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Review

# Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade

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## Abstract

The involvement of extracellular nucleotides and adenosine in an array of cell-specific responses has long been known and appreciated, but the integrative view of purinergic signalling as a multistep coordinated cascade has emerged recently. Current models of nucleotide turnover include: (i) transient release of nanomolar concentrations of ATP and ADP; (ii) triggering of signalling events via a series of ligand-gated (P2X) and metabotropic (P2Y) receptors; (iii) nucleotide breakdown by membrane-bound and soluble nucleotidases, including the enzymes of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, ecto-5'-nucleotidase/CD73, and alkaline phosphatases; (iv) interaction of the resulting adenosine with own nucleoside-selective receptors; and finally, (v) extracellular adenosine inactivation via adenosine deaminase and purine nucleoside phosphorylase reactions and/or nucleoside uptake by the cells. In contrast to traditional paradigms that focus on purine-inactivating mechanisms, it has now become clear that “classical” intracellular ATP-regenerating enzymes, adenylate kinase, nucleoside diphosphate (NDP) kinase and ATP synthase can also be co-expressed on the cell surface. Furthermore, data on the ability of various cells to retain micromolar ATP levels in their pericellular space, as well as to release other related compounds (adenosine, UTP, dinucleotide polyphosphates and nucleotide sugars) gain another important insight into our understanding of mechanisms regulating a signalling cascade. This review summarizes recent advances in this rapidly evolving field, with particular emphasis on the nucleotide-releasing and purine-converting pathways in the vasculature.

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## 1. Overview of extracellular nucleotide turnover

ATP and other nucleotides and nucleosides are found in all animal organ systems where they produce effects both by intracellular and extracellular mechanisms. Intracellular ATP is primarily utilised to drive energy-requiring processes such as active transport, cell motility and biosynthesis, whereas extracellular ATP is considered a powerful signalling molecule. The concept of purinergic neurotransmission was first proposed by G. Burnstock over three decades ago [1]. Since that time, clear signalling roles for ATP and other nucleotides (ADP, UTP, UDP) have been established in several tissues, including potent

neurotransmission in the central nervous system, non-adrenergic, non-cholinergic smooth muscle contractility and neuron–glia interactions [2,3], inotropic, chronotropic, and arrhythmogenic effects in the myocardium [4]; gastrointestinal and liver function [5], regulation of epithelial cell responses [6,7]; blood flow distribution and oxygen delivery [8,9]; immune responses and control of leukocyte trafficking between the blood and tissues [10,11], and activation and aggregation of platelets at sites of vascular injury [12,13]. In addition to acute signalling events, there is increasing awareness that purines and pyrimidines also have potent long-term (trophic) roles in cell proliferation and growth [14], induction of apoptosis and anticancer activity [10,15], atherosclerotic plaque formation [16], wound healing and bone formation and resorption [17].

All these effects are mediated via a series of nucleotide-selective receptors and, on the basis of pharmacological, functional and

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cloning data, two major receptor subfamilies, P2X and P2Y, have been described (Fig. 1). The P2X receptors are ligand-gated channels that gate extracellular cations in response to ATP and comprise seven receptor subtypes (P2X<sub>1</sub> through P2X<sub>7</sub>) [2,18]. The P2Y receptors are G-protein-coupled receptors that are categorized into a subfamily of receptors that predominantly couple to G<sub>q</sub> (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub>) and therefore activate phospholipase C-β, and into a subfamily of G<sub>i</sub>-coupled receptors (P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub>) that inhibit adenylyl cyclase and regulate ion channels [19]. Note, P2Y<sub>11</sub> receptor is dually coupled to phospholipase C and adenylyl cyclase stimulation. Pharmacologically, P2Y receptors can be subdivided into the adenine-nucleotide-preferring receptors mainly responding to ATP and ADP (human and rodent P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> and human P2Y<sub>11</sub>), the uracil-nucleotide-preferring receptors (human P2Y<sub>4</sub> and P2Y<sub>6</sub>) responding to either UTP or UDP, receptors of mixed selectivity (human and rodent P2Y<sub>2</sub> and rodent P2Y<sub>4</sub>), as well as nucleotide-sugar-preferring human P2Y<sub>14</sub> receptor responding to UDP-glucose and UDP-galactose [19,20].

Subsequent to the signal transduction, extracellular nucleotides need to be rapidly inactivated to adenosine (Fig. 2). General schemes of nucleotide hydrolysis have included a role for (i) ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family [21,22], (ii) ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family [23,24], (iii) ecto-5'-nucleotidase [25–27], and (iv) alkaline phosphatases (AP) [28]. Strikingly, along with the common view of extracellular ATP as a ligand for P2 receptors, substrate for ecto-nucleotidases and source of adenosine, this nucleotide was shown to concurrently serve as a phosphoryl donor for counteracting ecto-adenylate kinase (AK) and nucleoside diphosphate (NDP) kinase reactions [29,30]. The resulting adenosine in turn has a non-redundant counteracting role in the attenuation of inflammation and tissue damage and mediates diverse cardioprotective, neuroprotective, vasodilatory and angiogenic responses [10,31–33]. These effects are mediated through G-protein-coupled adenosine receptors (Fig. 1),

that function either by activating (A<sub>2A</sub> and A<sub>2B</sub>) or inhibiting (A<sub>1</sub> and A<sub>3</sub>) adenylyl cyclase and are classified according to their affinities for adenosine [2,31,33]. Extracellular adenosine is then transported into the cell either via equilibrative nucleoside transporters, which transport nucleosides, nucleobases and organic cations as facilitated diffusion-limited channels or through the concentrative nucleoside transporters which are selective for sodium and can also use lithium and proton gradients to drive concentrative fluxes of purine and pyrimidine nucleosides [34,35]. Alternatively, adenosine can be directly inactivated on the cell surface via inosine to hypoxanthine through sequential ecto-adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) activities [31,36] (Fig. 2).

