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Purinergic receptors in airway epithelia

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Abstract

Nucleotides and nucleosides within the airway surface liquid regulate mucociliary clearance (MCC) activities, the primary innate defense mechanism that removes foreign particles and pathogens from airway surfaces. Nucleotide and nucleoside actions in the airways are mediated mainly by two purinergic receptor subtypes, the G_q-coupled ATP/UTP-sensing $P2Y_2$ receptor and the G_s-coupled A_{2b} adenosine receptor. Activation of the A_{2b} receptor results in cyclic AMP-dependent activation of the cystic fibrosis transmebrane regulator (CFTR) Cl^- channel and stimulation of ciliary beat frequency. Agonist occupation of the $P2Y_2$ receptor promotes inhibition of Na^+ absorption as well as CFTR-dependent and CFTR-independent Cl^-secretion, ciliary beating, and mucin secretion.

Introduction

Almost two decades have elapsed since the initial observation that extracellular nucleotides promote Cl^- ion secretion from airway epithelial cells [1]. Extensive research has subsequently verified that a subset of purinergic receptors accounts for the physiological actions of ATP as well as other nucleotides and nucleosides within the airway surface liquid (ASL).

Extracellular nucleotide actions are mediated by two classes of broadly distributed cell surface P_2-purinergic receptors: the ligand-gated ion channel P2X receptors comprise seven species activated by ATP; and, the P2Y family of G protein-coupled receptors composed of eight species, activated by adenine and uridine nucleotides and nucleotide-sugars [2]. In addition, adenosine, the final product of ATP hydrolysis, activates a separate family of G protein-coupled receptors, the A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors [2]. The agonist selectivity and signaling properties of purinergic receptors are summarized in Table 1.

The Gq/phospholipase C-coupled $P2Y_2$ and $P2Y_6$ receptors and the Gs/adenylyl cyclase-coupled A_{2b} receptor are expressed on the apical surface of human airway epithelial cells [1; 3-7] as well as P2X receptors [8], suggesting that adenosine and adenine and uridine nucleotides are endogenous modulators of airway functions. Indeed, ATP, UTP, UDP, and adenosine are present in physiologically relevant concentrations in ASL both *in vivo* and *in vitro* [9-11]. Functional and biochemical evidence indicate that release of nucleotides into ASL represents a major mechanism of autocrine/paracrine signaling to regulate MCC activities [7;10;12-14].

This review discusses recent advances in the understanding of how purinergic receptors modulate MCC activities.

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ATP release provides a mechanism for MCC regulation

The MCC system ‘consists of three major components, all of which are regulated by extracellular nucleosides and nucleotides [9;14-16]: (i) ion transport elements in the epithelium, which produce an aqueous environment on the airway surface (i.e., ASL production); (ii) mucins, secreted by goblet cells or from submucosal glands, which mature into mucus, and (iii) cilia, which propel the mucus toward the mouth. Component failures may lead to airway inflammatory diseases. For example cystic fibrosis (CF) results from a failure in epithelial Cl^- and fluid secretion, primary ciliary dyskinesia results from structural failures in the ciliary axoneme, which negatively affect ciliary activity, and chronic bronchitis and asthma result, in part, from mucin hypersecretion [17-20].

The recognition that airway epithelial cells release ATP constitutively [10;11] suggests a mechanism for the control of basal MCC activities. *In vitro* studies demonstrated that resting airway epithelia release ATP at a rate of 300-500 fmol/min cm^2 [9;21]. Due to the action of ecto-ATPases, steady-state ATP concentrations on resting cells are in the 5-20 nM range, well below the EC_{50} value for $P2Y_2$ receptor stimulation [9;10;21]. However, ATP metabolism provides a source of adenosine, which reaches steady state concentrations capable of promoting A_{2b} receptor stimulation [9;22]. Cyclic AMP measurements in the presence or absence of adenosine deaminase verified that the A_{2b} receptor on resting airway epithelial cells is tonically stimulated by endogenous adenosine [9]. In addition to constitutive release, enhanced ATP release from airway epithelial cells is associated with mechanical stress that mimics physiological stimulus, e.g., shear stress provided by tidal breathing. Therefore, *in vivo* ASL ATP may reach concentrations capable of promoting $P2Y_2$ receptor activation (reviewed in [13;23]). Indeed, functional data demonstrated that ATP mediates acute MCC responses via $P2Y_2$ receptor stimulation [15;16]. In sum, adenosine and ATP are physiological relevant stimuli that impart cyclic AMP-regulated and phospholipase C-dependent MCC activities, respectively, to the airways.

