

Chapter 2

Mechanisms Regulating Airway Nucleotides

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Abstract In the respiratory system, extracellular nucleotides and nucleosides serve as signaling molecules for a wide spectrum of biological functions regulating airway defenses against infection and toxic material. Their concentrations are controlled by a complex network of cell surface enzymes named ectonucleotidases. This highly integrated metabolic network combines the activities of three dephosphorylating ectonucleotidases, namely nucleoside triphosphate diphosphohydrolases (NTPDases), nucleotide pyrophosphatase/phosphodiesterases (NPPs) and alkaline phosphatases (APs). Extracellular nucleotides are also inter-converted by the transphosphorylating activities of ecto adenylate kinase (ectoAK) and nucleoside diphosphokinase (NDPK). Different cell types use specific combinations of ectonucleotidases to regulate local concentrations of P2 receptor agonists (ATP, UTP, ADP and UDP). In addition, they provide AMP for the activity of ecto 5'-nucleotidase (ecto 5'-NT; CD73), which produces the P1 receptor agonist: adenosine (ADO). Finally, mechanisms are in place to prevent the accumulation of airway ADO, namely adenosine deaminases and nucleoside transporters. This chapter reviews the properties of each enzyme and transporter, and the current knowledge on their distribution and regulation in the airways.

Keywords Ectonucleotidase · CD39 · CD73 · Ectoenzyme · Alkaline phosphatase

2.1 Introduction

Ectoenzymes form a large diverse class of membrane proteins presenting their catalytic site to the extracellular environment. This chapter focuses on ectonucleotidases, which are specialized in the regulation of nucleotides and nucleosides.

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The original nomenclature based on their biochemical properties (i.e. substrate specificity, optimum pH and selective inhibitors) was recently refined based on cloning studies. For this reason, several ectonucleotidases were given more than one name over the past 50 years, which considerably hindered literature search. This chapter introduces each ectoenzyme family involved in the purinergic regulation of airway defenses, and reconciles the various aliases encountered in the literature. The detailed description of their structure and crystallography has been reviewed elsewhere and is beyond the scope of this discussion. Our intent is to provide discriminative tools for the study of ectonucleotidases, and a detailed description of their distribution and regulation in the airways.

There is increasing functional evidence that ectonucleotidases compete with P2 receptors for a limited pool of endogenously released nucleotides [1, 2]. In essence, the dephosphorylation of surface nucleotides terminates or limits the amplitude of the P2 receptor-mediated responses, either through autocrine or paracrine events. For instance, the expression level of NTPDase2 on rat portal myofibroblasts influences the proliferation of adjacent bile duct epithelial cells promoted by P2Y receptors [3]. The modulatory effects of ectonucleotidases will vary locally with the local sources of nucleotides and the substrate selectivity of the P2 receptors.

2.2 Three Families of ATP-Hydrolyzing Ectonucleotidases

The current literature recognizes three families of cell surface enzymes bound to the plasma membrane with their catalytic sites oriented toward the external milieu: the APs, the NTPDases and the NPPs (review: [4]). While APs have been known for >80 years, the other families were more recently defined following the establishment of molecular biology techniques which found associations to previously unrelated genes. This section provides a brief overview of this historical perspective, which will allow readers to search the literature efficiently using the various aliases assigned to each ectonucleotidase over time. This section focuses on the biochemical characteristics of each enzyme, their distribution in the airways and physiological roles. The impact of pathologies on their regulation will be discussed in [Chap. 4](#).

2.2.1 *Alkaline Phosphatases*

Alkaline phosphatases (APs; EC 3.1.3.1) have been investigated >80 years in human tissues (review: [5]). For decades, most studies focused on the measurement of AP activity released in body fluids as a diagnostic marker of pathologies and disease severity (review: [6]). Their properties emerged in the 1970s as techniques became available for the investigation of plasma membrane glycoproteins. Based on selective inhibitors, sequence mapping and tissue distribution, four different isoenzymes have been identified (Table 2.1): intestinal AP (IAP), tissue non-specific AP

Table 2.1 Mammalian alkaline phosphatases

Protein name	Aliases	Gene (name; accession #)	Inhibitors [IC ₅₀ ; mM] [7]
Intestinal alkaline phosphatase	IAP, IALP	Human: <i>ALPI</i> ; NM_001631 Mouse: <i>Akp3</i> ; NM_007432	Levamisole [6.8] L- <i>p</i> -bromotetramisole [>50] L-phenylalanine [1.0]
Non-specific alkaline phosphatase	NSAP, TNAP, TNSALP Liver-bone-kidney AP	Human: <i>ALPL</i> ; NM_000478 Mouse: <i>Akp2</i> ; NM_007431	Levamisole [0.03] L- <i>p</i> -bromotetramisole [0.01] L-phenylalanine [30.0]
Placental alkaline phosphatase	PLAP, PLALP	Human: <i>ALPP</i> ; NM_001632	Levamisole [1.7] L- <i>p</i> -bromotetramisole [0.3] L-phenylalanine [1.0]
Germ cell alkaline phosphatase	GCALP	Human: <i>ALPP2</i> ; NM_031313	n.d.
Embryonic alkaline phosphatase	EAP	Mouse: <i>Akp5</i> ; NM_007433	n.d.

(TNAP; NSAP), placental AP (PLAP) and germ-like AP (review: [8]). These enzymes are all encoded by chromosome 2, except for TNAP, which is derived from chromosome 1. They form homodimers bound to cell surfaces by a glycosyl phosphatidyl inositol (GPI) linkage limited to the outer leaflet of the bilayer, which confers lateral mobility [9]. It was suggested that GPI-linked proteins are targeted to the apical surface of polarized epithelia to participate in cell-cell interaction and signal transduction through rapid lateral redistribution. The cell surface density of APs is also locally influenced during infection by phosphatidyl inositol-specific phospholipases C or D released from bacteria to cleave the GPI linkage. Alternatively, the activation of bombesin receptors on nasal epithelia stimulated the release of AP [10], thereby supporting the existence of autocrine secretory mechanisms.

These highly versatile enzymes catalyze the removal of terminal phosphate groups from a variety of natural substrates, including nucleotides (ATP, ADP, AMP) and glucose-6-phosphate, as well as pyrophosphate and pyridoxal-5'-phosphate both involved in bone mineralization. An aberrant regulation of pyridoxal-5'-phosphate has been shown to provoke epileptic seizures in patients with hypophosphatasia (review: [8]). In addition, APs regulate signaling events through dephosphorylation of the receptor agonists: lysophosphatidate and ceramide 1-phosphate [11]. Synthetic substrates (β -glycerophosphate, *p*-nitrophenyl phosphate and *p*-nitrophenyl thymidine 5-monophosphate; PNP-TMP) are commonly used to measure AP activity in body fluids.

The AP inhibitors classically presented in biochemical studies should be used with caution, as non-specific interactions occur at high concentrations [7, 12–15]. For instance, 10–20 mM levamisole is routinely used to identify TNAP, which is a sufficiently high concentration to recognize other AP isoforms (Table 2.1). On the other hand, many studies use 1 mM levamisole to exclude the activity of all APs in complex enzyme preparations, which is nearly a log lower than the IC₅₀ of IAP.

Since several APs are often co-expressed, the inhibition conditions should be validated at the mRNA level. The recent development of highly specific inhibitors for TNAP [16, 17] and PLAP [18] may provide the necessary tools to elucidate their respective roles.

It is widely believed that APs constitute low affinity enzymes with limited ability to regulate low physiological nucleotide concentrations. This misconception was created because biochemists replicated the assay conditions originally used to quantify AP activity in body fluids. Accordingly, purified TNAP documented under alkaline conditions (pH 9–10) with PNP-TMP or ATP presented a low-affinity catalytic site, with K_m values in the 0.5–10.0 mM [19, 20]. More recently, Fortuna and collaborators compared the kinetic properties of purified TNAP under alkaline (pH 10.0) and physiological (pH 7.5) conditions. They confirmed that TNAP exhibits low affinity for ATP at alkaline pH ($K_m = 8.3$ mM), but also high-affinity at physiological pH ($K_m = 5$ μ M) [21]. These data, confirmed by independent groups [22–24], indicate that TNAP has the capacity to regulate endogenous ATP concentrations under physiological conditions.

