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Purinergic Regulation of Respiratory Diseases

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Nearly half a century has passed since the "Purinergic Nerve Hypothesis" was proposed by Burnstock. The demonstration that ATP is released by nerve endings in a regulated manner and plays a critical role as neurotransmitter raised worldwide controversy and paved the way for the exploration of nucleotides as extracellular messengers. To this date, the number of manuscripts published in the field of "Purinergic Signaling" continues to grow exponentially and has become the main focus of numerous research groups around the world. Families of cell surface receptors named purinoceptors were cloned and identified in most organs and cell types. At the same time, another field of research emerged with the initial studies of Drs. Beaudoin and Zimmermann, who showed that the availability of the purinoceptor agonists is regulated by cell surface enzymes. Twenty years ago, these world leaders launched the "International Symposium on Adenosine and Adenine Nucleotides", which led to the integration of both fields of purinergic research and groundbreaking discoveries from our basic understanding of cell signaling to therapeutic applications.

The growing list of medical institutions and pharmaceutical companies working together to develop therapeutic approaches based on purinergic signaling is a testimony to the now acclaimed critical importance of these messengers for homeostasis. This book is dedicated to the brave scientists who faced adversity to give birth to this new and exciting era of scientific endeavors.

Preface

Chronic respiratory diseases are complex disorders generally driven by genetic mutations weakening the airways' ability to respond appropriately to inhaled toxins or pathogens. In the case of cystic fibrosis (CF), functional mutations of the cystic fibrosis transmembrane resistance (CFTR) ion channel impair airway hydration, which fosters the formation of infected mucus plugs requiring mechanical stimulation for clearance. These patients suffer from a progressive and irreversible loss of lung function caused by overwhelming and damaging neutrophilic inflammatory responses. On the other hand, frequent exposure to the toxic components of cigarette smoke is considered the primary cause of chronic obstructive pulmonary disease (COPD). Subjects diagnosed with α1-antitrypsin deficiency are particularly vulnerable to lung destruction by elastases, and to the development of emphysema. Allergic asthma remains a serious challenge, as this disease incorporates multiple genetic factors and environmental stimuli. The patients experience recurrent episodes of breathless, wheezing, coughing and chest tightness triggered by airway hyperresponsiveness (AHR) to allergens. Over the years, the severity of AHR episodes increases, as chronic inflammatory responses to allergens induce extensive airway remodeling and narrowing of the airway passages. Recently, the discovery of significant overlap between the symptoms of these diseases raised serious concerns with respect to our ability to diagnose and treat the patients efficiently. The scientific community has been mandated to open new avenues for the development of discriminative diagnostic tools and customized therapies for these diseases.

For decades, the most common diagnostic method used to differentiate asthmatics from COPD patients was AHR induced by inhalation of methacholine. Yet, a third of the COPD patients present significant AHR to this drug. As an alternative, The European Respiratory Society Task Force recently endorsed AHR measurements after inhalation of adenosine monophosphate (AMP) as a more specific diagnosis for asthma than methacholine. This finding spiked the interest of the scientific community for the signaling pathways mediating the effects of AMP in the airways of asthmatic patients. We now know that aerosolized AMP must, first, be dephosphorylated by a cell surface enzyme named ecto 5′-nucleotidase (CD73) in order to

generate adenosine, a signaling molecule of the purinergic network. Upon binding to cell surface receptors, adenosine induces histamine release from mast cells, which initiates AHR in asthmatic patients. This narrow window was only the prelude to what would become a major endeavor to expose the purinergic regulation of airway defenses. In the past 15 years, the persistent exponential increase in the number of publications targeting the purinergic regulation of acute lung injury, mucociliary clearance, inflammation, wound healing, remodeling and lung fibrosis is a testimony to the extensive ramifications of this signaling network in chronic respiratory diseases. Clinical and fundamental studies support the existence of disease-specific aberrances in airway concentrations of the signaling molecules, as well as in expression levels of the receptors and related enzymes in lung tissues of asthmatic, COPD and CF patients. This book is a tribute to this exploding field of research, and promises to come for the development of specific diagnostic tools and therapies for the predominant chronic respiratory diseases affecting the general population.