Lung epithelia exhibit a complex cellular composition, and thus, several mechanisms and pathways likely are involved in the release of nucleotides into the airways. Circumstantial evidence supports the involvement of both the secretory pathway and plasma membrane channels in the cellular release of nucleotides from non-excitatory tissues (Fig. 1). However, unambiguous evidence for either vesicular or conductive mechanisms in airway epithelia and in most peripheral tissues is lacking. Moreover, the regulatory processes involved in ATP release are largely unknown [23]. The fact that airway epithelial cells release UDP-sugars constitutively, in addition to ATP [24], suggests that nucleotides involved in glycosylation reactions within the secretory pathway are released as cargo molecules during the export of glycoconjugates, i.e., via the constitutive secretory pathway (Fig. 1). Moreover, recent studies with goblet-like airway epithelial cells indicated that ATP and UDP-sugars are released concomitantly with MUC5AC, a secretory mucin, during Ca^{2+}-regulated exocytosis of mucin granules. This observation suggest that nucleotides are stored within and released from mucin granules in goblet cells [25] (Fig. 1). An important corollary derived from this observation is that nucleotide release is a mechanism by which mucin-secreting goblet cells produce paracrine signals for mucin hydration within the ASL. Less clear is how nucleotides are released from non-secretory (ciliated) cells in response to shear forces. Evidence for the involvement of either the secretory pathway or plasma membrane channels in the release of ATP from mechanically stimulated ciliated cells remains circumstantial.

Adenosine promotes CFTR Cl^- channel activity

A key component of MCC function involves the regulation of ASL volume by electrolyte transport. ASL volume production reflects a balance between Cl^- secretion and Na^+ absorption.

CFTR is a major element mediating liquid balance on normal airway surfaces. CFTR regulates Na^+ absorption and acts as a Cl^- channel [26]. Defective CFTR activity causes the syndrome of CF, characterized by enhanced Na^+ absorption and failure to secrete Cl^-, leading to ASL volume depletion and defective MCC [26].

CFTR is a cyclic AMP-regulated Cl^- channel [27]. Electrophysiological and biochemical evidence suggests that CFTR activity in normal airways is controlled, primarily, by the A_{2b} receptor (Fig. 2). Patch clamp studies with Calu-3 cells (an airway epithelial cell line that expresses large amounts of functional CFTR) revealed that basal CFTR Cl^- channel activity is abolished by adenosine receptor antagonists [12]. Inclusion of the 5' nucleotidase inhibitor AMPCP in the patch pipette also greatly reduced CFTR activity, indicating that adenosine was generated by hydrolysis of endogenous AMP, a product of ATP release and metabolism [12]. Subsequent studies indicated that the adenosine metabolizing enzyme adenosine deaminase (ADA) decreased cyclic AMP levels in resting cells [9]. Two major implications emanate from these observations: (i) in resting epithelial cells, adenosine accumulates in ASL at levels capable of promoting A_{2b} receptor activation; and, (ii) adenosine levels are maintained by the tonic release of ATP and its subsequent metabolism within ASL. These assumptions were corroborated by measurements of adenosine levels and ATP release and hydrolysis rates on resting airway epithelia. In studies combining chemical derivatization of adenyl purines with HPLC analysis of the resulting fluorescent ethenylated species, adenosine levels in ASL in the 180-350 nM range were found [9;22].