Two members of the AP family have been identified in human airway epithelia: TNAP and PLAP. The surface activity and protein expression of TNAP has been detected on the apical surface of the epithelial barrier, from the trachea down to alveoli [25–27]. While it constitutes 85% of the AP activity measured in broncho-alveolar lavage (BAL) fluid, the soluble enzyme is mainly derived from surfactant secretion [28, 29]. In the alveolar region, immunolocalization and functional assays showed that TNAP is only expressed on the apical membrane and lamellar bodies of Type II pneumocytes [30, 31]. In contrast, PLAP is restricted to Type I pneumocytes [14, 25, 32]. Consequently, these APs are used as cell markers during the purification and culture of specific pneumocytes [33].

The biochemical properties of TNAP support a role in the purinergic regulation of airway defenses [26, 27] (Fig. 2.1). On polarized primary cultures of human bronchial epithelial cells, the enzyme exhibits a low-affinity ($K_M = 717$ μ M) and a high-affinity ($K_M = 6$ μ M) toward AMP at pH 7.5 [26], as reported for purified APs [23, 34]. However, this enzyme competes with CD73 for the conversion of AMP into ADO, and accounts only for 15% of the overall hydrolytic activity toward physiological (<1 μ M) concentrations [26]. On the other hand, the contribution of TNAP increased with AMP concentration, suggesting that its primary role is to eliminate excess nucleotides in the ASL during stress situations, such as infection or tissue damage. Furthermore, assays conducted on purified TNAP demonstrated that hydrolytic rates increase with the degree of phosphorylation of the substrate (AMP > ADP > ATP) [19]. This intrinsic property suggests that TNAP may play a significant role in the regulation of physiological ATP concentrations on airway surfaces.

Other cell types encountered in the airway tissue express TNAP, including fibroblasts, lymphocytes [35], macrophages and neutrophils (reviews: [36, 37]). Ultracytochemical studies revealed that resting neutrophils restrict TNAP to intracellular membranes [38]. Cell stimulation by the bacterial products formyl-Met-Leu-Phe (fMLP) or lipopolysaccharide (LPS), or phorbol myristate acetate (PMA; protein kinase C activator), induces the mobilization of TNAP to the

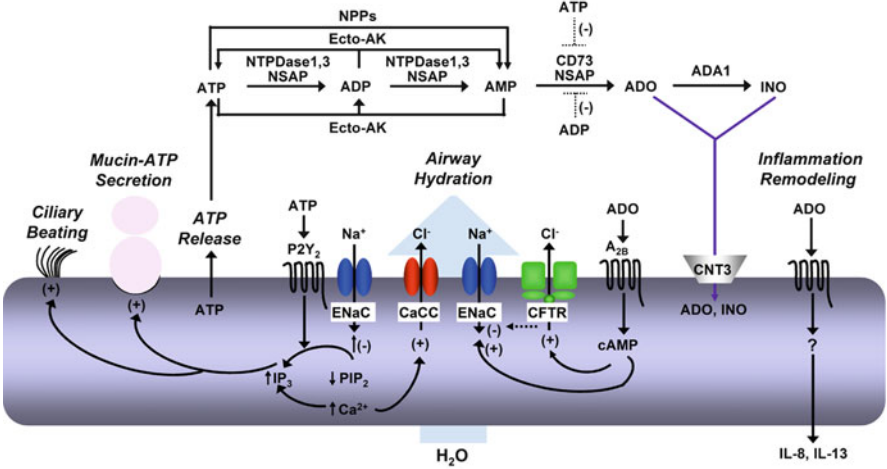


Fig. 2.1 The “Purinome” of human airway epithelia. The apical surface concentrates proteins specialized in the regulation of ATP and adenosine levels named ectonucleotidases. Although the enzymatic network is positioned above the surface for clarity, all enzymes are membrane proteins. This network regulates the availability of ATP for P2Y₂ receptors and adenosine for A_{2B} receptors, which mediate various epithelial functions involved in airway defenses against infection: ciliary beating activity, airway hydration and inflammation

surface, particularly at sites of cell-to-cell interaction with endothelial or Type I alveolar cells [38, 39]. This focal surface distribution is consistent with the GPI linkage allowing fluent lateral movement within the outer leaflet of the surface membrane.

The gram-negative bacteria invading the airways, including *Pseudomonas aeruginosa*, also exhibit AP activity. However, the enzyme is confined to the periplasmic region [40]. During normal growth, they slough off outer membrane vesicles with a fraction of their periplasmic content, including β -lactamase, hemolytic phospholipase C, AP and Cif (review: [41]). A recent study showed that these vesicles fuse with lipid rafts in the host plasma membrane and these virulence factors enter the cytoplasm via N-WASP-mediated actin trafficking, where they are distributed to specific sub-cellular locations to affect host cell biology [42]. This vesicular mode of delivery is particularly interesting given the postulated role of APs in the neutralization of LPS. This endotoxin elicits serious inflammatory reactions that may be lethal, particularly when the compound enters the circulation. The toxic moiety of LPS (lipid A) contains two phosphate groups highly conserved among gram-negative bacteria, and essential for their biological actions (review: [43]). The incubation of LPS with AP causes the formation of monophosphoryl lipid A, which is a weak macrophage activator of low toxicity, compared to diphosphoryl lipid A.

The first demonstration of endotoxin dephosphorylation by APs was provided by Holst et al. [44], who used commercially available calf IAP to release inorganic phosphate from the *Salmonella minnesota* and *Escherichia coli* LPS. Several *in vitro* and *in vivo* studies showed that TNAP and IAP have the capacity to reduce the cell

toxicity of LPS and associated inflammatory responses [45–48]. In 1997, Poelstra et al. published two studies showing that endogenous AP neutralizes *E. coli* LPS at physiological pH [45, 49]. Histochemical analysis of cryostat sections showed that intestinal and renal tissues dephosphorylate *E. coli* LPS. This enzymatic reaction was blocked by levamisole and the tissue distribution of the released phosphate corresponded to that generated by β -glycerophosphate. In addition, the local intra-dermal inflammatory reaction (PMN and macrophage infiltration) caused by systemic injection of LPS was strongly attenuated by pre-treatment of the endotoxin with exogenous AP. Since these initial studies, several groups demonstrated the importance of IAP for the maintenance of gut barrier integrity (review: [50]). The therapeutic potential of oral IAP was investigated in colitic rats, which responded by a significant reduction of colonic inflammation in terms of cytokines (TNF α , IL-1 β , IL-6) and inflammatory cell influx, and a restoration of normal intestinal wall morphology [51]. A study comparing wild-type and IAP knockout mice showed that IAP also prevents bacterial invasion across the gut mucosal barrier promoted by ischemia-reperfusion [52]. Interestingly, patients with Crohn's disease and ulcerative colitis exhibit low IAP mRNA levels in intestinal biopsies compared to healthy subjects [51]. These studies motivated the clinical trials currently conducted by AM-Pharma Inc with bovine IAP for the treatment of ulcerative colitis.

In the airways, a similar role was proposed for TNAP expressed on airway surfaces against the gram-negative bacteria omnipresent in chronic airway diseases: *P. aeruginosa*. Commercially available TNAP was found capable of dephosphorylating *P. aeruginosa* LPS [53]. In addition, pre-treatment of LPS with TNAP markedly reduced the inflammatory reaction of cultured human bronchial epithelial cells, measured by the release of nitric oxide [53]. These data suggest that the uniform expression of TNAP along airway surfaces may constitute a defense mechanism to reduce bacterial toxicity.