The term "Purinome" was recently ascribed to protein network mediating the effects of extracellular purines and pyrimidines. The composition of each "Purinome" is locally refined by different combinations of signaling molecules (ATP, ADP, Ap_4A, adenosine), purinergic receptors, cell surface nucleotide-metabolizing enzymes (ectonucleotidases), and nucleoside/nucleotide channels or transporters. These protein clusters mediate tightly concerted actions invested in the maintenance of homeostasis and airway defenses. In chronic disorders, alterations of their global and dynamic equilibrium contribute to the appearance and/or propagation of pathological states.

The vast majority of studies conducted on purinergic signaling are devoted to ATP and its metabolite, adenosine. In a nutshell, the local release of ATP constitutes an alarm signal perceived by surrounding cells through interaction with P2 cell surface receptors. This "communiqué" informs the cells to take action according to their specific roles in the restoration of homeostasis. The cells' alertness is maintained by the presence of local ectonucleotidases which promptly eliminate the ATP signal and restore receptiveness. The ingenuity of this communication network resides in the subsequent initiation of a negative feedback messenger from the dephosphorylation of ATP into adenosine. This signaling molecule binds P1 cell surface receptors to assist to restrain ATP-mediated responses and restore baseline activities. This sophisticated machinery works in concerts with other signaling networks, such as those supported by cytokines and growth factors, to maintain healthy lungs free of infection. However, chronic disorders associated with the maintenance of excess ATP or adenosine in the airways recruits surface receptors which induce or aggravate lung complications, including hyperinflammatory responses, tissue damage and airway remodeling leading to the loss of lung function.

This book was meticulously designed to systematically introduce the reader to each element of purinergic network, followed by their integration into a mathematical model. Then, evidence is presented for significant aberrances in the regulation of the signaling molecules in chronic respiratory diseases. Three chapters are dedicated to the detailed description of the major respiratory and inflammatory

functions regulated by purinergic signaling, and the aspects affected by chronic disorders. Finally, the reader is presented with the animal models and clinical applications currently used for the development of diagnostic and therapeutic approaches chronic respiratory diseases.

As editor-in-chief, I wish to thank all contributors for their efforts and the staff of Springer-Verlagh for their professionalism in overseeing this publication.

Chapel Hill, NC Maryse Picher

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Chapter 1
Nucleotide Release by Airway Epithelia

Eduardo R. Lazarowski, Juliana I. Sesma, Lucia Seminario, Charles R. Esther Jr., and Silvia M. Kreda

Abstract The purinergic events regulating the airways' innate defenses are initiated by the release of purines from the epithelium, which occurs constitutively and is enhanced by chemical or mechanical stimulation. While the external triggers have been reviewed exhaustively, this chapter focuses on current knowledge of the receptors and signaling cascades mediating nucleotide release. The list of secreted purines now includes ATP, ADP, AMP and nucleotide sugars, and involves at least three distinct mechanisms reflecting the complexity of airway epithelia. First, the constitutive mechanism involves ATP translocation to the ER/Golgi complex as energy source for protein folding, and fusion of Golgi-derived vesicles with the plasma membrane. Second, goblet cells package ATP with mucins into granules, which are discharged in response to $P2Y_2R$ activation and Ca^{2+}-dependent signaling pathways. Finally, non-mucous cells support a regulated mechanism of ATP release involving protease activated receptor (PAR)-elicited $G_{12/13}$ activation, leading to the RhoGEF-mediated exchange of GDP for GTP on RhoA, and cytoskeleton rearrangement. Together, these pathways provide fine tuning of epithelial responses regulated by purinergic signaling events.

Keywords ATP release · Airway epithelia · Ectonucleotidase · Thrombin · Mucin

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1.1 Introduction

1.1.1 Definitions: The Purinergic Receptors

The actions of extracellular nucleotides are mediated by two families of receptors: P2X receptors (P2XRs) and P2Y receptors (P2YRs) (reviews: [1–3]). The P2XR family includes seven ATP-gated non-selective cation channels modulated by extracellular Ca^{2+}, Na^{+}, Mg^{2+} and H^{+}. Their activation mobilizes intracellular Ca^{2+} and causes membrane depolarization. The P2YR family contains eight G protein-coupled receptors activated by ATP, UTP, ADP, UDP, and/or UDP-sugars (Table 1.1). The metabolite of ATP, adenosine, (ADO) mediates cellular responses through four G protein-coupled P1 receptors: A_1Rs, A_{2A}Rs, A_{2B}Rs and A_3Rs (reviews: [4–6]). Their agonist selectivity and signaling properties are summarized in Table 1.1.