These observations led to the hypothesis that, by promoting cyclic AMP-regulated CFTR activation, the A_{2b} receptor regulates ASL volume production. Experimental validation of this hypothesis came from confocal microscopy studies in which ASL volume production was assessed in normal and CF airway epithelia in the absence or presence of A_{2b} receptor antagonists or ADA. In normal cultures, the height of ASL at steady state is ~7 μm, the height of the extended cilia. Consistent with the notion that CFTR regulates ASL volume, ASL height in CF cultures (i.e., in the absence of functional CFTR) is reduced to < 4 μm. Addition of ADA or A_{2b} receptor antagonists to normal cultures caused a depletion of ASL volume, which displayed a height of ~4 μm at steady state [9]. Thus, in terms of ASL volume production, normal cultures behaved as CF cultures when activation of the A_{2b} receptor by endogenous adenosine was prevented.

An important element in the cascade that leads from A_{2b} receptor occupation to CFTR activation was revealed during early studies of protein kinase A (PKA)-mediated CFTR activation. Exposure of excised CFTR-containing membrane patches to cyclic AMP analogues increased CFTR open probability, indicating that PKA remained associated with excised membrane patches, i.e., physically close to CFTR. These studies also revealed that CFTR is selectively regulated by the membrane-associated isozyme of PKA, PKA-II, and that PKAII is anchored at specific subcellular sites by A kinase anchoring proteins (AKAPs) [28]. Altogether, these observations suggest that CFTR activity is controlled by adenosine generated locally, i.e., near the A_{2b} receptor site, and that A_{2b}-receptor-mediated cyclic AMP production efficiently activates PKA in close proximity to CFTR.

In addition to PKA-promoted CFTR phosphorylation and activation, activation of protein kinase C (PKC) results in enhanced CFTR-mediated Cl^- secretion. Two potential scenarios by which PKC promotes CFTR Cl^- channel activity include: (i) phosphorylation of CFTR by PKC, which facilitates subsequent PKA-mediated activation [29]; and (ii) enhanced cell surface expression of CFTR via inhibition of endocytosis [30]. These observations suggest a mechanism of activation of CFTR by the $P2Y_2$ receptor (Fig. 2).

Ca^{2+}-activated Cl^- channel

An alternative (i.e., CFTR-independent) Cl^- channel (named CaCC) is also present on the mucosal surface of the airway epithelium and is activated by elevation of cytosolic Ca^{2+} levels [31] (Fig. 2). Under normal, resting conditions, cytosolic Ca^{2+} levels are low, and therefore, CaCC activity is negligible. However, robust CaCC activation is associated with conditions promoting Ca^{2+} mobilization from intracellular stores or the influx of extracellular Ca^{2+}. In human airways, the $P2Y_2$ and $P2Y_6$ receptors and, likely, the $P2X_4$ receptor promote Ca^{2+}-dependent CaCC responses [4;7;8]. The identity of the CaCC had remained elusive for many years, but three independent groups have recently reported that members of the anoctamin (ANO)/TMEM16 family of membrane proteins are components of the CaCC [32-34]. The term anoctamin derives from the fact that these channels are anion selective and have eight (octa) transmembrane domains. $P2Y_2$ receptor activation promoted Ca^{2+}-mobilization and (ANO)/TMEM16-associated Cl^- current [32-34].

Regulation of Na^+ absorption

The epithelial Na^+ channel (ENaC) is expressed at the apical membrane of airway epithelial cells and is the major regulator of Na^+ absorption in the lung (Fig. 2). Airway epithelial ENaC is constitutively activated by proteolytic cleavage. The identities of the endogenous proteases that cleave ENaC on the airway epithelial cell surface have not been fully elucidated, but prostasin and other members of the channel-activating protein (CAP) family likely are involved [35-38]. A site in the extracellular loop of the γ-subunit of ENaC ($RKRK^{186}$) has been recently identified as a prostasisn-dependent cleavage site involved in ENaC activation [36;37].