2.2.2 Nucleoside Triphosphate Diphosphohydrolases

The existence of cell surface enzymes capable of dephosphorylating ATP and ADP at physiological pH has been documented for over five decades under various aliases: apyrase, ectoATPase, ectoADPase and ATP-diphosphohydrolase (reviews: [4, 54, 55]) (Table 2.2). The identity of these ectonucleotidases was clarified in the 1990s by studies conducted on purified preparations, which revealed that, in many cases, a single enzyme targeted both nucleotides. Given their broad substrate specificity toward purine and pyrimidine nucleotides, the general term nucleoside triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5) was adopted in 2000 by the scientific community [62]. Since then, the number of publications describing their properties and tissue distribution has grown exponentially. The identification of NTPDase1 as the lymphocyte cell activation antigen, CD39, was undoubtedly the most important event which raised the interest of the scientific community toward this family of ectonucleotidases.

Table 2.2 Mammalian nucleoside triphosphate diphosphohydrolases (NTPDases)

Protein name	Aliases	Gene mouse	HUMAN	Gene Locus human	Accession number	ATP/ADP ratio	K _m (ATP) μM	References
NTPDase1	Apyrase, ATPDase, CD39	<i>ENTPD1</i> <i>Entpd1</i>		10q24	NM_001776, U87967 NM_009848	1.1–1.3	10–17	[56, 57]
NTPDase2	CD39L1, ecto-ATPase	<i>ENTPD2</i> <i>Entpd2</i>		9q34	NM_203468, AF144748 NM_009849, AY376711	7.2–22	70–400	[58–60]
NTPDase3	CD39L3, HB6	<i>ENTPD3</i> <i>Entpd3</i>		3p21.3	NM_001248, AF034840 NM_178676, AY376710	3.6–4.2	75	[58]
NTPDase4	UDPase, LALP70	<i>ENTPD4</i> <i>Entpd4</i>		8p21	NM_004901, AF016032 NM_026174			
NTPDase5	CD39L4, ER-UDPase, PCPH	<i>ENTPD5</i> <i>Entpd5</i>		14q24	NM_001249, AF039918 NM_007647, AJ238636			
NTPDase6	CD39L2	<i>ENTPD6</i> <i>Entpd6</i>		20p11.2	NM_001247, AY327581 NM_172117			
NTPDase7	LALP1,	<i>ENTPD7</i> <i>Entpd7</i>		10q24	NM_020354, AF269255 NM_053103, AF288221	1.1	93	[61]
NTPDase8	Liver canalicular, ecto-ATPase, hATPDase	<i>ENTPD8</i> <i>Entpd8</i>		9q34	NM_198585, AY430414 NM_028093, AY364442			

Thus far, eight NTPDases have been cloned and functionally characterized using purified protein preparations and heterologous expression (Table 2.2). They share highly conserved sequence domains named “conserved apyrase regions” which define their catalytic properties. Among them, NTPDase1, 2, 3 and 8 are localized in the plasma membrane with their catalytic site facing the extracellular milieu. They are anchored by two transmembrane domains and readily form homooligomers (dimers, trimers and tetramers), which enhances their activity [63]. Other members of this family are sequestered in the Golgi (NTPDase4) and endoplasmic reticulum (NTPDase7), with their catalytic site facing the lumen of the organelle. In the Golgi, NTPDase4 preferentially uses UDP to assist in the import of nucleotide sugars. Finally, the NTPDase5 and 6 isoforms are secreted as soluble enzymes following heterologous expression.

The cell surface NTPDases are functionally differentiated based on substrate preferences. While a single catalytic site supports the hydrolysis of triphosphates and diphosphates, each enzyme hydrolyses these two classes of substrates with distinct efficiencies, which influences the transient accumulation of active intermediates (Table 2.2). For instance, NTPDase1 hydrolyses ATP and ADP at similar rates, resulting in little accumulation of ADP for the activation of P2Y₁ or P2Y₁₂ receptors. In contrast, NTPDase2 exhibits a strong preference for ATP, allowing ADP to accumulate for an extended period of time before being converted to AMP. NTPDase3 and 8 generate intermediate metabolic profiles. The readers are referred to the original manuscript of Seigny et al. [58] for a detailed review of the metabolic properties of human and murine cell surface NTPDases. The physiological consequences of these distinct properties on cell signaling depend on the composition of the local “Purinome” in terms of ectonucleotidases and receptors.

A considerable number of compounds are known to inhibit the cell surface NTPDases, although most of them also interfere with P2 receptor activation [64, 65] (Table 2.3). Azide was originally described as an inhibitor of the ATP-diphosphohydrolase [74] later identified as NTPDase1 (CD39) [75, 76]. Recent studies showed that azide inhibits NTPDase1 [66] and NTPDase3 [67], but not NTPDase2 and 8 [68, 69], TNAP [77] or NPPs [78]. However, azide is not suited for animal protocols or prolonged exposures, as the compound induces necrotic cell death [79]. The stable analogue of ATP, 6-*N,N*-diethyl-D- β , γ -dibromomethylene ATP (ARL67156; FPL67156), was the first compound developed to inhibit ectonucleotidases without affecting P2 receptors [80]. In fact, ARL67156 exhibits a weak antagonistic effect against P2X receptors and a weak agonist effect on P2Y receptors. This compound was used to uncover the purinergic elements involved in complex signaling events, such as neurotransmission *in vivo* [81]. However, recent studies showed that this weak competitive inhibitor is only effective in the presence of endogenous levels of nucleotides (<10 μ M) [82]. Also ARL67156 is nearly inactive against NTPDase2 [70, 82], and interacts with members of the NPP family [82]. Therefore, protocols using ARL67156 must be carefully designed to account for these limitations.

A capillary electrophoresis method was recently developed for the identification of selective NTPDase inhibitors [70]. The enzymatic reactions are performed within

Table 2.3 Inhibitors of surface nucleoside triphosphate diphosphohydrolases (NTPDases)

Inhibitor names	NTPDase1 Ki – % maximal inhibition	NTPDase2 Ki – % maximal inhibition	NTPDase3 Ki – % maximal inhibition	NTPDase8 Ki – % maximal inhibition	References
Azide	n.a. – 40–60%	No	n.a. – 40–60%	No	[66–69]
RB2	20.0 – 75%	24.2	1.1	Unknown	[70]
PPADS	46.0	44.2	3.0	Unknown	[70]
Suramin	300	65.4	12.7	Unknown	[70]
ARL 67156	27.0	>1,000 – 50%	112.0	Unknown	[70]
POM-1	2.6 – n.a.	28.8 – n.a.	3.3 – n.a.	Unknown	[71]
mabNTPDase3	No	No	^a 35 ng·ml ^{–1} – 70%	n.a. – 0%	[72]
PSB-06126	No	No	1.5 – 75%	Unknown	[73]
PSB-069	15.7 – 100%	18 – 100%	16.4 – 100%	Unknown	[73]

^aThis value corresponds to the concentration causing 50% inhibition (IC₅₀)

a capillary inlet using membrane preparations of transfected CHO cells, followed by electrophoretic separation of the reaction products. While this method allows for rapid screening of potent inhibitors, the substrate affinities (Michaelis constants; K_M) of NTPDase1, 2 and 3 in this expression system were 76, 203, and 311 μM , respectively. These K_M values are considerably higher than reported by classical test tube assays with respect to NTPDase1 and NTPDase3 (Table 2.2), suggesting that the inhibitory constants (K_i) of their inhibitors are also overestimated by this method. Nonetheless, this approach remains extremely useful for the identification of selective NTPDase inhibitors. For instance, the P2 receptor antagonists reactive blue 2, suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) inhibit NTPDase3 at least 15-fold more efficiently than NTPDase1. The capillary method also led to the identification of a novel class of NTPDase inhibitors, the polyoxometalates [71]. Among them, polyoxotungstate (POM-1) is very attractive for animal studies because of its high stability at physiological pH (>14 days). Nonetheless, it is important keep in mind that this compound is equally potent against NTPDase1 and NTPDase3, which are inhibited ten times more efficiently than NTPDase2. The usefulness of POM-1 would be limited to tissues or cell types expressing either NTPDase1 or NTPDase3. While these polyoxometalates were tested on rat NTPDases [71], POM-1 was also found effective in reducing extracellular ATP metabolism in mice [83, 84]. This technique was also used to evaluate the potential of anthraquinone derivatives of reactive blue 2 [73]. This study identified 1-amino-2-sulfo-4-(1-naphthylamino) anthraquinone as a potent and selective inhibitor of NTPDase3 ($K_i = 1.5 \mu\text{M}$) having no effect on NTPDase1 and 2 [73]. While these drugs were not tested against NTPDase8, this is a minor complication since the distribution of this isoform is limited to the liver [69, 85].