Collectively, extracellular purine turnover includes the release of ATP and other agonists, triggering of signalling events and subsequent inactivation. Despite the significant progress in our understanding of the purinergic machinery as a multistep cascade, current knowledge on the whole sequence of “release-signalling-inactivation” is rather limited. This review summarizes recent advances and some controversies in this rapidly evolving field, with particular emphasis on the nucleotide-releasing and inactivating pathways. Since our laboratory has had a long-lasting interest in various aspects of intravascular nucleotide turnover, special attention is also given to the role of nucleotides and adenosine in the immunomodulatory and thromboregulatory responses.

## 2. Release of ATP and other agonists into the extracellular milieu

### 2.1. Extracellular ATP and ADP release

The release of endogenous nucleotides represents a critical component for initiating a signalling cascade. Massive leakage of nucleotides might occur upon cell lysis, however this nonspecific mechanism is restricted by organ injury, traumatic shock or certain inflammatory conditions [10]. Non-lytic mechanisms of

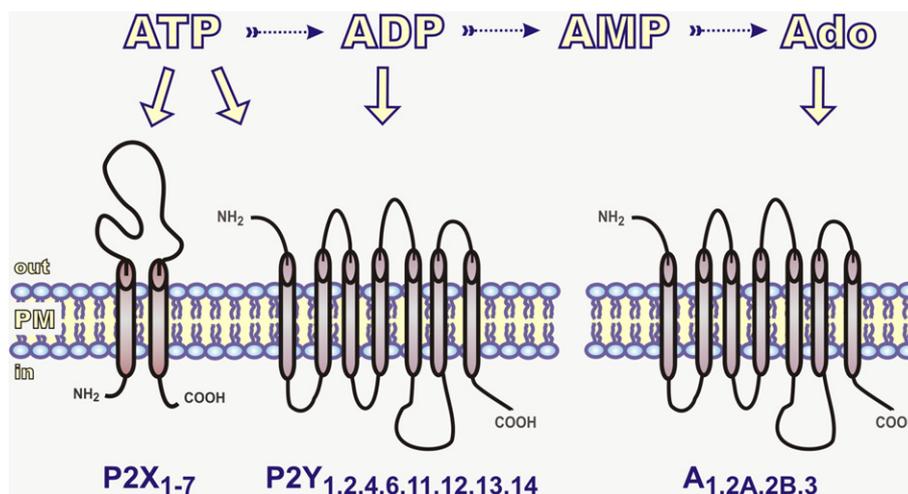


Fig. 1. Purinergic signalling pathways. Nucleotides mediate signalling effects through a series of ionotropic P2X receptors and metabotropic P2Y receptors, which are classified by their affinities towards ATP, ADP and other putative nucleotide and nucleotide-sugar agonists. The generated nucleotide-derived adenosine in turn acts at four own G-protein-coupled nucleoside-selective receptors.



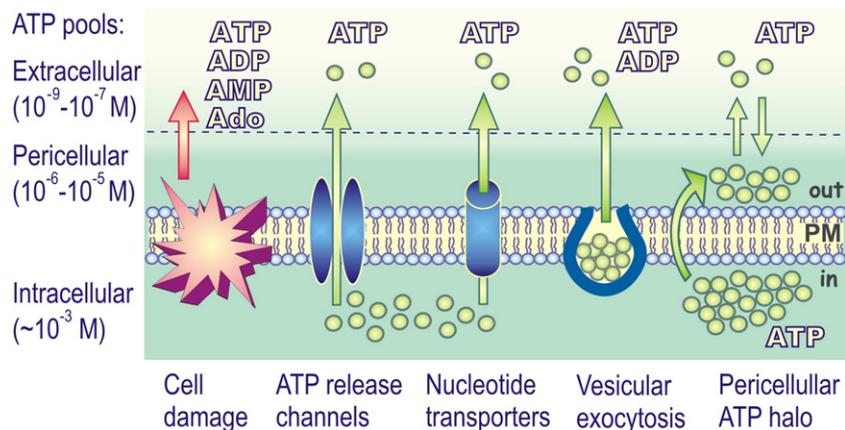


Fig. 3. Nucleotide-releasing pathways. Along with massive nucleotide leakage upon cell damage, nucleotides can appear in the external milieu via various non-lytic pathways, including electrodiffusional movement through ATP release channels, facilitated diffusion by nucleotide-specific transporters and vesicular exocytosis. Furthermore, preferential segregation of micromolar concentrations of “ATP halo” in the pericellular space and its exchange with bulk extracellular ATP pool via certain dissociation–association mechanisms has been proposed recently.

Likewise, by using highly sensitive high-performance liquid chromatographic (HPLC) analysis of fluorescent  $1,N^6$ -etheno-adenine derivatives, it has been shown that ATP release and metabolism constitutes a primary source for the accumulation of relatively high ( $\sim 10^{-7}$  mol/L) concentrations of adenosine, AMP and ADP within the bathing liquid covering cultures of human airway epithelial cells [57,58]. The latter studies additionally emphasized that continuous generation of adenosine provides sustained basal activity to the  $A_{2b}$  receptor and controls CFTR function and airway surface liquid homeostasis. Data on significantly decreased tissue adenosine levels and the impaired adenosine receptor-mediated signalling in carotid arteries [59,60] and myocardium [61] of ecto-5'-nucleotidase/CD73-deficient mice reasonably suggest that in the case of cardiovascular system the nucleotide-inactivating chain also represents the major route for generation of extracellular adenosine.

### 2.3. Release of UTP and nucleotide sugars

Identification of UTP/UDP-selective  $P2Y_4$  and  $P2Y_6$  receptors reasonably suggests that uracil nucleotides can also be released as extracellular signalling molecules. Indeed, the ability of various cell types to release low nanomolar UTP concentrations has been directly confirmed by developing a highly selective UDP-glucose pyrophosphorylase-coupled reaction with subsequent measurement of the UTP-dependent conversion of [ $^{14}C$ ]glucose-1-phosphate to UDP-[ $^{14}C$ ]glucose [20,62,63]. Studies with murine airway epithelial cells transfected with the  $P2Y_4$  receptor also demonstrated significant UTP release from the mechanically stimulated cells which, in turn, contributes to the generation of  $Ca^{2+}$  waves and coordination of the integrated epithelial stress response [39]. Induction of brain injury and neuronal death in rats induced by administration of an excitatory kainic acid caused marked releases of micromolar concentrations of extracellular UTP both in vivo and in vitro, followed by its immediate hydrolysis to UDP and subsequent activation of  $P2Y_6$ -receptor-mediated microglial phagocytosis [64]. Likewise, recent studies with humans revealed significant increase

in circulating UTP levels during acute myocardial infarction (which most likely occurs due to the transient nucleotide release by cardiomyocytes), and further demonstrated the important role for extracellular UTP and UDP as novel inotropic factors involved in the development of cardiac disease [65].

