous Cl^- transport pathways (Fig. 1).

A_2 receptor subtype functional characterization. Stimulation of both A_{2A} and A_{2B} receptor subtypes couple to G_s and produce cAMP. A_{2A} receptors are a high affinity subtype and bind to Ado at low (nM) concentrations, while A_{2B} receptors are a low affinity subtype and bind to Ado at higher (μM) concentrations. To determine which Ado receptor subtype stimulates cAMP production and activates CFTR in Calu-3 cells, we measured cAMP levels in the presence and absence of alloxazine (50 uM), a specific inhibitor of A_{2B} receptors (Fig. 2). Alloxazine (50 μM) blocked Ado (50 μM) stimulated cAMP production by 86% in Calu-3 cells (Fig. 2*A*). To compare these results to another

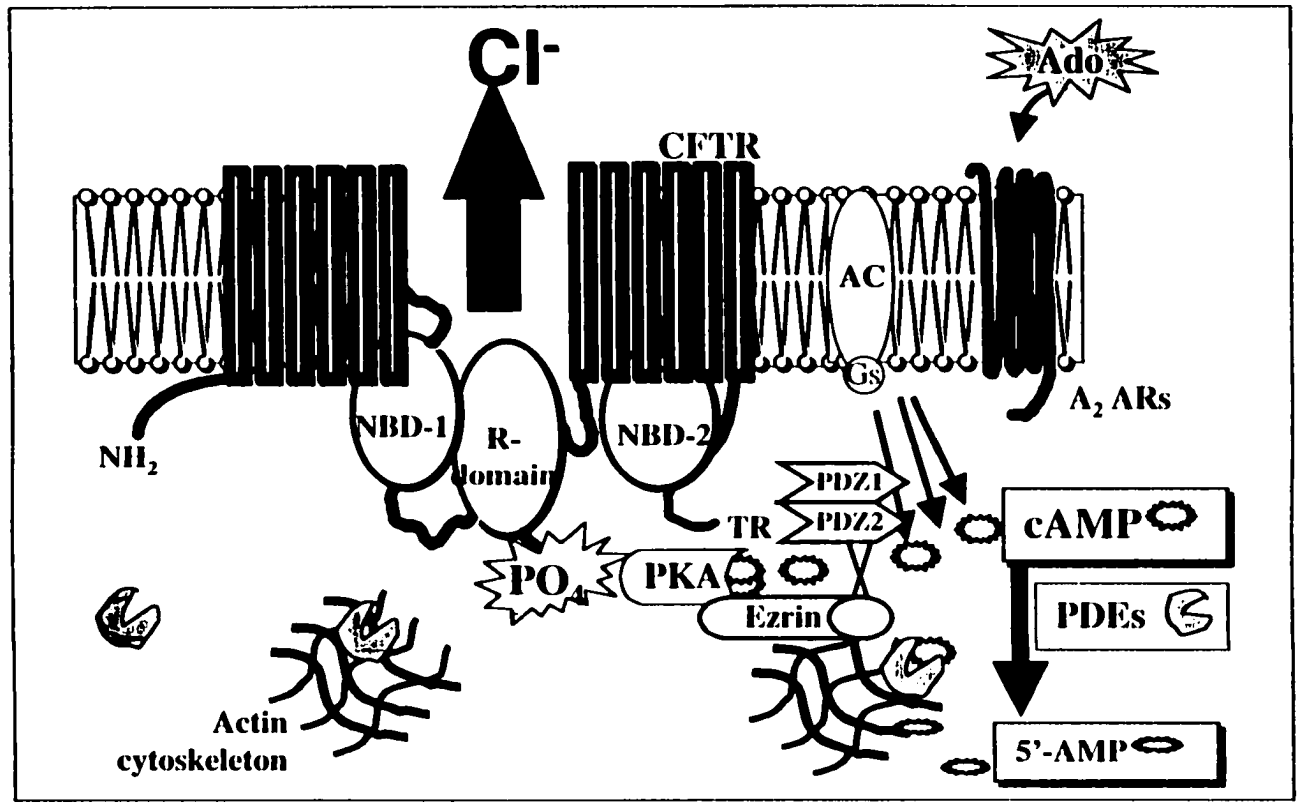

Fig. 1. Proposed model governing efficient coupling of A_2 receptors to CFTR activation through phosphodiesterases (PDEs), scaffolding proteins, and localized cAMP gradients. Ado stimulation of A_2 receptors leads to cAMP production, a process that is regulated by PDE-dependent cAMP cleavage into 5'AMP. PDEs have PDZ sequence on the carboxy terminal, allowing them to interact with proteins that have PDZ binding domains (see text). However, the specific role PDEs play in compartmentalization is unclear. CFTR also has a PDZ sequence (TRL) at the carboxy terminal and can interact with the PDZ binding domain on the Ezrin Binding Protein (EBP50). EBP50, in turn, binds to ezrin, an A-kinase anchoring protein (AKAP) that is tethered to the actin cytoskeleton. Isotype specific PDEs might localize preferentially to this region and serve to negatively regulate local pools of cAMP germaine to CFTR activation.