Sevigny and collaborators recently developed the first monoclonal antibody against human NTPDase3, as shown by immunolocalization of the enzyme in the human pancreas [72]. More importantly, this antibody also selectively inhibits the activity of NTPDase3 [72]. The advantage over synthetic drugs is the high

specificity and the lack of side-effects. Incidentally, POM-1 was recently found to inhibit central synaptic transmission in cerebral and hippocampal slices by a mechanism which does not involve NTPDase inhibition [86]. Ultimately, the choice of inhibitor will depend on whether the goal is to prevent the degradation of extracellular nucleotides or to target a specific NTPDase. In the latter, the most appropriate inhibitor would be dictated by the NTPDases co-expressed by the target cell or tissue. Unfortunately, in the case of NTPDase2 and NTPDase8, this field of research still relies on silencing protocols and genetically manipulated animal models to identify their physiological roles.

In the respiratory system, the presence of NTPDases was initially inferred by Northern blot analysis of human total lung RNA, which only presented a signal for NTPDase1 [75, 87, 88]. Since then, the expression of NTPDase1 in the lung was confirmed by immunohistochemistry in murine [89], bovine [90] and human [91] tissue, specifically on the endothelial and alveolar epithelial cells. More recently, NTPDase1 and 3 were immunolocalized on the epithelial barrier extending the entire respiratory system, except in the alveolar region for NTPDase3 [91]. These enzymes also exhibited unique epithelial polarities which shifted in opposite directions from the proximal to distal airways. In the tracheo-bronchial area, NTPDase1 was limited to the apical surface, whereas NTPDase3 labeled basolateral surfaces and basal cells. In the distal airways, NTPDase1 was detected on the basolateral surface, whereas NTPDase3 expression was bilateral. Such polarity shifts along the airways have been reported for other proteins, including the water channel, aquaporin3 [92]. The mechanisms regulating the epithelial polarities of the NTPDases in the airways have not been elucidated. However, LPS caused the relocation of liver canalicular NTPDase activity from the apical to basolateral surfaces [93], an enzyme recently identified as NTPDase8 [69]. The fact that thousands of LPS particles are inhaled every day and deposited mainly in the distal airways could explain the gradual polarity shifts of the two NTPDases.

The functional expression of NTPDase1 and 3 on airway surfaces was demonstrated by selective assays conducted on polarized primary cultures of human bronchial epithelial cells [91]. Fausther and collaborators developed an assay strategy to differentiate the activities of these two azide-sensitive NTPDases [91]. The same culture wells were assayed in the presence of the monoclonal antibodies against NTPDase3 that selectively inhibit this enzyme activity, then assayed in the presence of azide. The use of a wide range of nucleotide concentrations showed that NTPDase1 predominates (40%) under physiological conditions ($<1 \mu\text{M}$ ATP), while NTPDase3 predominates (70%) in the presence of excess nucleotides generated by cell lysis. These findings were consistent with their catalytic properties, as NTPDase1 is a low-capacity high-affinity enzyme ($K_M = 10\text{--}20 \mu\text{M}$ [56]), and NTPDase3 is a high-capacity low-affinity enzyme ($K_M = 90\text{--}130 \mu\text{M}$ [67]). Collectively, their distinct polarities and biochemical properties suggest that these two NTPDases regulate different epithelial functions on airway surfaces. For instance, human airway epithelia package nucleotides into mucin granules, which are released into the ASL by $\text{P2Y}_2\text{R}$ activation [94]. Since NTPDase3 is preferentially located on the mucin-secreting cells of superficial epithelia and submucosal

glands [91], this high-capacity enzyme may regulate the amplification cycles of P2Y₂R activation resulting from ATP-induced mucin and nucleotide secretion [95].

The presence of soluble NTPDases in the airway secretions has not been investigated. However, azide-sensitive ATPase activity has been detected in airway surface liquid (ASL) collected from human bronchial epithelial cultures (M. Picher 2004, personal communication). Likewise, shear stress was reported to induce the secretion of ectoATPases from endothelial cells in culture [96], a cell type known to express mainly NTPDase1 as ectonucleotidase (review: [55]). Since the airway surfaces are subjected to considerable mechanical stress, such as rhythmic breathing and coughing, they could potentially release NTPDases into airway secretions. Also, the epithelial cells lining the small ducts of rat pancreas have been shown to secrete particulate NTPDase1 in response to the endogenous peptide cholecystokinin (CCK-8) [97]. These studies raise the possibility that secreted NTPDases may participate in the regulation of airway nucleotides.

2.2.3 Nucleotide Pyrophosphatase/Phosphodiesterases (NPPs)

These ectonucleotidases have the unique ability to generate nucleoside monophosphates from a wide variety of nucleotides and dinucleotides. However, some members were already known under aliases based on their physiological roles. For instance, NPP1 was discovered as a plasma cell differentiation antigen (PC-1), NPP2 as an autocrine motility factor (autotaxin), and NPP3 as a monoclonal antibody RB13-6 against a subset of brain glial cells (gp130^{RB13-6}) (Table 2.4). The nomenclature was simplified in 2000 by numbering each NPP according to their order of discovery (review: [62]).

The NPP family now contains seven cell surface ectonucleotidases as single spanning transmembrane proteins in the type I (intracellular C-terminal; NPP4-7) or type II (intracellular N-terminal; NPP1 and NPP3) orientation (reviews: [98, 99]). The N-terminal hydrophobic sequence of NPP2 and NPP4-7 is a signal peptide that mediates uptake into the endoplasmic reticulum during translation. This sequence is removed by the signal peptidase, leaving NPP4-7 anchored to the plasma membrane by a C-terminal transmembrane domain. The NPP2 isoform is encoded by a different chromosome and synthesized as a pre-pro-enzyme. After removal of the N-terminal signal peptide and trimming by a furin-type protease, NPP2 is secreted as a soluble enzyme [100]. Three NPP2 isoforms generated by splicing were identified over the years and recently reviewed ([101]): ATX α was isolated from the human melanoma cell line A2058, ATX β from the Ntera2D1 human teratocarcinoma cell line, and ATX γ from rat brain (Table 2.5).