The purinergic network of human airway epithelia accommodates a discrete subset of purinergic receptors (review: [7]). The predominant nucleotide-sensing receptor in the airways is $P2Y_2R$, which is activated to a similar extent and potency by ATP and UTP. Receptor occupation initiates the breakdown of plasma membrane phosphoinositides by phospholipase C, resulting in the formation of two secondary messengers: inositol 1,4,5 tris-phosphate ($InsP_3$) and diacylglycerol (DAG). Whereas InsP3 triggers the Ca^{2+} release from intracellular stores, DAG activates protein kinase C (PKC)-dependent signaling cascades. These pathways promote the secretion of mucin (review: [8]), inhibition of the epithelial Na^{+} channel (ENaC) [8–14] and activation of the Ca^{2+}-activated Cl^{-} channel (CaCC) recently identified as (ANO)/TMEM16 [15–17]. Human nasal epithelial cells also express $P2Y_6$Rs,

Table 1.1 Agonist selectivity and signaling pathways regulated by purinergic receptors

Family	Natural agonist	Signaling
P2X receptors		
$P2X_1$–$P2X_7$	ATP	ATP-gated cation channel
P2Y receptors		
$P2Y_1$	ADP	Gq/PLC
$P2Y_2$	ATP = UTP	Gq/PLC
$P2Y_4$	UTP	Gq/PLC
$P2Y_6$	UDP	Gq/PLC
$P2Y_{11}$	ATP	Gq/PLC and Gs/AC
$P2Y_{12}$	ADP	Gi/AC inhibition
$P2Y_{13}$	ADP	Gi/AC inhibition
$P2Y_{14}$	UDP-sugars	Gi/AC inhibition and ERK activation
Adenosine receptors		
A_1	Adenosine	Gi/AC inhibition
A_{2A}	Adenosine	Gs/AC activation
A_{2B}	Adenosine	Gs/AC activation
A_3	Adenosine	Gi/AC inhibition

PLC phosphlipase C, *AC* adenylyl cyclase

which induces CaCC activity with a maximal effect about one-half of $P2Y_2Rs$ [18]. A study conducted in human A549 alveolar and BEAS-2B bronchial epithelial cells supports the expression of $P2Y_{14}R$ in human airway epithelial cells, where it participates in highly specific inflammatory responses [19] (see Chap. 7 for details).

The presence of P2XRs on airway epithelial surfaces has been documented in various species, including humans (reviews: [20]). Whereas normal bronchial epithelia express P2X4-6 receptors, primary cultures and cell lines from CF patients also express P2X7Rs [21]. The $P2X_4R$ was immunolocalized on the apical surface of rabbit tracheal epithelia, on the lower half of the cilia [22]. In the parenchyma, alveolar type 1 cells express P2X4R and P2X7R, the latter being associated with caveolae. The use of selective stable agonists and antagonists suggests that these P2XRs participate in the regulation of ion channels mediating airway hydration (see Chap. 5). However, their contribution remains highly debated because the affinity of the cloned receptors for ATP ($EC_{50} = 10–100\ \mu M$) [23] is several orders of magnitude above that of $P2Y_2Rs$ regulating the same cellular functions. On the other hand, P2XRs expressed on basolateral epithelial surfaces were found 100-fold more sensitive than those restricted to the apical surface [21]. Furthermore, Rettinger et al. published two detailed studies showing that receptor desensitization masks the nanomolar potency of ATP for P2X1Rs in *Xenopus* oocytes [24, 25]. These receptors also form heteromers, such as P2X4/6 and P2X4/7 [26], which generally raises their sensitivity to ATP (review: [27]). Clearly, much remains to be uncovered on the properties and roles of P2XRs in the respiratory system.

On airway epithelial surfaces, the vast majority of the studies describing ADO-mediated responses refer to the $A_{2B}R$. This P1 receptor has been shown to stimulate the conductance of CFTR [28] and ciliary beat frequency [9]. The $A_{2A}R$ has been identified on human airway epithelia by RT-PCR and using selective agonists and antagonists, where it promotes healing [29, 30] and inflammatory responses [31, 32] (see Chaps. 6 and 7 for details). This summary identifies the purinergic receptors expressed on airway epithelial surfaces, which will facilitate the discussion in the following sections.