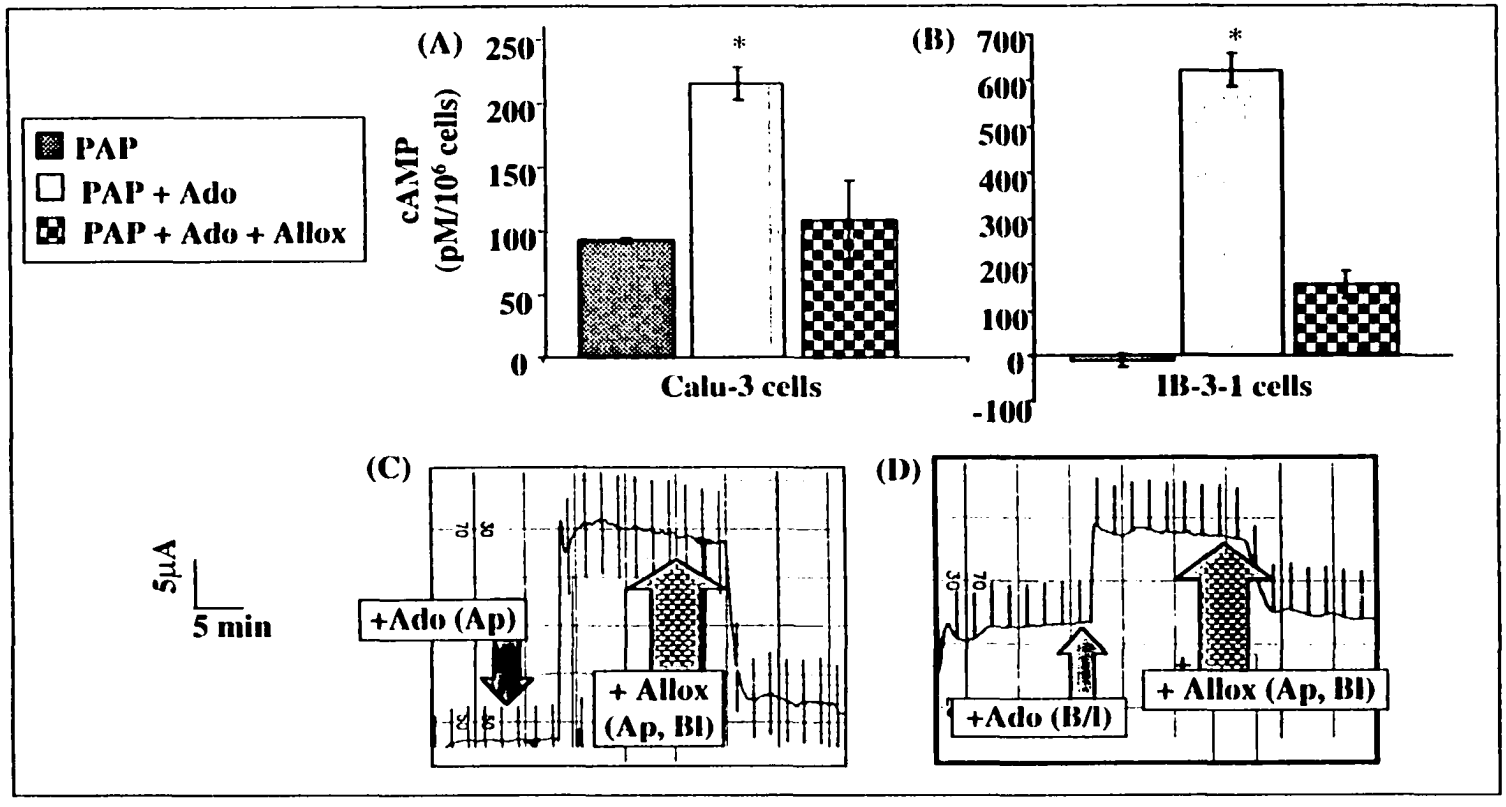

Fig. 2. Alloxazine (Allox), an A_{2B} Ado receptor antagonist, blocks Ado-stimulated cAMP production and I_{sc} response. cAMP levels were measured by ELISA as described (see METHODS in first manuscript). Cells were stimulated for 10 min before extraction with 100 μM papaverine (PAP), 50 μM Ado, and 50 μM alloxazine, (n = 6) 35 mm plates/condition. PAP was included in all conditions to enhance cAMP detection. Values are means ± SE for each condition (*P < 0.05) for *A*: Calu-3 cells and *B*: IB-3-1 cells. Allox blocked Ado-stimulated total cell cAMP production by 86% (Calu-3 cells) and 73% (IB-3-1 cells). For I_{sc} experiments (Fig. 4*C* and *D*) Calu-3 cell monolayers were grown at air-liquid interface, mounted in Ussing chambers and initially bathed with a Lactated Ringer solution on both membranes. The mucosal bath was switched to a low chloride concentration buffer + amiloride (100 μM) and then stimulated both on the mucosal (Fig. 4*C*) or serosal surface (Fig. 4*D*) with 25 μM Ado. Allox (25 μM) was added after 1,000 s on both sides (n = 6 wells/condition). Allox blocked Ado-stimulated I_{sc} on both surfaces by 77-87% (P < 0.01).

airway cell line, we found that alloxazine blocked Ado-stimulated cAMP production in IB-3-1 cells as well, by 73% (Fig. 2*B*, $P < 0.05$ for both cell types). To determine whether alloxazine could functionally interrupt Ado activation of Cl^- secretion, alloxazine was added to Calu-3 cell monolayers prestimulated with Ado (Fig. 2, *C* and *D*). Alloxazine (25 μM) was found to block Ado-stimulated