By developing a sensitive HPLC-based radiometric assay for detection of UDP-glucose mass, it has been demonstrated that mechanically stimulated 1321N1 human astrocytoma cells, primary airway epithelia and other cell types also release nanomolar concentrations ( $\sim 5$ – $10$  nM) of UDP-glucose [58,66]. Unlike transient releases of nucleotide agonists, UDP-glucose is released at a considerably slower rate, but its extracellular level remains stable for at least 3 h. The physiological significance of the regulated release of cellular UDP-glucose (and probably also of UDP-galactose) is heightened by observation of a novel  $P2Y_{14}$  receptor subtype (previously known as an orphan G-protein-coupled receptor KIA0001 or GPR105), which is expressed in placenta, adipose tissue, stomach, intestine and other tissues and specifically responds to UDP-glucose and related sugar-nucleotides [67].

### 2.4. Release of dinucleoside polyphosphates

Diadenosine polyphosphates ( $Ap_nA$ ;  $n=2$ – $6$ ) are a group of dinucleosides consisting of two adenosine molecules bridged by a variable number of phosphates, which have a relatively long half-life and, after degradation, may serve as a potential source of extracellular ATP and other purines.  $Ap_nA$  are produced and released from adrenal glands, platelets and chromaffin cells (where they are co-stored in granules with ATP and catecholamines), and substantial amounts have been also found in the human myocardium [4,68,69].  $Ap_nA$  serve as important neurotransmitter molecules in the nervous system [70] and in addition, stimulate different responses in the cardiovascular system, controlling vascular tone and preventing platelet aggregation [68,71]. Interestingly, certain amounts of other dinucleoside phosphates, adenosine (5') oligophospho-(5') guanosines ( $Ap_nG$ ;  $n=3$ – $6$ ) and oligophospho-(5') guanosines ( $Gp_nG$ ;  $n=3$ – $6$ ), can also be

released from human platelets during thrombin activation, thus contributing to the regulation of blood flow and vascular growth under physiological and pathological conditions [72]. Moreover, along with ATP and UTP, mechanically stimulated endothelial cells release nanomolar concentrations of uridine adenosine tetraphosphate ( $U_p4A$ ), with the latter dinucleotide being involved in endothelial-derived vasoconstriction [73].

Some dinucleoside polyphosphates have the capacity to potentiate signalling effects via P2 receptors (primarily, via  $P2X_1$ ,  $P2X_3$  and  $P2Y_1$  subtypes), although the existence of specific dinucleotide receptors has also been proposed [70,71]. However, the specificity of dinucleotide receptors is still poorly understood, primarily due to the complexity of purinergic signalling system. For example, the released  $Ap_nA$  can be hydrolysed by the enzymes of NPP family [74] so that the response of the cell should be the integral of various effects of dinucleotides and their biologically active metabolites, ATP, ADP or adenosine. Furthermore, the recent identification of membrane-bound and soluble forms of AK [75–77] and powerful inhibition of this catalytic reaction by  $Ap_5A$  [78] could provide another reasonable explanation of the agonistic effects of this bisubstrate analogue. Indeed, the ability of  $Ap_5A$  to stimulate the endocytosis of high-density lipoproteins in human hepatocytes was shown to be mediated via inhibition of ecto-AK mediated conversion of endogenous ADP, with subsequent stimulation of  $P2Y_{13}$ -receptors [46]. Likewise, data on the ability of  $Ap_4A$  to inhibit the pattern of extracellular ATP catabolism [79], should also be taken into consideration during evaluation of agonistic potency for this dinucleotide.

### 3. Detection of ATP in the vicinity of the cell using innovative ATP-sensing assays

“Classical” studies on ATP release generally involve either agitation of cell suspensions/monolayers followed by removal of bathing medium and bioluminescent detection of ATP content, or direct addition of luciferase/luciferin reagent to the cells for sensing ATP in real time [6]. Although highly specific and sensitive, such an approach suffers from a limited capacity, firstly because of the rapid ATP metabolism by surface-associated nucleotidases and, secondly, by the compromising effects of certain pharmacological reagents on luciferase activity and light output. Moreover, activation of most P2 receptors usually occurs at nucleotide concentrations within the range of  $10^{-6}$ – $10^{-4}$  M [10], that significantly exceeds the nanomolar ATP levels reported for extracellular fluids. Recent innovative nucleotide-sensing assays provide independent lines of evidence that most of the released ATP is retained in the pericellular space without significant convection into the bulk milieu (Fig. 3) and these cell-surrounding ATP concentrations are sufficient for triggering purinergic responses.

#### 3.1. Microscopic visualisation of ATP pools by using luciferin, quinacrine and other appropriate ATP sensors

By using atomic force microscopy in combination with a myosin ATPase-coated sensor tip (cantilever), accumulation of

micromolar ATP on surface of CFTR-transfected airway epithelial cells has been shown [80]. Fluorescence microscopic analysis of rat pancreatic acini loaded with the ATP sensor luciferin also revealed local release of 4–9  $\mu$ M ATP from the cells under conditions of cholinergic stimulation [38]. Yet another fluorescent microscopic technique for visualising ATP release in real time has been developed recently based on conversion of nicotinamide adenine dinucleotide phosphate,  $NADP^+$ , to its reduced form NADPH. Significant accumulation of micromolar concentrations of ATP has been detected with this approach at the leading edge of human neutrophils in response to chemotactic receptor stimulation with the chemoattractant fMLP [53,81], as well as in the immediate vicinity of Jurkat T cells under hyperosmotic conditions or stimulation with anti-CD3/CD28 antibodies and phytohemagglutinin [81]. Perfusion of murine thalamic slices with a solution containing luciferase and D-luciferin and simultaneous microscopic bioluminescent imaging of the emitted photons also revealed transient release of micromolar ATP concentrations from the exposed cortex in response to high-frequency electric stimulation [56]. While these techniques allow discerning of micromolar ATP in the microenvironment surrounding living cells, the relatively low sensitivities of semi-quantitative microscopic analyses limit their application for routine measurements.