Heterologous expression confirmed that NPP1-3 exhibit phosphodiesterase I (EC 3.1.4.1) and pyrophosphatase (EC 3.6.1.9) activities [106] using a single nucleotide binding site [107]. Such versatility allows them to support the metabolism of ATP, UTP, NAD, dinucleotides (i.e. Ap₄A), the phosphodiester bond of nucleic acids and the pyrophosphate linkage of nucleotide sugars (i.e. UDP-glucose). The synthetic

Table 2.4 Mammalian nucleotide pyrophosphatase/phosphodiesterases (NPPs)

Name	Additional name	Tertiary structure	Gene locus	Accession numbers	Substrates	Distribution
NPP1 <i>ENPP1</i>	PC-1, PC-1, MAFP, NPPase, NPP γ <i>PDNPI</i> , <i>NPPS</i> , <i>Pca-I</i>	Type II	6q22-23		Mononucleotides Dinucleotides	Lymphocytes, bones, cartilage, heart, liver intestine, testis, placenta
NPP2 <i>ENPP2</i>	NPP α , <i>PDNP2</i> , Autotaxin (ATX), NPP2, ENPP2	Secreted	8q24.1	BC034961	Mononucleotides Dinucleotides LPC	Brain, placenta, ovary Intestine
NPP3 <i>ENPP3</i>	B10, gp130 ^{RB13-6} , PD-1 β , NPP β , <i>PDNP3</i> , <i>pdnpno</i>	Type II	6q22	NM_005021	Mononucleotides Dinucleotides Unknown	Basophils, prostate, uterus, colon, hepatocytes
NPP4 <i>ENPP4</i>		Type I	6p12.3	NM_014936		
NPP5 <i>ENPP5</i>		Type I	6p11.2-21.1	NM_021572	Unknown	
NPP6 <i>ENPP6</i>		Type I	4q35.1	NM_153343	Choline phosphate esters (LPC)	
NPP7 <i>ENPP7</i>	Alkaline sphingomyelinase	Type I		NM_178543	Choline phosphate esters (LPC) Sphingomyelin	

Table 2.5 Nucleotide pyrophosphatase/phosphodiesterase 2 isoforms (NPP2s)

Name	Additional name	Accession #	Source for original cloning	Splice variant
ATX α	ATX NPP2 α , ATX _{mel} , PDN2 α	NM_006209 Transcript variant 1	Human melanoma cell line A2058 [102]	Lacks exon 21
ATX β	NPP2 β , ATX _{ter}	NM_001040092 Transcript variant 2	Human teratocarcinoma cell line Ntera2D1 [103] Human retina cDNA library [104]	Lacks exons 12, 21
ATX γ	PD-1 α , NPP2 γ	NM_001130863 Transcript variant 3	Rat brain cDNA [105]	Lacks exon 12

compound *p*-nitrophenyl-thymidine 5-monophosphate (*p*NP-TMP) is commonly used as a selective substrate of NPPs. Partially purified NPPs from rat C6 glioma cells [108] and human fetal serum [109] were also reported to hydrolyze ADP. But, since most cell types co-express APs and/or NTPDases with NPPs, the identification of ADP as NPP substrate remains to be verified in transfected cells.

Recent investigations identified additional natural substrates providing a high degree of specificity among the NPP isoforms (review: [99]). NPP2 exhibits lysophospholipase-D activity toward lysophosphatidylcholine, which generates the signaling molecule lysophosphatidic acid (LPA). This enzyme also generates the cell mobility factor sphingosine-1-phosphate (S1P) from sphingosylphosphorylcholine. As for the other NPPs, the natural substrates of NPP4-5 remain unknown, whereas NPP6 and 7 hydrolyze phosphodiester bonds from lysophospholipids and sphingomyelin, respectively. To date, the physiological consequences of NPP6 activity have not been established. Regarding NPP7, since sphingomyelin metabolism generates the signaling molecule ceramide, this isoform may influence cell proliferation. On a general note, NPPs experience considerable feedback inhibition, as they bind the phosphorylated products (AMP and LPA) with higher affinity than the substrates. Finally, only NPP1-3 have the capacity to regulate nucleotides, and are therefore relevant for purinergic signaling.

The first agents tested as inhibitors of the NPP family were known inhibitors of other ectonucleotidases and P2 receptors as a means to test for specificity and cross-inactivation of signaling events on intact cells and tissue. The biochemical characterization of rat heart NPP showed that the enzyme activity is not inhibited by blockers of TNAP (levamisole) or NTPDase1 and 3 (sodium azide), but reduced to about 50% by the P2 receptor antagonist, suramin [78]. The RT-PCR analysis of this preparation supported the expression of NPP2 and NPP3, but not NPP1 in the heart. Likewise the NPP activity of rat C6 glioma cells was inhibited by the P2 receptor antagonists suramin, reactive blue 2, PPADS [110] and β,γ -methyleneATP [111]. In contrast, the P1 receptor antagonists CGS15943, 8-phenyl-theophylline, cyclo-pentyltheophylline (CPT) and iso-butylmethylxanthine (IBMX) had no significant effect. These agents were further tested by capillary electrophoresis using NPP1 and NPP3 transfected in COS-7 cells [112]. Suramin inhibited NPP3 activity seven times more efficiently than

NPP1, with K_i values in the sub-micromolar range. In contrast, reactive blue inhibited both NPP activities with comparable efficiency. These studies suggest that P2 receptor antagonists affect the availability of their agonists through competitive inhibition. For instance, PPADS raised by threefold the inhibitory effects of ATP on isoproterenol-induced cAMP synthesis in rat C6 glioma cells by a mechanism involving NPP inhibition [110]. Therefore, the scientific community turned to unrelated compounds for the inhibition of this family of ectonucleotidases. More recently, a series of 1,3,4-oxadiazole-2(3*H*)-thiones and 1,3,4-thiadiazole-2(3*H*)-thiones were synthesized and evaluated for their inhibitory effects on recombinant NPP1 [113]. While this study presented the first non-competitive inhibitors against NPPs, the most effective compound exhibited a K_i value of 360 μ M. With such low efficiency, these compounds should be tested for specificity and possible side-effects.

A number of drugs already on the market, or currently tested in clinical trials, have been shown to affect the activity of NPPs. For instance, statins are described as cholesterol-reducing agents with anti-inflammatory properties [114]. The treatment of human blood basophils with cerivastatin and atorvastatin led to the down-regulation of NPP3 and reduced their capacity to release histamine in response to IgE [115]. This response is not surprising since NPP3 was first characterized as a basophil-activation antigen whose expression is raised by IgE-mediated cell activation [116]. Whether statins interact with the ectonucleotidase is unknown. Interestingly, the anti-depressant drugs, imipramine, fluoxetine and moclobemide, have been shown to raise the NPP activity of cultured cells from rat salivary glands [117]. Since ATP promotes salivary flow [118], NPP activation may contribute to the common side-effect associated with these drugs, which is dry mouth [119]. Also, FTY720 (fingolimod) is currently in Phase three clinical trial for the treatment of multiple sclerosis [120]. Structurally similar to sphingosine, the phosphorylated form generated *in vivo* (FTY720-P) interacts with the receptor of SIP, product of NPP2 activity. This drug inhibits the activating effects of SIP on lymphocytes by triggering the internalization and degradation of the receptor, which then fail to migrate from the lymphoid organ to the site of inflammation [121]. Interestingly, FTY720-P also inhibits the activity of NPP2 with a K_i of 200 nM [122], which may explain its anticancer activity [123]. To date, no selective inhibitor of NPP3 has been reported likely due to the lack of evidence for pathological conditions associated with functions or deregulations of this isoform.

The presence of NPPs in the respiratory system was first inferred by Northern blot analysis of total lung RNA, which combines genetic material from pneumocytes, fibroblasts, endothelial cells and inflammatory cells. This approach supported the presence of NPP2 and NPP3, but not NPP1, in lung parenchyma [104, 124–126]. In the upper airways, NPP1, NPP2 and NPP3 were all detected in primary cultures of human bronchial epithelial cells by RT-PCR [127]. The cultures exhibited surface NPP activity toward dinucleotides, ADP-ribose and UDP-glucose, as well as competitive activities between dinucleotides and ADP-ribose [128]. While these reactions were concentrated on the apical surface, the basolateral surface populated by basal cells also presented significant NPP activity. The surface expression of NPPs on the epithelial barrier was confirmed by immunolocalization using a polyclonal

antibody developed against NPP proteins purified from bovine intestinal mucosa [129], which express NPP2 and NPP3, but not NPP1 [103, 126, 130]. This antibody labeled the basal cells forming the basolateral surface, as well as columnar cells on the apical surface [129]. In another study, NPP1 was immunodetected in human airway epithelia only as a weak signal in submucosal glands [131]. Collectively, these studies suggest airway epithelial functionally express NPP2 and/or NPP3. Since NPP2 is secreted as a soluble enzyme [100], NPP3 would be responsible for the membrane-bound activity detected on the apical surface of human airway epithelia [128].