I_{sc} by 77-87% ($P < 0.01$) in Calu-3 cells when stimulated with Ado on the mucosal (Fig. 2*C*) or serosal surface (Fig. 2*D*). From these results, we conclude that A_{2B} receptors appear to be the primary subtype responsible for Ado-stimulation of CFTR.

Ado-stimulated Cl^- secretion was shown in the first manuscript of this dissertation to be sensitive to $cPLA_2$ inhibition both in vitro and in vivo. Since $cPLA_2$ requires Ca^{2+} to translocate to cell membranes and releases AA, additional experiments were performed that investigated the role of Ca^{2+} in the Ado-stimulated signal transduction pathway. First, Calu-3 cells grown on impermeable supports were stimulated with Ado and intracellular Ca^{2+} levels were measured using Fura 2, a ratiometric fluorescent dye (Fig. 3*A*). Ado (100 μM) increased intracellular Ca^{2+} levels 238% above unstimulated conditions ($P < 0.001$). To determine if stimulation of β_2 adrenergic receptors also involved increases in intracellular Ca^{2+} concentrations, Calu-3 cells were stimulated with albuterol (100 μM, Fig. 3*B*). Albuterol had no effect on raising intracellular Ca^{2+} concentrations. Next, studies were conducted to determine if Ado-stimulated Ca^{2+} production contributed to activation of Cl^- secretion in Calu-3 cell monolayers. Pretreatment with (BAPTA-AM) (50 μM for 45 min), a Ca^{2+} chelator, blocked Ado-stimulated I_{sc} by 55% (25 μM mucosal, $P < 0.05$, Fig. 4*A*). In contrast, β_2 adrenergic receptor-stimulated I_{sc} with albuterol (2.5 μM) was not effected by BAPTA-AM pretreatment (Fig. 4*B*).