Cellular ATP can also be visualised by using the fluorescent dye quinacrine, which belongs to the quinoline–acridine class, binds to nucleic acids and is considered as the preferred available marker for detecting releasable ATP stores. Labelling of rabbit ciliary epithelial cells [82], human umbilical vein endothelial cells (HUVEC) [42], rat pancreatic acini [38], human leukemic cell lines [83] and human airway epithelial cells [58] with quinacrine revealed the accumulation of this acidotropic dye within cytoplasmic granules beneath the plasma membrane and further demonstrated marked decreases in its fluorescence intensities under various stimuli (hypotonic stress, shear stress, cholinergic stimulation, and ionomycin treatment).

#### 3.2. Indirect quantification of pericellular ATP by using biosensor-based approaches

Upon stimulation of a single pancreatic  $\beta$ -cell with glucose or sulfonyleurea glibenclamide, local ATP concentrations of over 25  $\mu$ M were recorded using the patch-clamp technique with a “biosensor” PC12 cell expressing  $P2X_2$  receptors [84]. However, correct interpretation of biosensor cell-based data is hampered by receptor inactivation/desensitization or potential self-activation of  $P2X$  receptors during ATP release from the sensor cell [85] and, in addition, this sophisticated technique requires three-dimensionally fine-controlled micromanipulators to bring the sensor into the close proximity of the target cell. Yet another electrochemical approach for in vivo sensing of ATP release from the tissues has been developed recently by using microelectrode sensors coated with an ultrathin bilayer containing glycerol kinase and glycerol-3-phosphate oxidase [86]. By inserting these biosensors into the *Xenopus* embryos, transient releases of low micromolar ATP concentrations were recorded from spinal cord during locomotor activity [86] and from the anterior neural plate during eye-field development [87].

Given the high substrate specificity for AK for reversible reaction  $\text{ATP} + \text{AMP} \leftrightarrow 2\text{ADP}$ , cell surface-associated AK was exploited as an intrinsic probe for sensing pericellular ATP. Thin-layer chromatography (TLC) autoradiographic analysis revealed the ability of human lymphocytes, but not vascular endothelial cells, of slightly converting [ $^3\text{H}$ ]AMP into high-energy  $^3\text{H}$ -phosphoryls even without exogenous  $\gamma$ -phosphate-donating ATP [30,88]. Subsequent kinetic studies confirmed that various lymphoid cells constitutively retain micromolar concentrations of pericellular ATP, which can be quantified by the extent of its ectoenzymatic transphosphorylation with exogenous  $^3\text{H}/^{32}\text{P}$ -labelled AMP [83]. This work additionally emphasized that cell-surrounding ATP might provide an important signalling loop sufficient for self-activation of lymphocytes and also for triggering purinergic responses in a paracrine fashion. Note, due to the prolonged dynamics of transphosphorylation of tracer AMP with endogenous ATP, this enzyme-coupled technique is not appropriate for detecting transient ATP fluxes.

### 3.3. Bioluminescent ATP-sensing assays with cell surface-targeted luciferase

Another approach for detecting ATP release into localized membrane microenvironments includes selective targeting of luciferase to the cell surface. By using biotinylated cell surface-immobilized firefly luciferase, dynamic and local thrombin-mediated ATP efflux was monitored in the vicinity of human pulmonary artery endothelial cells [89]. The experiments with membrane-attached protein-A luciferase also revealed transient release of 15–20  $\mu\text{M}$  ATP from thrombin-activated platelets [90], and further demonstrated that micromolar ATP concentrations are transiently segregated in the vicinity of human astrocytoma cells [91] and at the apical membrane surfaces of primary airway epithelia [41] upon cells challenging with  $\text{Ca}^{2+}$ -mobilizing agonists or hypotonic stress, respectively. Transfection of recombinant plasma membrane-targeted luciferase into HEK293 cells stably transfected with the human or rat P2X<sub>7</sub> receptor also revealed transient and local release of 100–200  $\mu\text{M}$  ATP in response to membrane stretching or during P2X<sub>7</sub> activation by a potent agonist benzoyl-ATP (BzATP) [92]. A limitation of these assays is the need for specially designed surface antigens or cell lines appropriate for luciferase targeting. Moreover, by measuring the relative increases in light output or second messenger production, most of the above techniques can detect only transient ATP fluxes under certain stimulated conditions.

## 4. Nucleotide breakdown to adenosine via ecto-nucleotidase reactions

### 4.1. Ecto-nucleoside triphosphate diphosphohydrolase (E-NTDPase) family

The presence of specific nucleotide-hydrolysing activities at the surface of many cell types had been recognized for a long time, and information about these enzymes may be found in the early literature under different names, including ecto-ATPase,

ATP-diphosphohydrolase, apyrase, ATPDase, nucleoside diphosphatase, etc. (for review see [37,93,94]). As a common feature, these ectoenzymes are capable of hydrolysing nucleoside tri- and/or diphosphates, but not monophosphates, require millimolar concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for maximal activity, and remain insensitive to specific inhibitors of P-type, V-type, and F-type ATPases. While the mechanism of sequential ATP hydrolysis via ADP into AMP was initially thought to be mediated via two separate ATPase and ADPase activities [95,96], more recent studies suggested that a single enzyme ATP-diphosphohydrolase can dephosphorylate both ATP and ADP as substrates [37,97]. Later, given the broad substrate specificity of ecto-ATPase/ATPDase towards different purine and pyrimidine nucleoside tri- and diphosphates, a more accurate term NTPDase has been introduced for this ectoenzyme [98,99]. According to the current nomenclature, all NTPDase family members should be termed as NTPDase proteins and classified in order of discovery and classification [22]. Namely, eight different ENTDP genes encode members of the NTPDase protein family, with four of the NTPDases (NTPDase1, 2, 3, and 8) being expressed as cell surface-located enzymes. NTPDases 5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression, while NTPDases 4 and 7 are entirely intracellularly located, facing the lumen of cytoplasmic organelles.