Maurice and collaborators provided key information on the distribution of NPP1 and NPP3 in polarized epithelia [132–134]. Using selective antibodies, they detected NPP3 on the apical surface of hepatocytes and enterocytes, whereas NPP1 was confined to the basolateral surface of hepatocytes [132]. These data are consistent with the proposed apical polarity of NPP3 in human airway epithelia [129]. This group studied the membrane trafficking of NPP3 transfected in two epithelial cell lines, Madin Darby Canine Kidney (MDCK) and human colon adenocarcinoma (Caco-2) cells, by pulse-chase ^{35}S -labeling and immunoprecipitation [134]. Their study revealed cell type specificity in membrane trafficking, as NPP3 was targeted directly to the apical surface in MDCK cells, whereas 50% of the proteins reached the basolateral membrane of Caco-2 cells before being transcytosed to the apical membrane. Numerous studies documented the functional similarities between respiratory and intestinal epithelia (review: [135]), including the polarity of ADO transporters [136]. In human airway epithelia, an indirect trafficking route for NPP3 would reconcile the bilateral distribution of the membrane-bound NPP activity [128] with the absence of NPP1 [131]. On the apical surface, high-affinity NPP3 activity could participate in the purinergic regulation of airway clearance through local regulation of nucleotides (ATP, UTP) and dinucleotides (Ap_4A) known to activate P_2 receptors [137, 138].

The possible contribution of secreted NPP in human airways should not be neglected, as the presence of NPP2 was confirmed in primary cultures of human bronchial epithelial cells and non-small-cell lung cancer (NSCLC) cell lines by quantitative PCR and Northern blot [139]. Regarding the different splice variants, the expression level of $\text{ATX}\alpha$ correlated negatively with the differentiation state of NSCLC cultures. Accordingly, *in situ* hybridization positioned $\text{ATX}\alpha$ only in the poorly differentiated basal cells of superficial epithelia, with weak staining of type II pneumocytes. In another study, $\text{ATX}\alpha$ and $\text{ATX}\gamma$ were both detected in human lung tissue by RT-PCR [105]. However, quantitative analysis revealed that $\text{ATX}\gamma$ represents <10% of the total NPP2 mRNA. Taken together, these studies suggest that the isoform immunodetected in the basal cells of bronchial epithelia corresponds to $\text{ATX}\alpha$. On the other hand, the authors point out that it is impossible to design primers specific for $\text{ATX}\beta$ because of the organization of the isoforms within the NPP2 gene [105]. Therefore, the presence of $\text{ATX}\beta$ in the respiratory system can not be excluded. Incidentally, NPP2 was immunolocalized by confocal microscopy in human bronchial tissue using an antibody which recognizes all three isoforms [140]. The epithelial barrier exhibited a strong signal in the columnar cells, concentrated beneath the apical plasma membrane (Picher 2002, personal communication).

As such, the secretion of apical NPP2 (ATX γ) would allow the airways to regulate the concentrations of P2 receptor agonists within airway secretions, which contain a variety of receptive inflammatory cells under pathological conditions.

The literature is also surging with original studies and reviews on LPA and SIP-1, which occur naturally in the surfactant and BAL fluid (reviews: [141–143]). It is widely accepted that NPP2 stimulates proliferation, migration and survival by its ability to generate LPA. This potent bioactive phospholipid activates G protein-coupled receptors (LPA₁₋₄) on human airway epithelia, which promote fibronectin and cytokine release, cell proliferation, filopodia formation and barrier integrity. On adjacent fibroblasts, LPA stimulates proliferation and collagen gel contraction. It is, therefore, not surprising that this phospholipid was shown to accelerate wound healing *in vivo* [144]. The second bioactive lipid generated by NPP2, SIP-1, was shown to activate a different family of G protein-coupled receptors (SIP1–5) [145]. These receptors stimulate cytokine secretion from the epithelial barrier [145, 146], promote the activation and recruitment of immune and inflammatory cells, and the proliferation of fibroblasts (review: [147]). Since LPA and SIP-1 concentrations increase dramatically in the BAL fluid under various pathological conditions, these studies highlight the critical importance of NPP2 for the non-purinergeric regulation of airway defenses.

2.3 The Cell Surface Transphosphorylating Enzymes

The regulation of extracellular nucleotides by cell surface kinases represents a logical extension of their role in the regulation of intracellular nucleotides, RNA and DNA synthesis. Within mammalian cells, nucleoside diphosphate kinases (NDPKs; E.C. 2.7.4.6) and nucleoside monophosphate kinases (NMPKs) support transphosphorylation reactions in the *de novo* and salvage pathways (reviews: [148, 149]). The ubiquitous intracellular NDPKs catalyze the reaction: $\text{NTP} + \text{NDP} \leftrightarrow \text{NDP} + \text{NTP}$ [149]. Among the NMPKs, the activities of adenylate kinases (AKs; E.C. 2.7.4.3) are restricted to adenine-based nucleotides by the reaction: $\text{ATP} + \text{AMP} \leftrightarrow \text{ADP} + \text{ADP}$ [148]. These enzymes have been proposed to form long distance conduits for ATP from the production sites (i.e. mitochondria) to various consumption sites, including actin-myosin fibers and plasma membrane ATP-sensitive K⁺ channels [150].

Over the past decade, few studies reported the existence of extracellular AK (ectoAK) and/or NDPK (ectoNDPK) activities at the surface of endothelial cells [151, 152], lymphocytes [152], hepatocytes [153], glomeruli [154], keratinocytes [155], astrocytoma cell [156] and the airway epithelial cells [157–159]. Both enzyme activities were also detected in ASL of human bronchial epithelial cultures and nasal secretions from healthy subjects [158]. Whether these soluble enzymes are different from the membrane-bound enzymes remains to be determined.

The human bronchial epithelial cell line, 16HBE14o-, supports an ectoNDPK activity regulating the relative concentrations of P2 receptor agonists by the

reaction: $\text{ATP} + \text{UDP} \leftrightarrow \text{ADP} + \text{UTP}$ [157]. In addition, primary cultures of human bronchial epithelial cells express an ectoAK supporting the reaction: $\text{ATP} + \text{AMP} \leftrightarrow \text{ADP} + \text{ADP}$ [158, 159]. Interestingly, the substrate affinities of these epithelial ectoAK and ectoNDPK are within the range of high-affinity ectonucleotidases (5–25 μM), such as NPPs [108, 128] and NTPDase1 [58], thereby supporting a role in the regulation of P2Y₂-mediated airway defenses. Functional assays involving the selective inhibitor, Ap₅A, demonstrated that ectoAK significantly prolongs the effective concentrations of ATP and ADP (0.1–1.0 μM) for P2Y₂Rs on airway surfaces, and reduces the rate of ADO production by sixfold [159]. These results suggest that the transphosphorylase activity of ectoAK propagates P2 receptor agonists along airway surfaces, much like the ATP conduits within the cytoplasm. Following stimulated ATP release, ectoAK competes with the dephosphorylating ectonucleotidases to maintain effective concentrations for P2Y₂R activation, which enhances MCC through Ca^{2+} -dependent Cl^- secretion, mucus secretion and cilia beating (review: [138]). The physiological contribution of ectoAK was demonstrated by a study describing the purinergic regulation of mucin secretion [160]. Human bronchial epithelial cells, grown as xenografts on the back of nude mice, responded to ADP (not UDP) by enhanced mucin secretion. These cells did not respond to 2-MeS-ADP, thereby ruling out the contribution of ADP receptors. Alternatively, the effects of exogenous ADP could be mediated following transphosphorylation into ATP by ectoAK.