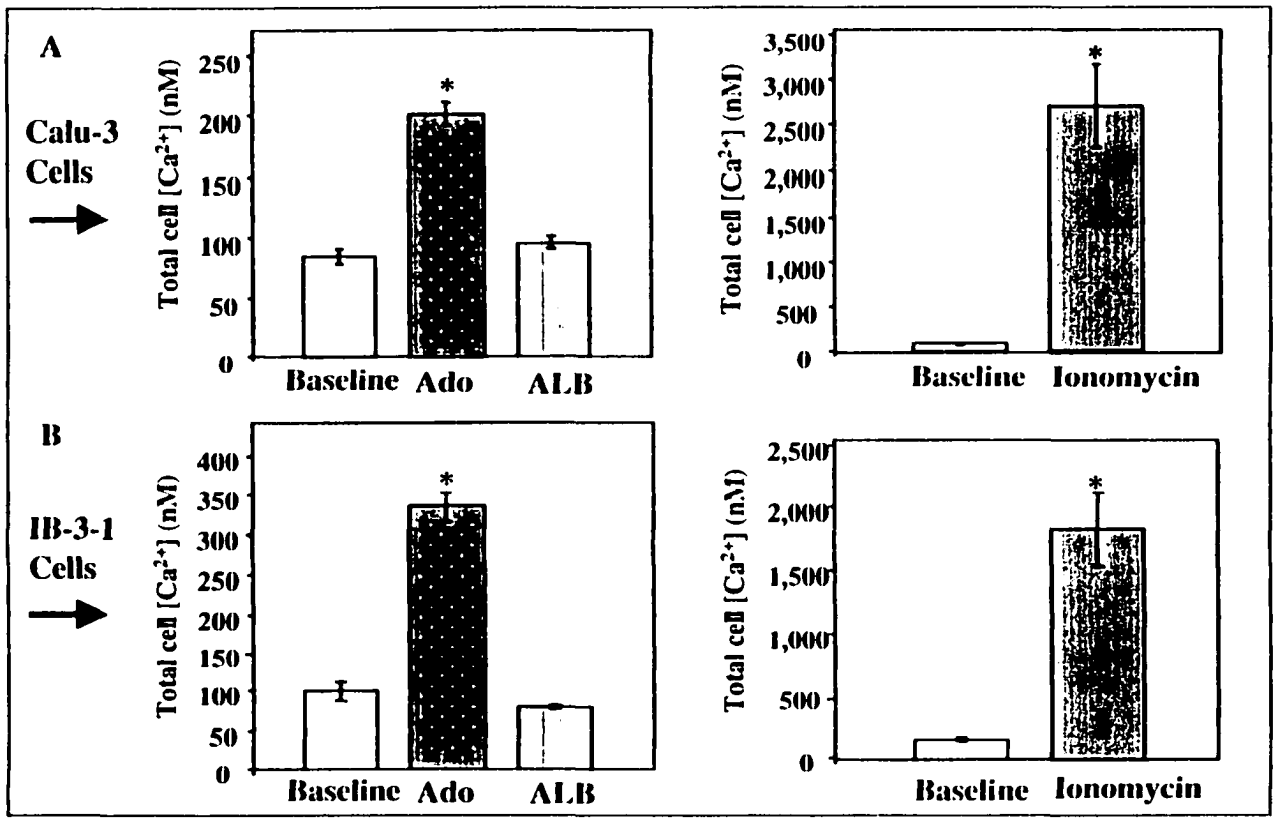

Fig. 3. Ado raises total cell [Ca^{2+}] in Fura-2 loaded Calu-3 (*A*) and IB-3-1 (*B*) cells. Cells were grown in 24-well plates loaded with 2 μM Fura-2-AM for 30 min and unloaded for an additional 30 min in regular media, and ratiometric analysis was performed using a fluorescent microplate reader. Ca^{2+} concentrations were determined approximately 3-5 min after stimulation using a standard equation and the known K_d for the dye. Concentrations were 100 μM for Ado and albuterol, and 2 μM for ALB [Baseline was in the absence of stimulation, and ionomycin (2 μM) was used as a positive control]. Values are mean ± SE for each condition, $^*P < 0.01$ (n = 18 wells/condition). Ado raised total cell Ca^{2+} concentrations to 238% above unstimulated conditions, while ALB had no effect on Ca^{2+} at this time point.