1.1.2 Nucleotide and Nucleoside Concentrations on Airway Surfaces

Mucociliary clearance (MCC) represents the first line of defense of the airways against infection, a mechanism known to be tightly regulated by purinergic events taking place within the airway surface liquid (ASL). The signals are initiated by the release of the signaling molecule, ATP, from airway epithelial cells. *In vitro* studies indicated that resting airway epithelia release ATP at a rate of 300–500 $fmol \cdot min^{-1} \cdot cm^{-2}$ [33, 34]. The rate of ATP release is counterbalanced by the rate of surface metabolism mediated by ectonucleotidases. This dynamic flow of nucleotides maintains steady-state ASL ATP concentrations around 5–20 nM, which is below the activation threshold of $P2Y_2Rs$ [33–35]. However, the metabolism

of surface ATP continuously generates the signaling molecule ADO, which is maintained at steady-state concentrations capable of activating A_{2B}Rs. Indeed, measurements of cyclic AMP production conducted in the presence or absence of ADO-degrading enzymes, revealed that A_{2B}Rs are tonically stimulated by endogenous ADO on resting airway epithelial cells in culture [33]. This hypothesis was further validated using sensitive assays for the quantification of ADO within the ASL. Endogenous nucleotides and nucleosides display low intrinsic fluorescence, and UV-based approaches require relatively high concentrations (>0.1 μM) for detection. This limitation was overcome using the chloroacetaldehyde derivatization technique, which quantitatively converts the adenine ring of ADO and its nucleotides into fluorescent 1,N^6-etheno(ε)-adenine derivatives, i.e., ε-ADO, ε-AMP, ε-ADP, and ε-ATP. The (ε)-adenyl purines are separated by HPLC and readily quantified with low nanomolar sensitivity. Using this technique, the concentration of ADO within the ASL of resting epithelial cultures was found in the 100–400 nM range [33], which is above the activation threshold of A_{2B}Rs.

The recognition that the constitutive[1] release of ATP results in physiologically relevant levels of ADO in the ASL suggests a mechanism for the purinergic control of basal MCC activities in resting epithelia, i.e., via A_{2B}R activation. However, the rate of ATP release from airway epithelial cells is enhanced threefold by mechanical stresses, such as rhythmic shear and compression, experienced during tidal breathing or coughing. The resulting transient increase in ASL ATP concentration (100–1,000 nM) is capable of activating $P2Y_2$Rs [36]. Functional studies (e.g. measurement of ASL volume regulation) demonstrated that ATP released from mechanically-stimulated epithelia mediates acute MCC responses via $P2Y_2$R stimulation [36–39]. In summary, airway surfaces initiate purinergic events critical for MCC via ATP release and surface conversion into ADO by ectoenzymes, both inducing specific cell surface receptors and signaling pathways.

1.2 Mechanisms of Nucleotide Release

1.2.1 Constitutive Release of Nucleotides from the Secretory Pathway

1.2.1.1 Lessons Learned from the Yeast

The complex cellular composition of the airways, i.e., ciliated cells and mucin-secreting goblet cells, suggests that several mechanisms and pathways are involved in the release of nucleotides into the airways. Circumstantial evidence supports the involvement of both exocytosis and plasma membrane channels in the cellular

[1] We use the term “constitutive” to refer to a release process that occurs in non-stimulated cells.