The molecular identity of the first member of the E-NTPDase family (NTPDase1) was unravelled over one decade ago. The prototypic member of the enzyme had been cloned, sequenced and identified as a cell activation antigen CD39, a putative B-cell activation marker [100]. Subsequent experiments with purified and cloned soluble ATP-diphosphohydrolase (apyrase) from potato tubers [101] and from different mammalian tissues [102] confirmed the significant homology of this enzyme to human CD39. Data on the close correlation between CD39 expression level and nucleotide-hydrolysing activity in lymphocytes and CD39-transfected COS-7 cells further strengthened the hypothesis that NTPDase1 is primarily attributed to the surface antigen CD39 [102,103]. Further studies revealed the abundant expression of NTPDase1/CD39 on vascular endothelial and smooth muscle cells [12,104,105], exocrine pancreas [106], dendritic cells [107], lymphocytes [108] and in a variety of other cells [22,109].

NTPDase2 is particularly associated with the adventitial surfaces of the muscularised vessels [105,110], cultured astrocytes, non-myelinating Schwann cells and other glial cells of the central and peripheral nervous system [22,111]. Recent immunohistochemical studies with adult and developing murine brain revealed that NTPDase2, together with alkaline phosphatase, plays an important role in purinergic control of embryonic, postnatal and adult neurogenesis [111]. The experiments with *Xenopus* embryos also demonstrated the abundant expression of NTPDase2 in the mesodermal and endodermal layers and further emphasized the key regulatory role for this nucleotidase in the triggering of purinergic signalling and initiation of eye development [87]. The expression of NTPDase3 is mainly associated with axon-like neuronal structures in the brain, where it may act as a pre-synaptic regulator of extracellular ATP levels and coordinates multiple homeostatic systems, including

feeding and sleep–wake behaviours [112]. Recent cloning and characterisation of the last mammalian membrane-associated NTPDase, named NTPDase8, revealed its predominant expression in the liver, with lower enzyme levels being detected in jejunum and kidney [113]. Abundant expression of NTPDase8 in bile canaliculi and large blood vessels of liver suggests the involvement of this ecto-nucleotidase in the regulation of bile secretion and/or nucleoside salvage [114].

All cell-surface members of NTPDase family are highly glycosylated proteins with molecular masses ~70–80 kDa, which show close immunological cross-reactivity [21] and may exist either in monomeric or in higher homooligomeric (dimeric to tetrameric) states [115]. These enzymes contain two predicted transmembrane domains at the N- and C-terminus with a large extracellular loop containing a more central hydrophobic region with five highly conserved sequence domains known as “apyrase conserved regions” (ACR), with two of them (ACR1 and ACR4) sharing common sequence homology with members of the actin/HSP70/sugar kinase superfamily [22,116]. The NTPDases can hydrolyse a variety of nucleoside di- and triphosphates, although preferences vary considerably among individual enzymes. In particular, ATP:ADP hydrolysis ratios for NTPDases 1, 2, 3 and 8 are of ~1–1.5:1, 10–40:1, 3–4:1 and 2:1, respectively [21,117]. Notwithstanding, due to the ability of NTPDases to sequentially dephosphorylate ATP via ADP to AMP (with concomitant cleavage of two phosphates), measurement of catalytic activity using conventional  $P_i$ -liberated photometric assays can lead to overestimation of actual ATP-hydrolysing activity. Indeed, more reliable radio-TLC analyses of the rates of tracer ATP and ADP conversions into their corresponding dephosphorylated metabolites by human endothelial cells [76,118], lymphocytes [30] and rat pancreatic juice [119] revealed that the ADP-hydrolysing capacity of NTPDase1/CD39 may even exceed ATPase activity by up to ~1.5–2.0-times. NTPDases may also have other functions distinct from their catalytic properties alone, particularly via intracellular interactions with a membrane scaffolding protein RanBPM and subsequent direct modulation of ERK/Ras signalling pathways [22,120].

In terms of the vascular system, endothelial E-NTPDase1/CD39 has been implicated in playing critical role through the termination of prothrombotic and proinflammatory effects of circulating ATP and ADP thus keeping the hemostatic process tightly regulated to prevent excessive clot formation and vessel occlusion [12,22,96]. This ectoenzyme is expressed by endothelium and endocardium at the luminal surface of blood vessels and efficiently inhibits and reverses platelet aggregation in the presence of ATP and/or ADP [97,110]. In contrast to NTPDase1, vascular NTPDase2 is mainly expressed by microvascular pericytes and adventitial cells in muscularised vessels, where it promotes platelet aggregation in the presence of ATP and further facilitates aggregation in the presence of ADP [110]. Among the circulating blood cells, the NTPDase1/CD39 has been identified as major nucleotide-inactivating enzyme on neutrophils, monocytes and certain T- and B-cell subsets, but not on platelets and red blood cells [121,122]. Strikingly, recent data have shown that constitutive expression of CD39 by a population of murine CD4<sup>+</sup>/CD25<sup>+</sup>/Foxp3<sup>+</sup> T regulatory (Treg) cells can increase

their suppressive activity via dual mechanism, including scavenging of proinflammatory ATP [123] and simultaneous generation (in concert with CD73) of the immunosuppressive adenosine with subsequent activation of A<sub>2A</sub> adenosine receptors [108]. On human Treg cells, the expression of CD39 is restricted only to a specific subset of effector/memory-like cells and its level can be significantly decreased in patients with the remitting/relapsing form of multiple sclerosis (which are characterised by a reduced suppressive capacity of their Treg cells) [123]. Probably, in humans the lymphoid NTPDase1/CD39 is also important for the removal of ATP and coordinate regulation of immune responses.