CF airway epithelia absorb Na^+ at two to three times the normal rate. In normal airway epithelia, stimuli that raise intracellular cyclic AMP have no effect on the rate of Na^+ absorption, but in CF, airway epithelial cyclic AMP further stimulates the already elevated rate of Na^+ absorption. Little is known about how CFTR controls Na^+ absorption. It has been hypothesized that CFTR expression restrains the activity of ENaC (Fig. 2), but the molecular basis for a functional connection between CFTR and ENaC remains elusive. One speculation is that reduced or absent functional CFTR results in decreased expression of a signaling pathway that inhibits constitutively activated ENaC or, conversely, permits the expression of a stimulatory pathway that is normally repressed in normal airway epithelial cells [39].

It has been known for a while that activation of the airway epithelial $P2Y_2$ receptor results in inhibition of Na^{2+} absorption (Fig. 2). While early studies excluded the role of intracellular Ca^{2+} or protein kinase C, recent evidence suggests that inhibition of ENaC by $P2Y_2$ receptor activation requires phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2). For example, Kunzelmann et al. illustrated that ATP-promoted inhibition of Na^+ absorption in tracheal epithelial cells was suppressed by neomycin, which binds to PIP_2, and by inhibitors of PIP kinase. These authors also showed that PIP_2 co-immunoprecipitated the β subunit of ENaC and that co-expression of $P2Y_2$ receptor and α,β,γ-ENaC in *Xenopus* oocytes resulted in ATP-promoted ENaC inhibition [40]. A series of studies with renal epithelia provided further support for the notion that the $P2Y_2$ receptor regulates ENaC activity through PIP_2 depletion [41]. Eaton and co-workers illustrated that PIP_2 binding to the NH3-terminal region of the β subunit of ENaC increased the open probability of the channel [42-44]. Pochynyuk et al. demonstrated that conserved positive-charged regions at the NH3-terminal of β and γ ENaC are important for inhibition of ENaC activity in response to $P2Y_2$ receptor-promoted PIP_2 depletion [45;46].

$P2Y_2$ receptor regulation of mucin secretion

Mucins are released from goblet cells via regulated exocytosis. In contrast to submucosal glands, which are innervated richly and regulated primarily by acetylcholine and possibly other neurotransmitters, goblet cells of the superficial epithelium are sparsely innervated and are regulated primarily by local mediators in the airway lumen, acting on their apical surfaces. The current body of data indicates that the $P2Y_2$ receptor on apical membranes of surface goblet cells constitutes the major regulatory system for mucin secretion. The $P2Y_2$ receptor promotes mucin secretion via complex Ca^{2+} and dyacylglycerol (DAG)-regulated mechanisms. Excellent reviews have been recently published on the regulation of mucin secretion in goblet cells [14;47;48] and, therefore, we will not expand on this topic.

Regulation of ciliary beat by P2Y and A_{2b} receptors

Airway epithelial cells contain hundreds of motile cilia per cell that beat together to propel the mucus over the epithelial surface. Thus, in normally hydrated airways, the rate of MCC is determined by ciliary beat frequency (CBF). Coordinated ciliary beat is achieved by the co-orientation of ciliary structure and direction of beating, both within each cell and between individual ciliated cells, and is essential to the physiological functions of the epithelia (reviewed in [49]). Extracellular nucleotides regulate ciliary beating in the airways by mechanisms that involve Ca^{2+} mobilization via $P2Y_2$ (and to a lesser extent $P2Y_6$, [7]) receptor activation. P2Y receptor-regulated Ca^{2+}-dependent CBF involves two components: (i) a transient Ca^{2+} signal associated with Ca^{2+} release from intracellular stores; and, (ii) a more sustained Ca^{2+} influx via plasma membrane Ca^{2+}channels. In a recent study, Lorenzo et al. have elegantly shown that at non-saturating concentrations, ATP generates oscillatory Ca^{2+} signals in ciliated cells, which in turn increases CBF. At maximally effective concentrations, ATP elicited a robust CBF response, which was associated with a sustained Ca^{2+} influx via receptor operated Ca^{2+} entry mechanisms [50]. It has been proposed that an oscillatory Ca^{2+} signal increases CBF by acting directly on a ciliary target, most likely a calcium-binding protein [51;52]. An indirect mechanism for Ca^{2+}-promoted CBF increase, e.g., phosphorylation of axonemal proteins, may be involved in response to sustained Ca^{2+} elevation [52].