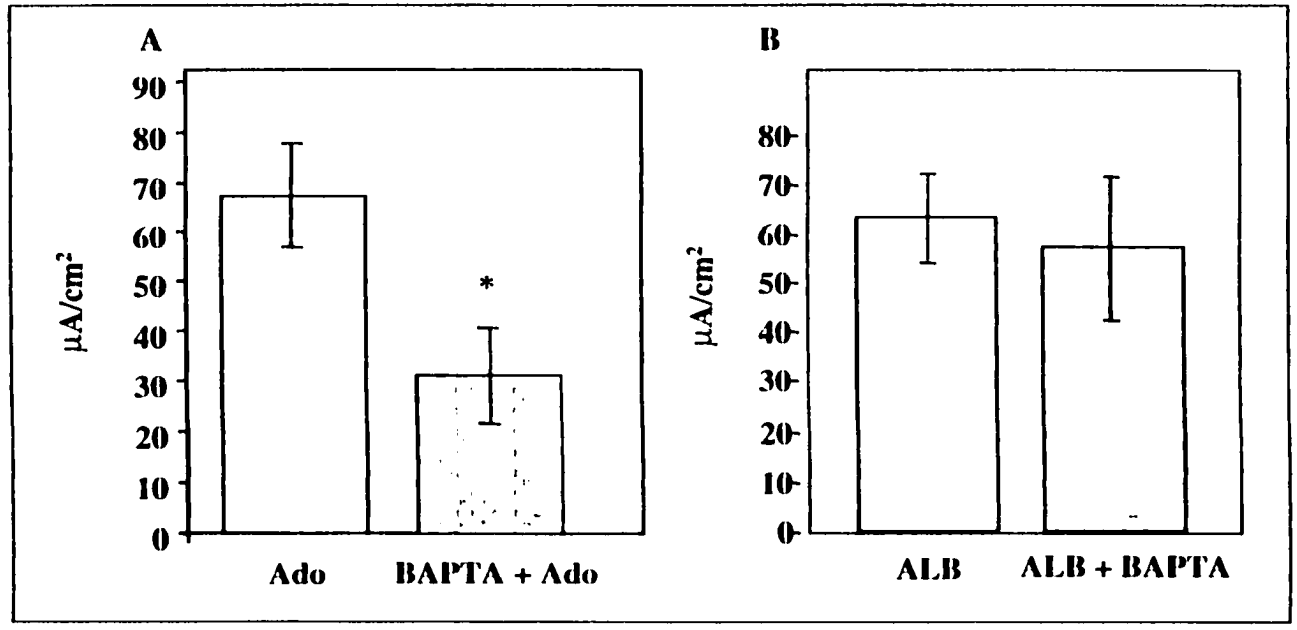

Fig. 4. Pretreatment with BAPTA reduces Ado-stimulated (*A*) but not β_2 adrenergic receptor-stimulated (*B*) I_{sc} in Calu-3 cell monolayers grown at air-liquid interface. Cells were preloaded with and without 2 μM BAPTA-AM for 45 min and then mounted in Ussing chambers, initially bathed with a Lactated Ringer solution on both membranes. The mucosal bath was switched to a low chloride concentration buffer + amiloride (100 μM) and then stimulated both on the mucosal and serosal surfaces with 25 μM Ado (*A*) or 2.5 μM ALB (*B*). Glybenclamide(200 μM) was added to the mucosal surface at the end of each experiment. Values represent mean I_{sc} measurements ± SE for each condition, $^*P = 0.043$ ($n = 6$ wells/condition). Treatment with BAPTA inhibited Ado-stimulated I_{sc} by 55%, while treatment with BAPTA had no effect on ALB-stimulated I_{sc}.