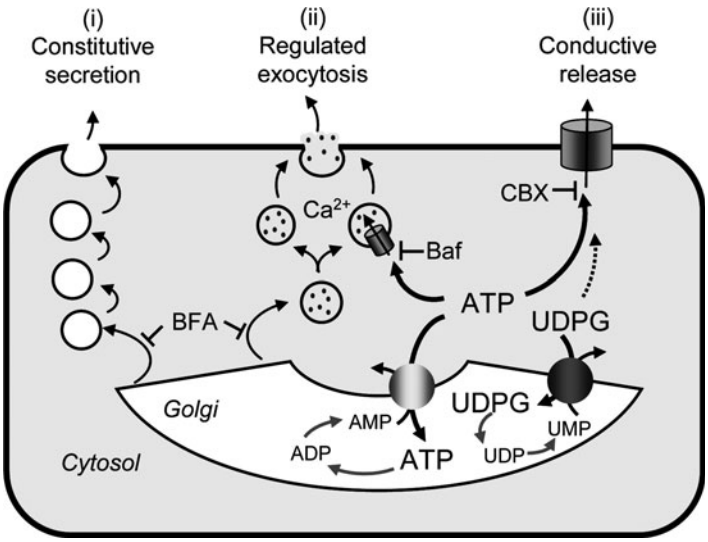

Fig. 1.1 Pathways for airway epithelial nucleotide release. Several scenarios account for the constitutive and stimulated release of nucleotides from airway epithelial cells. (**i**) Nucleotides entering the Golgi lumen via specific transporters are released as a residual cargo product of the constitutive secretory pathway. The fungal venom brefeldin A (*BFA*) blocks this pathway by disrupting the Golgi apparatus. (**ii**) Secretory granules (e.g., mucin granules) containing ATP are competent for Ca^{2+}-regulated exocytosis. Bafilomycin A_i (*Baf*), an inhibitor of the H^+-ATPase that loads ATP into specialized granules in secretory cells, impairs mucin secretion-associated ATP release in goblet cells. (**iii**) A plasma membrane connenxin/pannenxin-like channel effluxes cytosolic ATP out of the cells

release of nucleotides (Fig. 1.1), but unambiguous support for either vesicular or conductive mechanisms of release in epithelia has only recently begun to surface.

Initial evidence for the involvement of the secretory pathway in the release of nucleotides from non-excitatory cells was provided by studies of glucose-dependent ATP release in the yeast *Saccharomyces cerevisia* [40, 41]. Glucose-dependent ATP release was enhanced in yeast overexpressing Mcd4p, a Golgi-resident transporter postulated to transports ATP to the lumen of the secretory pathway. This mechanism of ATP release was inhibited by the Golgi-disrupting agent brefeldin A [40, 41]. Since ATP release from yeast dramatically decreased when glucose was omitted from the extracellular medium, it was hypothesized that ATP release from these cells reflected an exocytotic mechanism initiated by the activation of a glucose-sensing cell surface receptor [40]. The HPLC analysis of adenyl purines released by yeast indicated that, in the absence of glucose, extracellular AMP levels increased robustly as ATP levels decreased, maintaining the net mass of extracellular AMP/ADP/ATP unaffected by glucose [41]. Moreover, short-term removal of glucose from the extracellular medium did not affect the ability of yeast to secrete invertase, a marker of exocytosis [41]. The simplest interpretation of these data is that yeast releases nucleotides constitutively, via vesicle exocytosis, and that the

energy balance of the cell determines the relative levels of ATP and AMP within the releasable vesicular pool.

Additional evidence supporting the involvement of the secretory pathway in the release of nucleotides was generated from the observation that, in most cells, ATP release is accompanied by the release of UDP-sugars. Interest in UDP-sugar release followed the realization that one member of the P2YR family, the $P2Y_{14}R$, is selectively and potently stimulated by UDP-glucose (UDP-Glc) and other UDP-sugars [42]. A series of studies indicated that UDP-Glc is released from a number of cells, including simple cell systems such as yeast and highly differentiated airway epithelial cells [41, 43–45]. The fact that UDP-sugars participate in glycosylation reactions within the secretory pathway suggested that these molecules are released as cargo molecules during the export of glycoconjugates to the plasma membrane, i.e., via the constitutive pathway (Fig. 1.1).

This hypothesis has been recently tested at the molecular level. It has been shown that UDP-Glc, UDP-N-acetylglucosamine (UDP-GlcNAc), and other UDP-sugars are synthesized in the cytosol and concentrated in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus to serve as sugar donor substrates for glycosyltransferase reactions [46–48]. UDP, a byproduct of this process, is in turn hydrolyzed to UMP [48]. The entry of UDP-sugars to the ER/Golgi is mediated by ER/Golgi-resident UDP-sugar transporters, which use luminal UMP as antiporter substrate (Fig. 1.1). The UDP-sugar/UMP translocators are multi-transmembrane helices that belong to the family of solute carrier SLC35 ER/Golgi nucleotide-sugar transporters [49]. Studies in yeast suggest that the cellular release of UDP-Glc is preceded by its uptake into the ER/Golgi. That is, UDP-Glc release was enhanced in yeast missing the putative nucleotide-sugar transporter encoding gene *YMD8* (*ymd8*Δ cells), and this release was significantly reduced in *ymd8Δ* cells in which Yea4p or HUT1p, the ER-resident UDP-GlcNAc transporter and Golgi UDP-galactose transporter, respectively, were deleted. Thus, UDP-Glc release from yeast is influenced by the rates of ER/Golgi uptake of UDP-GlcNAc and UDP-galactose [41]. Diminished entry of UDP-Glc to the ER/Golgi of *YEA4*- or *HUT1*-deficient cells likely reflected the decreased availability of the antiporter substrate UMP in the ER/Golgi lumen [48]. However, since Yea4p or HUT1p are not primarily UDP-Glc transporters, the interpretation of these results has remained speculative.