In addition, a Ca^{2+}-independent mechanism of CBF regulation operates in response to adenosine, i.e., via A_{2b} receptor-elicited cyclic AMP formation and PKA activation [53]. The putative substrate for PKA phosphorylation that regulates CBF in human airway epithelial cells has not been identified. However, there is good evidence that cyclic AMP regulates CBF in mammalian cells via PKA-promoted phosphorylation of dynein light chain, which in turn possibly mediates a switch from the slow to the fast dynein-duty cycle and thus increases CBF (reviewed in [51]).

Conclusions and prospective

The above studies highlight the importance of purinergic signaling in the airways. While it is now evident that adenosine and ATP are crucial regulators of MCC activities in normal airways, the $P2Y_2$ receptor has promising perspectives as a therapeutic target to promote CaCC activity and ENaC inhibition in CF lungs, improving the otherwise poor ASL volume production associated with defective CFTR. Therapeutic use of a $P2Y_2$ receptor agonist to promote lung hydratation in CF is in late phases of clinical testing. Of note, the potential role of purinergic agonists in the pathophysiology of inflammatory airway diseases, e.g., chronic obstructed/inflammed lungs, has been incompletely unexplored. For example, adenosine receptors, P2X receptors, and the $P2Y_2$, $P2Y_{11}$, and $P2Y_{14}$ receptors are expressed in inflammatory cells such as macrophages, lymphocytes, and granulocytes. Rrecent measurements of nucleotides in bronchoalveolar secretions from patients with lung inflammation indicated levels of adenosine,

ATP, and UDP-sugars in range of promoting robust activation of their cognate receptor ([54] and data not shown). An interrelated area of active research focuses on the mechanisms of nucleotide release, in particular, the identification of biochemical signals that transduce mechanical forces into ATP release.

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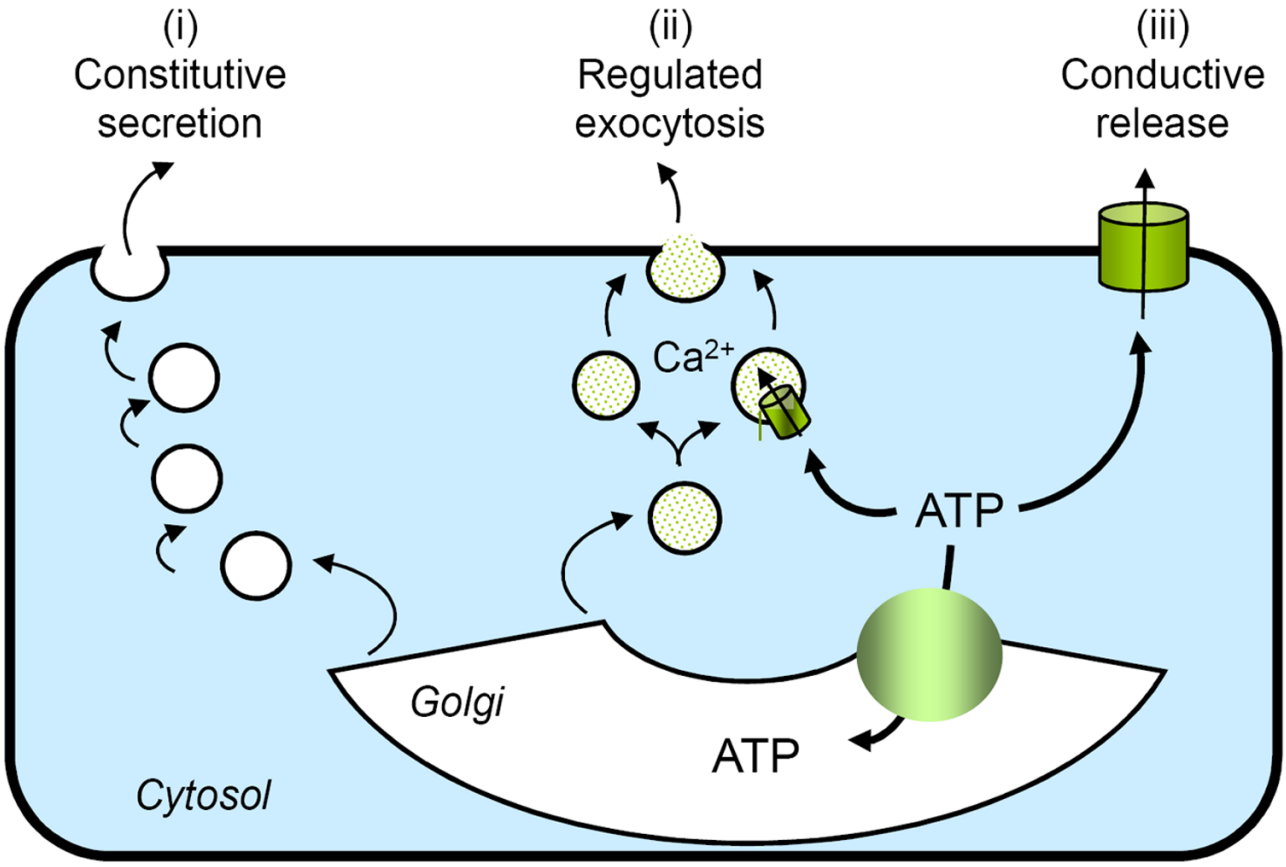