Concluding remarks. The results from both manuscripts address the specific aims described in this dissertation, which entail characterizing A_2 receptor coupled activation of CFTR in living cells, the mechanisms by which these receptors signal, and the ability of A_2 receptor agonists combined with other signaling pathways to activate wt and mutant CFTR. Based on these studies, it can be concluded that A_2 receptors activate CFTR through signaling pathways that include cAMP, AA, and Ca^{2+}. The nature in which Ca^{2+}, $cPLA_2$ signaling, AA release, and cAMP contribute to Ado-stimulated Cl^- secretion is unclear; however, possible mechanisms are outlined in Fig. 5. Although β_2 adrenergic receptors also stimulate cAMP production and activate CFTR, the results in this dissertation distinguish the signaling pathway utilized by this receptor, which was shown to be Ca^{2+}-independent and insensitive to $cPLA_2$ inhibition, from signaling pathways used by A_2 receptors. It is likely that the predominant receptor subtype responsible for coupling to CFTR is the A_{2B} subtype in Calu-3 cells in that a subtype-specific antagonist (alloxazine) blocks Ado-stimulated I_{sc} and cAMP production. These studies also indicate that efficient receptor coupling might be the result of local cAMP production that is regulated by specific PDE isotypes expressed in this region. This is supported by data in this report that Ado-stimulated Cl^- secretion is in the setting of low cAMP production compared to forskolin-stimulated I_{sc}. Forskolin produced significantly larger amounts of total cell cAMP production compared to Ado at the lowest concentrations that each agonist activates CFTR (0.1 μM and 10 μM, respectively). This observation, coupled to the differences in stimulated I_{sc} by various isotype-specific PDEis, suggests that Ado stimulation of cAMP efficiently activated PKA and CFTR, with specific PDE isotypes controlling the duration and magnitude of the stimulus. Ado, through A_2 receptors, also