The recent identification of the gene encoding ectoNDPK led to the development of an anti-NDPK antibody (Nm23-H1; Insight Biotechnology) which inhibits the surface enzyme activity [153]. Together, with the selective ectoAK inhibitor, Ap₅A, these pharmacological tools are being used to elucidate the roles of these extracellular kinases. In human hepatocytes, the inhibition of ectoNDPK and ectoAK suppressed the endocytosis of high-density lipoprotein (HDL) mediated by exogenous ADP on P2Y₁₃ receptors [153]. However, their physiological roles should be ascertained under conditions reflecting the relative concentrations of their substrates in the extracellular milieu. The impact of ectoNDPK on cell functions may be lower than anticipated due to the >10-fold lower concentrations of uridine, compared to adenine, nucleotides within the ASL layer. For instance, the addition of Ap₅A, but not the anti-NDPK antibodies, stimulated HDL endocytosis in the absence of exogenous substrates [153]. Future studies, using these tools, should reveal their roles in the regulation of airway defenses.

2.4 The Regulation of Airway Adenosine: A Balancing Act

2.4.1 *Ecto 5'-Nucleotidase*

Extracellular ADO stems predominantly from the activities of ectonucleotidases on nucleotides released by activated or damaged cells. Two ectonucleotidases support the last dephosphorylation step: TNAP and ecto 5'-nucleotidase (ecto 5'-NT; CD73;

EC 3.1.3.5). Whereas TNAP dephosphorylates ATP, ADP and AMP, the activity of CD73 is limited to monophosphates. Furthermore, the gene encoding CD73 is on a different chromosome (6q14-q21), which generates a dimer attached to cell surfaces by a GPI anchor (reviews: [161, 162]). This enzyme was also named low K_m -nucleotidase due to its high affinity for AMP ($K_m = 3\text{--}19\text{ }\mu\text{M}$) compared to its intracellular counterparts (review: [161]). This ectonucleotidase operates preferentially at neutral pH and is inhibited by the stable ADP analogue named AMPCP ($K_i = 5\text{--}19\text{ nM}$). However, since ADP also inhibits the activity of TNAP toward AMP [26], AMPCP is expected to affect both CD73 and TNAP. On cell types expressing both ectonucleotidases, their relative contributions to the production of extracellular ADO should be determined as levamisole-sensitive (TNAP) and levamisole-insensitive (CD73) activities [26]. It is important to mention that CD73 is reported on all mammalian cell types, except granulocytes and monocytes (review: [163]), which has important repercussions for the regulation of ADO-mediated inflammatory responses (see [Chap. 7](#) for details).

Polarized epithelia have been documented exhaustively with regard to the properties and physiological roles of CD73, especially in the regulation of fluid transport and barrier integrity (review: [162]). In 2003, the discovery that this ectonucleotidase is also expressed on airway epithelial surfaces [26] paved the way for numerous *in vivo* studies establishing the importance of ADO for airway defenses [84, 89, 164]. On human bronchial epithelial cultures, CD73 exhibits a bilateral polarity and a substrate affinity within the range of the ATP-hydrolyzing ectoenzymes expressed on these cells ($K_M = 14\text{ }\mu\text{M}$) [26]. Furthermore, CD73 accounts for $>80\%$ of the total ectoAMPase activity toward physiological ($<10\text{ }\mu\text{M}$) AMP levels, which identified this enzyme as the predominant regulator of airway ADO production.

2.4.2 Adenosine Deaminase

The capacity of human tissue to eliminate extracellular ADO and 2'-deoxyadenosine has been investigated for decades owing to the early discovery that congenital defects in the gene encoding adenosine deaminase (ADA; EC 3.5.4.4) causes severe combined immunodeficiency disease (SCID) (reviews: [165–168]). The cytosolic enzyme is released in the extracellular milieu and has the capacity to bind to cell surfaces on the serine protease known as CD26 (dipeptidyl peptidase IV; E.C. 3.4.14.5). On T lymphocytes, the CD26-ADA₁ complex was found essential for cell proliferation by local removal of inhibitory ADO [169]. The ADA-deficient children also exhibit high plasma levels in 2'-deoxyadenosine, which causes lymphocyte apoptosis. Hence, both substrates participate in the pathology of SCID. On the other hand, the immunodeficiency virus (HIV-1) has the ability to prevent ADA-CD26 complex formation via interference by the envelope protein gp120, which contributes to the defective T-cell activation noticed in patients with acquired immunodeficiency syndrome (AIDS) [170]. These discoveries strongly motivated the exploration of ADA

correction therapies (see [Chaps. 8 and 9](#) for details). However, the following paragraphs highlight the importance of fundamental research to identify the potential and limitations of these therapeutic applications.

The early nomenclature related to ADA and CD26 was derived from SDS-PAGE and Western blot analysis of membrane preparations. The authors identified a band of 35 kDa they named ADA-S, and a band of 280 kDa they named ADA-L (review: [\[171\]](#)). Further analysis revealed that the latter was a protein complex composed of two ADA-S and one glycoprotein called the adenosine deaminase complexing protein (ADCP). This glycoprotein was later identified as CD26 (review: [\[172\]](#)). This information will allow readers to reconcile the early literature with the current notions of ADA-CD26 complexing on cell surfaces.

In human tissue, two ADA isoforms were identified based on their distribution, catalytic properties and gene mapping. The gene that encodes ADA₁ was mapped to chromosome 20 (20q13.12), which yields a protein essential for the cytosolic purine salvage pathway. This ubiquitous ADA has the ability to bind CD26 [\[173\]](#) and to regulate physiological ADO levels ($<10\ \mu\text{M}$) with a K_M in the 20–50 μM range [\[166\]](#). In addition, some inflammatory and immune cells secrete a second isoform (ADA₂) which exhibits a weak affinity for ADO ($K_M = 2\ \text{mM}$) [\[174\]](#) and lacks the ability to bind cell surface proteins [\[173\]](#). The ADA₂ isoform was originally isolated from the conditioned medium of activated rat macrophages [\[173\]](#). In the plasma, HIV-infected T-cells [\[175\]](#) and B-cells [\[176\]](#) were also proposed as sources of plasma ADA₂. This isoform was recently purified and identified as the cat eye syndrome critical region candidate 1 (CECR1), member of a novel family of ADA-related growth factors [\[174\]](#). The gene encoding ADA₂ was mapped to the same locus as CECR1 (22q11), and sequence analysis revealed that ADA₂ is synthesized as a pre-protein containing a signal peptide released during secretion. While ADA₂ may not regulate extracellular ADO, it was recently found to affect the differentiation and proliferation states of immune and inflammatory cells [\[177\]](#).

A number of natural and synthesized ADA inhibitors have been developed based on the structure of ADO (review: [\[167\]](#)). For instance, naturally-occurring coformycin and 2'-deoxycoformycin (pentostatin) form nearly irreversible associations with ADA₁ ($K_i = 10^{-11}$ – $10^{-12}\ \text{M}$). Pentostatin is commonly used to inhibit cell surface ADA activity *in vitro* and to investigate lymphoproliferative disorders (review: [\[178\]](#)). However, this compound is also a potent inhibitor of ADA₂ activity ($K_i = 10^{-9}\ \text{M}$) [\[179\]](#), which may cause serious side-effects. To date, the best inhibitor available to distinguish the two ADA isoforms remains the specific ADA₁ inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), which has been shown not to interfere with ADA₂ activity [\[180, 181\]](#). This molecule inhibits ADA₁ by a particular two-step mechanism composed of an initial classic competitive behavior, followed by rearrangement of the two molecules into a tight enzyme-inhibitor complex. In fact, EHNA is currently used to validate novel inhibitors screened from natural extracts by ADA₁-immobilized capillary electrophoresis [\[182\]](#). Since ADA₂ inhibitors have not been identified, the activities of the two isoforms are generally determined in terms of EHNA-sensitive (ADA₁) and EHNA-insensitive (ADA₂) activities measured with a saturable ADO (1–2 mM) concentration.