Figure 1. Pathways for ATP release

Several scenarios possibly account for the basal and stimulated release of nucleotides from airway epithelial cells. (i) ATP entering the Golgi lumen via specific transporters may be released as a residual cargo product of the constitutive secretory pathway. (ii) Secretory granules (e.g., mucin granules) containing ATP may be competent for Ca^{2+}-regulated exocytosis. (iii) A not yet identified ATP conductance effluxes cytosolic ATP out of the cells.

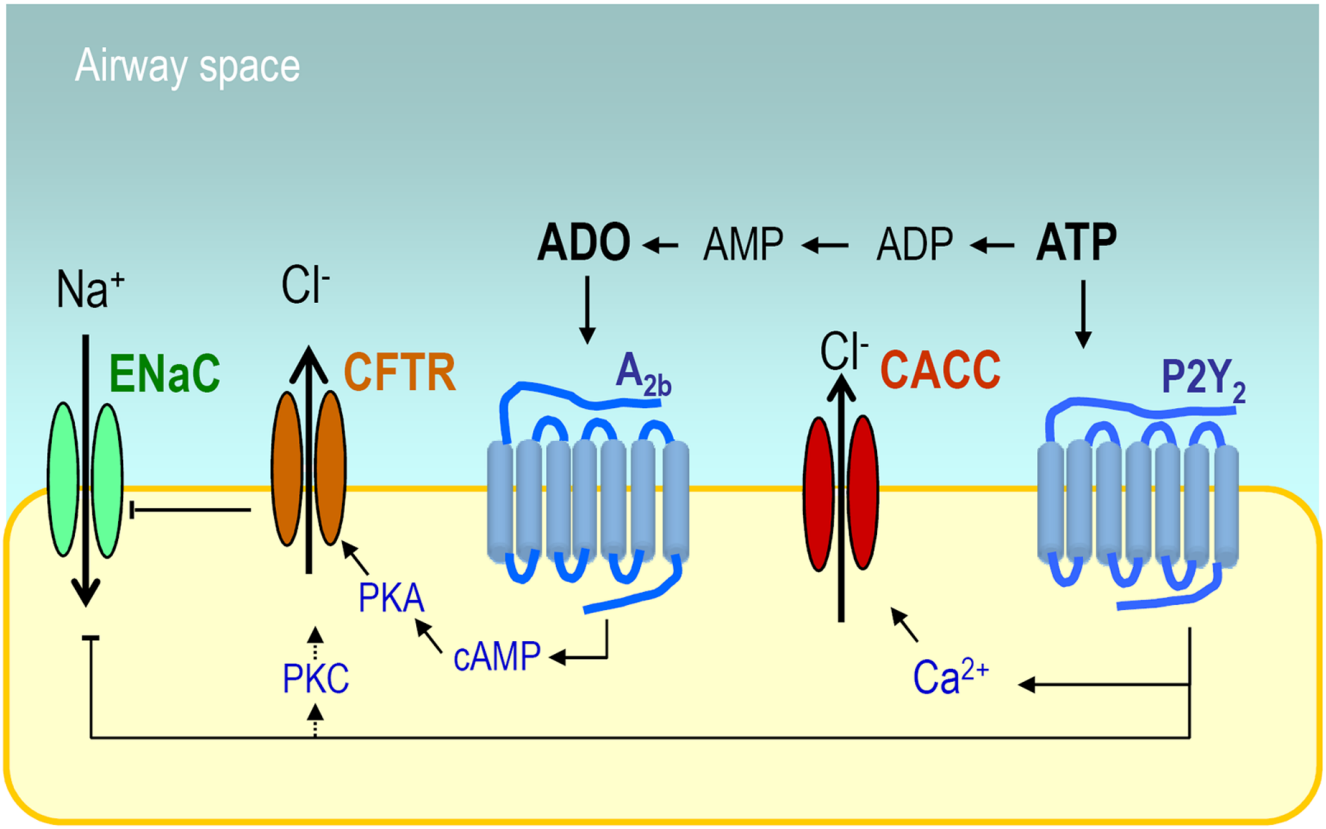

Figure 2. Purinergic regulation of ion transport

ASL ATP promotes $P2Y_2$ receptor-mediated Ca^{2+}-activated Cl^- channel (CaCC) activity, and inhibition of the epithelial Na^+ channel ENaC (via PIP_2 depletion, not shown). Potentially, the $P2Y_2$ receptor promotes PKC-mediated CFTR activation. ATP hydrolysis results in adenosine accumulation (ADO), which in turns activates the A_{2b} receptor, leading to cyclic AMP (cAMP) and protein kinase A (PKA)-mediated CFTR activation. CFTR inhibits ENaC by mechanisms that are not well understood.