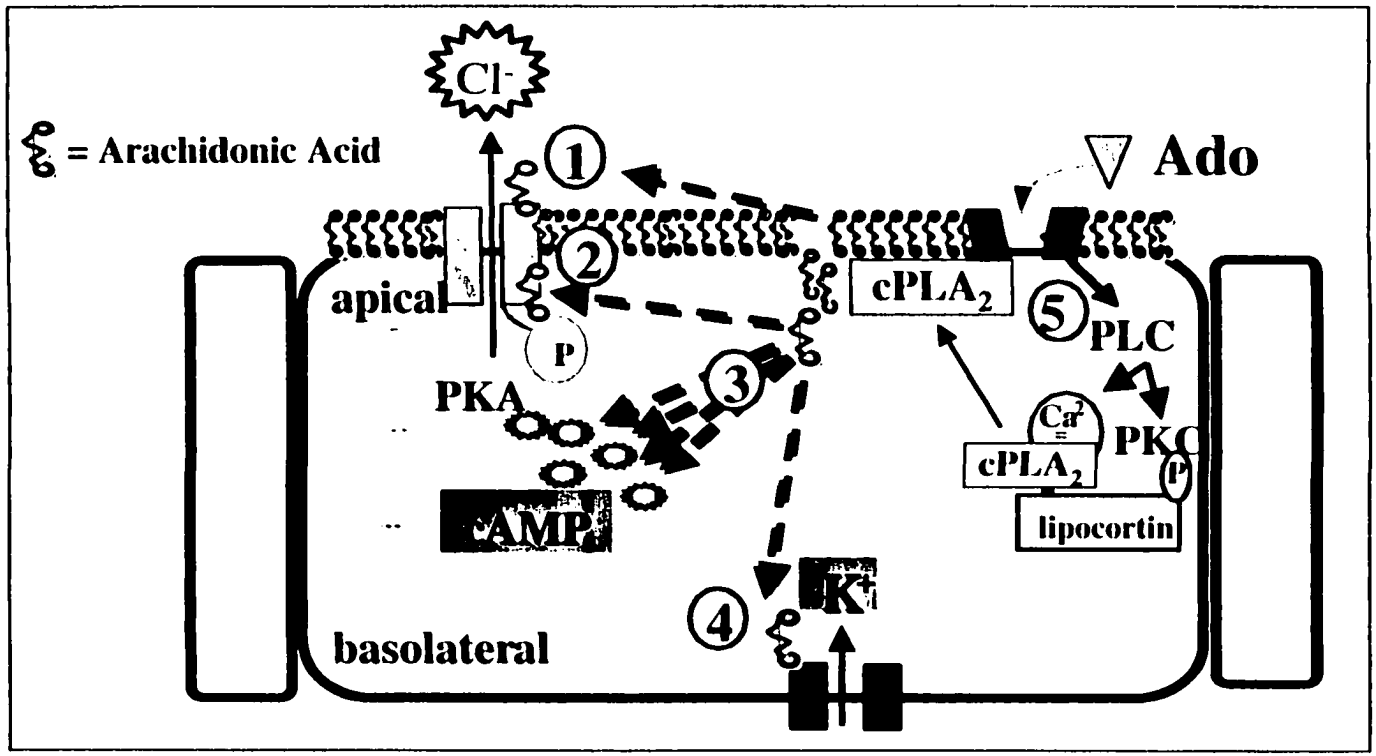

Fig. 5. Proposed model linking A_2 Ado receptors and $cPLA_2$ activity to Cl^- secretion. Ado stimulates Cl^- secretion in Calu-3 cell monolayers (depicted in this diagram) as well as AA release and inhibiting $cPLA_2$ blocks this response. Ado stimulation may (1) release AA into the extracellular milieu or intracellularly or (2) have direct effects on channel activity through a physical interaction with CFTR. AA or one of its metabolites might also contribute to either the local pool of cAMP that activates CFTR (3) or interact directly with a basolateral K^+ channel (4) that hyperpolarizes the membrane and stimulates transepithelial Cl^- secretion. Finally, A_2 Ado receptors might couple to the phospholipase C (PLC) effector coupling system (5) that involves both increasing intracellular Ca^{2+} concentrations and activation of PKC. Lipocortin is a negative regulatory protein that binds and inhibits $cPLA_2$ activity. PKC-dependent phosphorylation of lipocortin, shown to disrupt binding to $cPLA_2$, coupled with increases in intracellular Ca^{2+} concentrations that might recruit $cPLA_2$ to cell membranes might explain the mechanisms by which A_2 Ado receptors activate CFTR. Alternatively, the PKA/cAMP signaling pathway linked to A_2 receptors may be entirely distinct from the $cPLA_2$ pathway.

proved to be a more predictable and potent pathway to activate Cl^- secretion in vivo compared with β_2 adrenergic receptor stimulation with isoproterenol. In light of these observations, Ado may indeed represent a more effective agonist to stimulate CFTR-dependent Cl^- secretion in diagnostic nasal PD assays.

The most important objective underlying these studies was to test strategies that involve manipulation of cell signaling pathways that enhance the activity of partial function CFTR mutants. In this dissertation, class II, III, and IV mutations were targeted for this purpose. Ado was found to activate ΔF508 CFTR (class II mutant, under surface localizing conditions) expressed in Cos-7 cells. Multiple PDEis alone were capable of activating R117H CFTR (class IV) expressed in a bronchial epithelial cell line. Finally, the severe mutant G551D CFTR (class III) expressed in Cos-7 cells was activated by a combination of PDE inhibition, AA, and Ado stimulation. The signaling molecules, agonists, and/or inhibitors used in these studies to augment mutant, surface localizing CFTR molecules lend credence to investigating the efficacy of these agents in human studies.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

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