Data from mutant mice deficient in NTPDase1/CD39 or over-expressing human CD39 further confirmed an important role for this ectoenzyme in the control of hemostasis, platelet reactivity, thrombotic reactions and vascular growth in vivo and further support its therapeutic potential in clinical vascular diseases and during transplantation [104,105,124,125]. Furthermore, studies with CD39-null mice demonstrated an important role of CD39 in preventing exacerbated skin inflammation triggered by irritant chemicals [107], activation of integrin-associated signalling pathways and abrogation of tumor angiogenesis and metastasis [126], maintenance of pulmonary integrity during acute lung injury [127] and in renal [128] and myocardial [129] protections from ischemia–reperfusion injury. Interestingly, CD39-deficient mice showed reduced T cell-dependent immune responses to antigens [107] and more recent studies further demonstrated that Treg cells from these mice display impaired suppressive properties in vitro and fail to block allograft rejection in vivo [108].

#### 4.2. Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family

The E-NPP family consists of seven structurally related ectoenzymes (NPP1 through NPP7) that are numbered according to their order of discovery. Members of this multigene family possess surprisingly broad substrate specificity capable of hydrolysing pyrophosphate and phosphodiester bonds in (di) nucleotides, nucleic acids, nucleotide sugars, as well as in choline phosphate esters and lysophospholipids [74]. Only the first three members of this family, NPP1 (previous name plasma cell differentiation antigen-1, PC-1), NPP2 (autotaxin, phosphodiesterase 1 $\alpha$ ) and NPP3 (gp130<sup>RB13-6</sup>, B10, phosphodiesterase 1 $\beta$ ), are capable of hydrolysing various nucleotides and are therefore relevant in the context of the purinergic signalling cascade [23]. In addition, NPP1–3 hydrolyse Ap<sub>3</sub>A, Ap<sub>4</sub>A and Ap<sub>5</sub>A at comparable rates and are considered to be the major enzyme candidates for metabolism of extracellular diadenosine polyphosphates in vertebrate tissues [130]. NPP6–7 hydrolyse only phosphodiester bonds in lysophospholipids or other choline phosphodiester, while natural substrates for NPP4–5 still remain unknown. Notably, an unambiguous distinction between the members of NPP and NTPDase families is often complicated by their co-expression to a variable extent among the mammalian tissues and shared similarities in substrate specificity [21,109]. The contribution of these two nucleotidases could be estimated by autoradiographic analysis of the relative amounts of

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -generated  $^{32}\text{P}_i$  and  $^{32}\text{PP}_i$  (for example, [131]). However, it has to be kept in mind that, due to broad substrate specificity, some NPP can also dephosphorylate ATP via ADP to AMP in a “NTPDase-like” fashion.

The mammalian members of the NPP1–3 family are thought to be a type II (intracellular N-terminus) transmembrane glycoproteins of 110 to 125 kDa that share a highly homologous extracellular domain containing two somatomedin B-like domains of vitronectin and a highly conserved catalytic site [23]. More recent data revealed that NPP2 is not a transmembrane protein but is synthesized as a pre-pro-enzyme. Following the removal of the N-terminal signal peptide and further trimming by a furine-type protease, NPP2 is secreted [24]. NPP2 (autotaxin)-deficient mice die at embryonic day 9.5 with profound vascular defects, while heterozygous mice appeared healthy but showed half-normal NPP2 activity and low plasma level of lysophosphatidic acid (LPA) [132]. These studies demonstrated that NPP2/autotaxin primarily functions as a lysophospholipase-D, converting lysophosphatidylcholine into the lipid mediator LPA and further underlined vital role of the autotaxin-LPA axis in vascular development.

Human NPP1 is highly expressed in bone and cartilage, with intermediate enzyme expression in heart, liver, placenta, kidney and testis [23]. In bone tissue, NPP1 activity is mainly associated with osteoblast- and chondrocyte-derived membrane structures termed “matrix vesicles”, where it has been identified as the key  $\text{PP}_i$ -generating enzyme ensuring normal bone matrix mineralization and soft tissue calcification [133]. Mice lacking NPP1 spontaneously develop articular cartilage, perispinal and aortic calcification at a young age [134]. These knockout animals share similar phenotypic features with a human disease, idiopathic infantile arterial calcification, where a deficiency in NPP1-mediated production of  $\text{PP}_i$  has been postulated to cause arterial calcification and periarticular calcifications of the large joints [135]. Note,  $\text{PP}_i$  has a dual physiological role: it can function as a promoter of mineralization at low concentrations (0.01–0.1 mmol/L), but also acts as an inhibitor of mineralization at higher concentrations [133]. There is increasing evidence that the extracellular concentration of  $\text{PP}_i$  is maintained within a narrow physiological range via the opposing and reciprocal actions of NPP1, which generates  $\text{PP}_i$ , and tissue-nonspecific alkaline phosphatase (TNAP), which further degrades it to  $\text{P}_i$  [28]. Indeed, data with double knockout mice revealed that deletion of both NPP1 (*Enpp1*<sup>-/-</sup>) and TNAP (*Akp2*<sup>-/-</sup>) genes leads to a less severe phenotype than single knockouts where only one gene was deleted [136].

#### 4.3. Ecto-5'-nucleotidase/CD73

To date, seven human 5'-nucleotidases have been isolated and characterised, with five of the enzymes located in the cytosol, one in the mitochondrial matrix and one attached to the outer plasma membrane. The intracellular nucleotidases generally dephosphorylate 5'-ribonucleoside monophosphates with low affinity ( $K_m > 10^{-3}$  M), share an ability to hydrolyse 5'-(deoxy)NMPs and 2'- and 3'-NMPs as substrates and mainly participate in salvage pathways and/or de novo nucleotide synthesis [26].

Surface-associated ecto-5'-nucleotidase, otherwise known as CD73, efficiently hydrolyses 5'-AMP with  $K_m$  of 3–50  $\mu\text{M}$  and shows no activity towards nucleoside 2'- and 3'-monophosphates [25,26]. The mammalian ecto-5'-nucleotidase consists of two (glyco)protein subunits with apparent molecular masses of 60–70 kDa, which are tethered by non-covalent bonds [137], binds zinc and other divalent metal ions at the N-terminal domain [138] and is anchored to the plasma membrane at the C-terminus by glycosyl-phosphatidylinositol (GPI) [25,26]. This ectoenzyme belongs to a large superfamily of dinuclear metallophosphoesterases acting on diverse substrates such as Ser/Thr phosphoproteins, various nucleotides and sphingomyelin [138].