Table 1

Purinergic receptors, their agonists and signaling properties

Nineteen purinergic receptor species have been identified at the molecular level. The A_{2b}, $P2Y_2$, $P2Y_6$, and $P2X_4$ receptors are present in airway epithelial cells [1;3-8]. Abbreviations: PLC, phospholipase C; PKC, protein kinase C; AC, adenylyl cyclase; cAMP, cyclic AMP; ↓, inhibition.

	Agonist	Signaling
P2X receptors		
$P2X_1$-$P2X_7$	ATP	ATP-gated cation channel
P2Y receptors		
$P2Y_1$	ADP	Gq/PLCβ → Ca^{2+}/PKC
$P2Y_2$	ATP = UTP	Gq/PLCβ → Ca^{2+}/PKC
$P2Y_4$	UTP	Gq/PLCβ → Ca^{2+}/PKC
$P2Y_6$	UDP	Gq/PLCβ → Ca^{2+}/PKC
$P2Y_{11}$	ATP	Gq/PLCβ → Ca^{2+}/PKC and Gs → AC/cAMP
$P2Y_{12}$	ADP	Gi → ↓AC/↓cAMP
$P2Y_{13}$	ADP	Gi → ↓AC/↓cAMP
$P2Y_{14}$	UDP-glucose	Gi → ↓AC/↓cAMP
Adenosine receptors		
A_1, A_3		Gi → ↓AC/↓cAMP
A_{2a}, A_{2b}		Gs → AC/cAMP