The passage of UDP-sugar species through the ER/Golgi before their release was more conclusively established by correlating the amount of UDP-GlcNAc transporter in the ER/Golgi with the cellular release of its substrate, UDP-GlcNAc. By developing an assay which quantifies UDP-GlcNAc concentrations within the low nanomolar range, Sesma et al. demonstrated that yeast, in which the *YEA4* gene (*yea4*Δ cells) was deleted, display reduced release of UDP-GlcNAc, the natural substrate of Yea4p [45]. Consistent with the role of Yea4p as major ER/Golgi UDP-GlcNAc transporter, the *yea*Δ cells also displayed reduced synthesis of chitin, a glucosamine-rich cell wall component. The reduced UDP-GlcNAc release rate and chitin content of the *yea*Δ cells were returned to normal by complementing the mutant strain with the WT *YEA4* gene [45]. The most parsimonious conclusion

from these studies is that, by facilitating UDP-GlcNAc entry into the ER/Golgi, Yea4p mediates the release of its UDP-sugar substrate from the secretory pathway.

1.2.1.2 Vectorial Release of Nucleotide Sugars from Resting Cells

The above-mentioned studies in yeast paved the way to investigate the role of Golgi-derived vesicles in the constitutive release of nucleotide-sugars from airway epithelia. Three gene products, SLC35A3, SLC35B4 and SLC35D2, have been characterized as Golgi-resident UDP-sugar/UMP translocators in humans [49–51]. SLC35D2, also known as HFRC1, is the human homologue to the fruit fly fringe connection (Frc) transporter and may facilitate the transport of UDP-Glc and UDP-GlcNAc into the Golgi [50]. By controlling the entry of UDP-GlcNAc into the Golgi, HFRC1 modulates the surface expression of *N*-acetylglucosamine-rich glycoconjugates on airway epithelial cells [50]. Consistent with this concept, the human bronchial epithelial cell line 16HBE14o$^-$, stably overexpressing HFRC1, displayed enhanced cell surface immunoreactivity towards heparan sulfate and increased apical surface binding of WGA, a lectin that recognizes *N*-acetylglucosamine and sialic acids in airway epithelia. The overexpression of HFRC1 resulted in an increased rate of mucosal release of UDP-GlcNAc and, to a lesser extent, UDP-Glc release, relative to vector-transformed cells [45]. These studies provide compelling evidence for the contribution of Golgi-derived vesicles to the release of nucleotide-sugars from airway epithelial cells.

Measurements of ATP and UDP-sugars release rates in polarized monolayers of human airway epithelial Calu-3 and 16HBE14o$^-$ cells revealed that the nucleotides accumulate predominantly in the mucosal bath [33, 34, 45], suggesting a membrane specialization for nucleotide release. Similarly to UDP-sugars, ATP is translocated to the ER/Golgi via ATP/AMP antiporters, where it serves as an energy source for protein folding reactions [52, 53]. Therefore, the tonic release of ATP, UDP-sugars, as well as ADP and UDP, likely supports the continuous ("constitutive") recycling of proteins and glycoconjugates on the apical plasma membrane and the exocytotic release of co-cargo nucleotides (Fig. 1.1).

1.2.2 Calcium-Promoted Release of Nucleotides from Goblet Cells

In addition to the above-described constitutive release, recent studies conducted with goblet-like airway epithelial cells indicated that ATP and UDP-sugars are released concomitantly with MUC5AC, a secretory mucin, during the Ca^{2+}-regulated exocytosis of mucin granules. Electron microscope analysis of polarized Calu-3 cultures indicated that up to 40% of the cells within the monolayer express ~1 μm-diameter electron-translucent granules that resemble the mucin granules of airway mucous

goblet cells. Subsequent immunostaining and slot blot analysis revealed the presence of MUC5AC granules in the Calu-3 cultures, which were competent for Ca^{2+}-regulated exocytosis in the mucosal compartment. The Ca^{2+}-promoted mucin secretion was accompanied by enhanced ATP release into the mucosal bath [44]. The kinetics of ATP release and mucin-granule secretion were similar and triggered by identical stimuli, suggesting that nucleotides are stored within, and released from, mucin granules in goblet cells [44]. Furthermore, bafilomycin A_1, which depletes ATP storage granules [54], markedly impaired ionomycin-promoted ATP release from Calu-3 cells [44]. These data are consistent with the possibility that a vesicular/granular ATP pool contributes to Ca^{2+}-stimulated ATP release. An important corollary derived from these observations is that ATP/mucin-secreting goblet cells produce paracrine signals for $P2Y_2R$-mediated mucin hydration and MCC activities.