Ecto-5'-nucleotidase is expressed to a variable extent in different tissues, with abundant expression in the colon, kidney, brain, liver, heart and lung [25,37,139,140]. In the vasculature, ecto-5'-nucleotidase is predominantly associated with the vascular endothelium of large vessels such as the aorta, carotid and coronary artery [59]. In the case of circulating T- and B-lymphocytes, the enzyme expression is restricted by certain cell types and strongly correlates with cell maturity, while neutrophils, erythrocytes, platelets and other blood cells express little or no CD73 [141–143]. Specifically, ecto-5'-nucleotidase/CD73 is co-expressed with NTPDase1/CD39 on the surfaces of  $\text{CD4}^+/\text{CD25}^+/\text{Foxp3}^+$  Treg cells and comprises an important constituent of the suppressive machinery via conversion of ATP/ADP-derived AMP further into the anti-inflammatory mediator adenosine and subsequent inhibition of T cell proliferation and secretion of cytokines [108,144]. While the major physiological role of ecto-5'-nucleotidase seems to be the regulation of purinergic signalling cascade [27], other non-enzymatic functions of the CD73 molecule have also been proposed, such as induction of intracellular signalling and mediation of cell–cell and cell–matrix adhesions [141,145].

A number of substances were shown to regulate ecto-5'-nucleotidase expression in vascular endothelial cells and hematopoietic cells in a cell type-specific manner, including the cytokine interferon- $\gamma$  [146], PMA [147], inhibitor of HMG-CoA reductase lovastatin [148], adenosine analogue 5'-(*N*-ethylcarboxamido)adenosine (NECA) and other cAMP-activating agents forskolin and prostaglandin- $\text{E}_2$  [149]. In intestinal epithelia, ecto-5'-nucleotidase is predominantly localized on the apical cell surface [51] and its expression can be further up-regulated by hypoxia-inducible factor-1 $\alpha$ , leading to increased extracellular adenosine formation that in turn, counteracts hypoxia-induced intestinal permeability and protects the epithelial barrier [150]. Interestingly, the efficacy of the anti-inflammatory drugs methothrexate and sulfasalazine in the treatment of rheumatoid arthritis has been well documented for a long time. However, it was only recently reported that both compounds could mediate their anti-rheumatic effects by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides and subsequent activation of adenosine receptors [151,152].

Recently, three separate groups have generated the mice with a genetic deficiency of CD73. These mice have been particularly characterised in the context of a role for ecto-5'-nucleotidase in

the mediation of tubuloglomerular feedback and renal function [153,154], pulmonary integrity and lung function [127,155], immunomodulatory and thromboregulatory responses [27,59,60,139], and cardioprotection during myocardial ischemia [61]. Specifically, data with ecto-5'-nucleotidase/CD73<sup>-/-</sup> mice have identified CD73-catalyzed production of adenosine as one of the control points for maintenance of vascular barrier function in various tissues during hypoxia [27,139]. CD73-mediated adenosine formation was also shown to protect pulmonary integrity in murine models of lung injury induced by the drug bleomycin [155] or mechanical ventilation [127]. Studies from another group who generated CD73-deficient mice found an increase in neointimal plaque formation and macrophage accumulation in CD73<sup>-/-</sup> mice after wire injury of carotid arteries and further emphasized the importance of CD73 in finely tuned constitutive regulation, balancing proinflammatory and anti-inflammatory mechanisms in the vasculature [60].

#### 4.4. Alkaline phosphatases (AP)

AP are ubiquitous enzymes present in many organisms from bacteria to man. With few exceptions, AP are homodimeric enzymes and each catalytic site contains three metal ions (two Zn and one Mg), necessary for enzymatic activity [28,156]. Mammalian AP display broad substrate specificity towards different phosphomonoesters and other phosphated compounds, including adenine nucleotides, pyrophosphate, phosphatidates with various fatty acid chains, inorganic polyphosphates, glucose-phosphates,  $\beta$ -glycerophosphate, bis(*p*-nitrophenyl) phosphate, with release of inorganic phosphate and with a pH optimum for this catalytic reaction lying in the alkaline range from 8 to 11 [156,157]. Three isozymes are tissue-specific and 90–98% homologous, i.e. intestinal AP, placental AP and germ cell AP, while the fourth isozyme, TNAP, is ~50% identical to the other three isozymes and is abundantly expressed in bone, liver, kidney and, at lower levels, in some other tissues [28].

A major role for TNAP in bone tissue is to hydrolyse PP<sub>i</sub> to maintain a proper concentration of this mineralization inhibitor ensuring normal bone mineralization [136,158]. The clearest evidence that AP are important *in vivo* has been provided by studies of human hypophosphatasia, which is associated with poorly mineralized cartilage (rickets) and bones (osteomalacia) and spontaneous bone fractures. The primary biochemical defect in this condition is a deficiency of the TNAP isozyme caused by missense mutations in the human ALPL (TNAP) gene, which leads to greatly elevated levels of extracellular PP<sub>i</sub> and increased urinary excretion of pyridoxal-5'-phosphate, PP<sub>i</sub> and phosphoethanolamine [28]. Two TNAP-deficient mouse models (Akp2<sup>-/-</sup>) have been developed independently [159,160], and both strains of knockout animals were characterised by marked bone and cartilage abnormalities that generally phenocopied the human disease known as infantile hypophosphatasia.