In the respiratory system, the bulk of the literature on ADA is derived from measurements of total ADA activity in the BAL fluid. The enzyme activity is reported abnormally elevated in various disorders, including pulmonary interstitial lung disease, pneumonia, tuberculosis and sarcoidosis [183–188]. While *in vivo* studies measure total lung ADA in mice (review: [189]), only one study documented the presence of membrane-bound ADA on airway surfaces. Hirsh and collaborators provided a systematic biochemical characterization of ADA activities bound to the apical surface of human bronchial epithelial cultures [136]. The RT-PCR analysis showed that human nasal and bronchial epithelial cells only express the ADA₁ isoform. The conversion of ADO into inosine was completely inhibited by EHNA, and exhibited a high affinity ($K_M = 24 \mu\text{M}$) consistent with ADA₁. In contrast, both ADA isoforms were detected in lung parenchyma at the mRNA level, likely owing to the presence of monocytes and macrophages. The glycoprotein binding ADA₁ to airway surfaces has not been formally identified. Yet, CD26 has been immunolocalized on human airway epithelia [190–193] and alveolar epithelial cells [190]. Together, these data demonstrate that airway surfaces have the capacity to eliminate ADO in close proximity to the receptors located on the epithelial surfaces. In addition, a fraction of the secreted ADA₁ remains soluble in the ASL, where it regulates ADO around other cells, including inflammatory cells recruited during infection. In fact, the only information available on the mechanisms mediating ADA₁ secretion is derived from inflammatory cells. Monocytes exposed to a PKC activator (PMA) or a calcium ionophore (A23187) exhibit a fourfold decrease in ADA₁ secretion in conditioned medium [194]. This signaling mechanism was also demonstrated in neutrophils [195] and thymocytes [196], supporting a major role for the PKC-dependent signaling cascades initiated by P2Y₂Rs on inflammatory and epithelial cells. Following ATP secretion, a P2Y₂R-mediated stimulation of ADA₁ secretion could allow local ADO levels to transiently rise to promote A_{2B}R-mediated cellular responses.

2.4.3 Nucleoside/Nucleobase Transporters

Polarized epithelia counterbalance the accumulation of luminal ADO and inosine using a dual transport system maintaining vectorial flux in the apical-to-basolateral direction. This system requires the concerted efforts of two gene families: Soluble Carrier Family 28 (SLC28) and Soluble Carrier Family 29 (SLC29) (reviews: [197, 198]). The SLC28 family comprises high-affinity Na⁺-dependent concentrative nucleoside transporters (CNTs) which facilitate ADO uptake against a concentration gradient. In contrast, SLC29 designates equilibrative nucleoside transporters (ENTs) acting as low-affinity facilitated carrier proteins. The SLC28 and SLC29 glycoproteins are all oriented with the N-terminus in the cytoplasm and the C-terminus facing the extracellular milieu, and accommodate 13 and 11 membrane spans, respectively. They exhibit a variety of permeant selectivity, kinetic parameters and distributions. For instance, ENTs are ubiquitous transporters,

whereas CNTs are restricted to the polarized epithelia (reviews: [199, 200]). In the intestinal epithelium, the vectorial flux of ADO is maintained by CNTs promoting ADO uptake on the apical surface, and basolateral ENTs facilitating the release of excess ADO into the bloodstream (review: [201]).

In the respiratory system, Hirsh et al. demonstrated that polarized cultures of human bronchial epithelial cells functionally express CNT2 and CNT3 on the apical surface [136]. A survey of their mRNA levels along the airways showed that CNT2 is restricted to nasal passages, whereas CNT3 regulates ADO in nasal and bronchial epithelia [136]. The fact that CNT3 exhibited an affinity for ADO ($K_M = 17 \mu\text{M}$) comparable to that of ADA₁ ($K_M = 24 \mu\text{M}$), suggested that both mechanisms contribute to the elimination of airway ADO. However, the bronchial cultures regulated ADO primarily through ADA₁ (>80%) due to differences in kinetic efficiencies, which takes into account the maximum velocity of each mechanism. In the nasal epithelia, CNTs play a larger role in the regulation of airway ADO owing to the co-expression of CNT2 and CNT3 [136]. Thus, regional differences in the predominant elimination mechanism are expected along the airways. The functional expression of ENTs on the basolateral surface of airway epithelia has not been reported, but mRNA was detected for ENT1 and ENT2 in the BEAS-2B bronchial epithelial cell line [202]. The active uptake of airway ADO and inosine would supply nucleosides for the nucleotide salvage pathways in these cells lacking the *de novo* mechanism. On the other hand, the immune and inflammatory cells accumulating on either sides of the barrier during infection would also constitute major sources and sinks for extracellular ADO through ADA₁ and ENTs expressed at their surface.

2.5 Functional and Spatial Enzymatic Microenvironments

The integration of all information available on the ectonucleotidases regulating ATP and ADO on airway surfaces revealed the presence of two groups targeting different pools of nucleotides. We learned from *in vitro* assays conducted on human bronchial epithelial cultures that physiological nucleotide levels (<1 μM) are regulated by a high-affinity low-capacity metabolic chain including NTPDase1, E-NPPs, CD73 and ADA1. Stress situations associated with massive nucleotide release (i.e. emphysema, mechanical ventilation or chronic bacterial infection) may allow low-affinity high-capacity NTPDase3 and TNAP to prevent exaggerated inflammatory responses and apoptosis.

From a morphological point of view, high-affinity and low-affinity ectonucleotidases appear to maintain opposite expression gradients along the airways. High-affinity NTPDase1 [91] and CD73 [26] are particularly abundant in the upper airways, whereas low-affinity NTPDase3 [91] and TNAP [26] are concentrated in distal airways. These regional differences suggest that the composition of the enzymatic network is adjusted along the airways according to local requirements in nucleotide regulation and/or to regulate different defense mechanisms.

This model was further refined by considering the various cell types expressed in the respiratory epithelia, which may host different populations of ectonucleotidases. For instance, the immunolocalization of NTPDase3 revealed a preferential association with secretory cells throughout the airways, the highest expression being detected in the luminal membrane of submucosal glands [91]. This association appears specific for mucin-secreting cells because NTPDase3 is not expressed in the alveoli, including surfactant-secreting cells. The recent discovery that mucin granules accumulate high nucleotide concentrations [203] suggests that NTPDase3 may locally regulate the amplification cascades of P2Y₂R-mediated mucin secretion. In fact, the expression gradient of NTPDase3 follows that reported for mucin-secreting cells along the airways, their relative abundance in the superficial epithelium increasing toward the alveoli [204]. This morphological feature was confirmed functionally in terms of NTPDase3 activity and mRNA levels measured on the apical surface of cultured nasal, bronchial and bronchiolar epithelial cells [91]. These data suggest that selective inhibitors of NTPDase3 may be beneficial for the treatment of mucus hypersecretion, since this enzyme does not target the physiological nucleotide concentrations regulating airway hydration.

The second most important airway defense mechanism, mucociliary clearance (MCC), takes place on the ciliated epithelial cells, which express the epithelial sodium channel (ENaC) [205], CFTR [206], and the water channel aquaporin-5 (AQP5) [92]. Interestingly, these channels share the same cell specificity and expression gradient than high-affinity NTPDase1 [91] and CD73 [26]. Since these enzymes have the capacity to regulate sub-micromolar purine concentrations, they could regulate MCC via P2Y₂R-mediated ENaC activation, A_{2B}R-mediated CFTR activation and cilia beating activity [95], as well as CFTR-mediated ENaC inhibition [207]. These epithelial functions will be reviewed in detail in Chap. 5. This type of integrative data analysis introduces the concept of “Purinomes” for airway defenses, which incorporate ectonucleotidases, purinoceptors and effector proteins into functional protein clusters.

In conclusion, the distinct biochemical and catalytic properties, cell type specificities and expression gradients exhibited by the ectonucleotidases expressed along the airways alleviate the redundancy anticipated for the co-expression of such closely-related proteins. The information provided in this chapter allows the readers to fully appreciate the complexity and sophistication of the multi-enzyme network regulating ATP and ADO concentrations in the airways.

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