The Ca^{2+}-dependent mechanism of ATP release identified in Calu-3 monolayers also stimulated the apical release of UDP-Glc [44], which has pathophysiological implications. High concentrations of UDP-Glc (100–1,000 nM) were recently detected in lung secretions from patients with cystic fibrosis (CF) or other goblet cell hyperplasic lung diseases [44, 45]. These concentrations were demonstrated to activate $P2Y_{14}Rs$, which are highly expressed on inflammatory cells (lymphocytes and neutrophils) [55–57]. Therefore, the sustained accumulation of UDP-Glc in the ASL in conditions associated with goblet cell hyperplasia and/or mucin hypersecretion (asthma, chronic obstructive pulmonary disease or CF) may amplify inflammatory responses.

1.2.3 Regulated ATP Release from Non-Mucous Cells

1.2.3.1 Rho-Dependent Signaling Participates in ATP Release

The mechanisms supporting nucleotide release from non-mucous lung epithelial cells (i.e. ciliated or alveolar cells) remains unclear due to the scarcity of pharmacological approaches known to promote ATP release. While most studies rely on mechanical or osmotic stimuli, the biochemical signals mediating ATP release remain poorly defined.

Recent data suggest that in A549 alveolar cells, hypotonicity-induced ATP release involves the mobilization of intracellular Ca^{2+} [58–60]. However, in human bronchial epithelial (HBE) cultures dominated by ciliated cells, the chelation of intracellular Ca^{2+} resulted in only a minor inhibition of hypotonic shock-elicited ATP release [34]. However, Ca^{2+} mobilizing agents, such as ionomycin and UTP, promoted only minor release of ATP relative to hypotonic shock, in both HBE and A549 cells [34, 59–61]. These observations suggest that additional and/or alternative signals to Ca^{2+} mobilization are required upstream of ATP release in non-mucous cells. This notion appears not to be restricted to epithelial cells. For example, studies in 1321N1 human astrocytoma cells demonstrated that Ca^{2+} mobilization is not sufficient to reach maximal ATP release in response to

pharmacological stimulation. In these cells, the serine protease thrombin promoted a robust Ca^{2+}-dependent nucleotide release response via activation of the protease-activated receptor-1 (PAR1). In contrast, carbachol induced a robust Ca^{2+} mobilization via the muscarinic receptor M3, but modest ATP release from these cells [62, 63]. A more recent study by Dubyak et al. indicated that G protein coupled receptor-elicited ATP release involves the activation of RhoA, consistent with the notion that PAR1 couples to $G_{12/13}$ in addition to Gq [64]. Studies conducted on human umbilical vein endothelial cells also support a role for Rho in mechanical stress-induced ATP release. In these cells, both hypotonic stress- and lysophosphatidic acid (LPA)-induced ATP release were abolished by the Rho kinase inhibitor Y27632 [65]. Together, these studies suggested that Rho is a central regulator of ATP release in response to physical and pharmacological stimuli.

The presence of Rho-stimulating thrombin receptors in lung epithelia [61, 66] provided a physiological approach to investigate the involvement of this GTPase in epithelial ATP release. Consistent with the above-mentioned studies on astrocytoma and endothelial cells [64, 65], thrombin promoted ATP release from A549 cells, which was accompanied by a rapid (1–3 min) activation of RhoA [61]. In contrast, activation of the Gq/PLC/Ca^{2+}-mobilizing signaling pathway by UTP on $P2Y_2Rs$ resulted in negligible ATP release from these cells, and a delayed onset (>15 min) of Rho activation [61]. The involvement of Rho was supported by the observation that ATP release from thrombin-stimulated A549 and HBE cultures decreases in the presence of ROCK inhibitors [61]. These observations were further validated by mutational studies. The transfection of A549 cells with the RhoA mutant RhoA(T19N), which tightly binds RhoGEF (guanine exchange factor of Rho) without promoting downstream effector activation, markedly inhibited thrombin-elicited ATP release [61]. Similar results were obtained in cells transfected with p115RGS, a $G_{12/13}$-inhibitory protein derived from the RGS (regulatory G-protein signaling) domain of p115-RhoGEF [67]. These studies suggest that thrombin-promoted ATP release on non-mucous lung epithelial cells encompasses PAR-elicited $G_{12/13}$ activation, leading to the RhoGEF-mediated exchange of GDP for GTP on RhoA.

How Rho/ROCK modulates ATP release remains unclear. However, the well-established control that ROCK exerts on the phosphorylation status of the myosin regulatory light chain (RLC) supports the attractive hypothesis that, by promoting cytoskeleton rearrangements, Rho facilitates the plasma membrane insertion and/or activation of an ATP channel, such as connexin/pannexin hemichannels.

1.2.3.2 Connexin/Pannexin Hemichannels: A Pathway for ATP Release

A gap junction, also named intercellular channel, allows direct communication between the cytosol of adjacent cells. In mammalian cells, the hemichannel contributed by each cell is formed by connexin or pannexin proteins (review: [68]). Recent evidence suggested that, unlike connexins, pannexins form single plasma membrane hemichannels (pannexons) rather than junctional channels [69].

Connexin and pannexin hemichannels have been proposed as diffusion pathways for ATP release under various experimental conditions [70, 71]. First, connexin hemichannels are highly dependent on extracellular Ca^{2+} ($[Ca^{2+}]_{ex}$), as they close in the presence of millimolar $[Ca^{2+}]_{ex}$ and open when the $[Ca^{2+}]_{ex}$ is lowered [72–74]. Exposure to low levels of extracellular divalent cations is a well-known procedure to potentiate or trigger the opening of connexin hemichannels leading to ATP release [75, 76]. In contrast, pannexin hemichannels are not gated by $[Ca^{2+}]_{ex}$ [77]. However, they were both reported to conduct ATP [70, 71, 78].

Exposure of A549 cells to thrombin resulted in a robust ATP release response that was inhibited by the non-selective blockers of connexin and pannexin hemichannels, anandamide, flufenamic acid and carbenoxolone [61]. Thrombin-evoked ATP release was accompanied by an increase in cellular uptake of the hemichannel permeable reporter dye, propidium iodide, which was inhibited by the same hemichannel blockers. These results suggest that ATP release from thrombin-stimulated lung epithelial cells occurs via connexin or pannexin hemichannels. A recent study by Ransford and coworkers provides additional support to this hypothesis in airway epithelial cells. These authors reported that ATP release from hypotonic shock-stimulated HBE cells was reduced in the presence of non-selective inhibitors of pannexin or after silencing pannexin-1 via shRNA [79].

An important unresolved issue regarding the putative involvement of connexin or pannexin in ATP release is the identification of the signaling pathways that regulate the gating of these hemichannels. Studies in *Xenopus* oocytes co-expressing pannenxin-1 with $P2Y_1Rs$ or $P2Y_2Rs$ suggested that pannenxin opens in response to intracellular Ca^{2+} elevation [80]. This mechanism, however, may not be relevant to airway epithelia since, as explained above, (i) the chelation of intracellular Ca^{2+} did not inhibit ATP release in hypotonic shock-stimulated HBE cells [34], and (ii) $P2Y_2R$ activation does not promote ATP release or propidium iodide uptake in lung epithelial cells [61]. Thus, assuming an involvement of pannexin and/or connexin in ATP release from airway epithelial cells, mechanisms other than intracellular Ca^{2+} mobilization should regulate the activity of these hemichannels. In this regard, it is noteworthy that the influx of propidium iodide uptake in thrombin-stimulated A549 cells was inhibited by Rho kinase inhibitors [61].

1.3 Conclusions and Future Directions

Nearly two decades have elapsed since the initial observation that extracellular nucleotides and nucleosides promote Cl^- secretion from airway epithelial cells [81, 82], the initial finding that led to the identification of $P2Y_2Rs$ and $A_{2B}Rs$ as major purinergic receptors regulating MCC. 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. While clues are emerging, several questions remain unanswered: How do epithelial cells control the constitutive release of nucleotides? To what extent does the Golgi compartment

contribute to ATP release in resting cells and what is the contribution of conductive/transporter mechanisms to this release? What are the molecular sensors for shear and compressive stress? How does mechanical stress impart a secondary messenger-mediated response? How do non-mucous lung epithelial cells release ATP? Finally, what is the contribution of connexin and pannexin hemichannels to ATP release in response to physiologically relevant stimuli and how are they regulated in epithelial cells? These questions are bound to unravel, yet, new avenues of research for the purinergic regulation of airway defenses.

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