While the important regulatory role of bone TNAP during the development and mineralization of the skeleton has been well established, the functions of AP in other organs are less understood. Given that one of the natural substrates of TNAP is pyridoxal-5'-phosphate (a phosphated form of vitamin B<sub>6</sub>), it is possible that a

role of liver TNAP is to participate in the metabolism of this vitamin [28,156]. Intestinal AP isozyme is likely to be involved in the intestinal absorption and detoxification of lipids via its association with surfactant-like particles, while the placental AP might be involved in mediating immunoglobulin-G transport through the placenta and growth and remodelling of fetal tissues [28]. Experiments conducted on cultured human nasal epithelial cells and freshly excised bronchial tissues indicated that TNAP could also contribute, in conjunction with ecto-5'-nucleotidase, in the regulation of adenosine-mediated epithelial functions [161]. Specifically, although ecto-5'-nucleotidase still remains more efficient at producing adenosine from physiological AMP concentrations (10<sup>-6</sup>–10<sup>-5</sup> mol/L), the high-capacity TNAP can protect the mucosal surface against the deleterious effects of acutely elevated nucleotide levels, thus playing an important role in airway defences during periods of infection and inflammation. Recent data on selective expression of TNAP on certain subsets of progenitor cells in the murine brain and modulation of its nucleotide-hydrolysing activity during embryonic and postnatal development also suggests the important role of this ectoenzyme in the regulation of purinergic signalling and embryonic neurogenesis [111].

## 5. Ecto enzymatic inactivation of adenosine and other nucleosides

### 5.1. Adenosine deaminase (ADA)

ADA is another important enzyme of the purine-inactivating chain, which catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine respectively and is widely expressed in intestine, thymus, spleen and other lymphoid and non-lymphoid tissues [32,140]. Along with cytosolic localization, ADA can be expressed as an ectoenzyme on the surfaces of lymphocytes [36,162] and dendritic cells [163,164]. Interestingly, liver, monocytes/macrophages and serum also contain another isozyme, ADA2, with a low substrate affinity ( $K_m$  for adenosine ~2 mM). ADA2 can be active at sites of inflammation during hypoxia and in areas of tumor growth and in addition, it binds to heparin and belongs to a new family of ADA-related growth factors [165].

On lymphoid cells, extracellular ADA is often associated in larger complexes with CD26/dipeptidyl peptidase IV [166,167] or via alternative anchoring mechanism by interacting with A<sub>1</sub> or A<sub>2b</sub> adenosine receptors [163,168]. Recent radio-TLC assays with [<sup>3</sup>H] adenosine as substrate confirmed cell-surface expression of catalytically active ADA on human lymphocytes [30]. HPLC data shown in Fig. 4A provide another line of evidence for the ability of lymphocytes to directly deaminate exogenous adenosine, and similar results have been reported recently in studies with mouse bone marrow-derived dendritic cells [164]. Taking into account the important anti-inflammatory role for adenosine, abundant expression of ecto-ADA in the lymphoid tissues may provide an efficient means for scavenging cell-surrounding adenosine with subsequent sustained activation of dendritic cells and T-lymphocytes during inflammation, even despite the general state of immune suppression [164]. In addition, lymphoid ecto-ADA, in association with CD26, has been proposed to have a catalytic-independent function as a co-

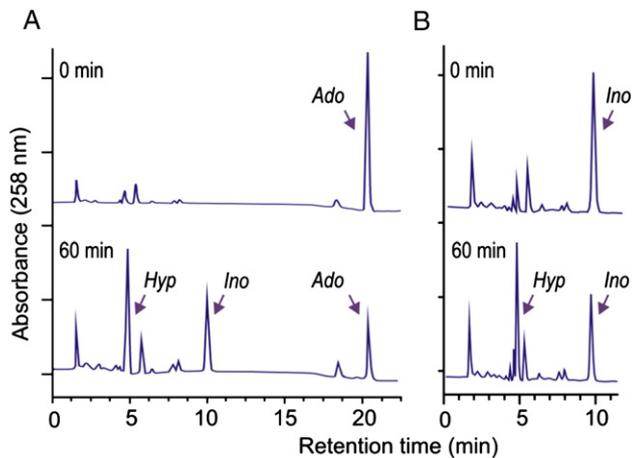


Fig. 4. Metabolism of exogenous nucleosides by human lymphocytes. Human leukemic T cells Jurkat were incubated with 50  $\mu$ M adenosine (A) and inosine (B) for 0–60 min. Exogenous nucleoside substrates and their metabolites were separated by reverse-phase HPLC and are indicated in the chromatograms shown. For experimental details, see [77,119].

stimulatory molecule during T cell antigen receptor–CD3 complex engagement [166], and during the immunological synapse formation [163], thus promoting an augmented T cell activation and production of proinflammatory cytokines. Compared with lymphocytes, vascular endothelium displays relatively low ecto-ADA activity [30], however endothelial cell-surface expressions of ADA and its counter-ligand CD26 can be coordinately up-regulated in areas of ongoing inflammation and diminished oxygen supply [169].

ADA deficiency in humans arises from naturally occurring mutations that account for approximately 20% of cases of human severe combined immunodeficiency disease and affects both cell-mediated and humoral immunity. Genetic deficiencies in ADA in humans result in a severe lymphopenia and immunodeficiency and in addition, exhibit phenotypes in other physiological systems including the renal, neural, skeletal and pulmonary systems [36,170]. ADA-deficient mice have been generated by two groups with similar phenotypes noted [171,172]. The ADA-deficient fetuses die perinatally, around 18.5 days post coitum, in association with severe liver impairment and failure of lung expansion presumably due to marked accumulations of cytotoxic 2'-deoxyadenosine and adenosine. This prenatal lethality of ADA-deficient fetuses was overcome by the ectopic expression of an ADA minigene in the gastrointestinal tract of otherwise ADA-deficient mice [173]. ADA-deficient mice develop a severe pulmonary phenotype and combined immunodeficiency similar to that seen in ADA-deficient humans, and experiments in these mice have confirmed the key role of adenosine signalling in severe immunodeficiency diseases, including chronic inflammatory lung diseases [36]. Along with severe pulmonary insufficiency, ADA-deficient mice also exhibited bone abnormalities and kidney pathogenesis [36,173]. Noteworthy, in spite of the obvious beneficial effects of daily enzyme replacement (by injecting a polyethylene glycol (PEG)-modified form of bovine ADA) in ADA-deficient patients and ADA<sup>-/-</sup> mice, relatively high doses are required to correct the phenotypic abnormalities [36], presumably reflecting

the inability of PEG-conjugated ADA to leave the circulation, enter the cell or penetrate the blood-brain barrier.

## 5.2. Purine nucleoside phosphorylase (PNP)

PNP is the enzyme next to ADA in the purine catabolic pathway that is mainly localized in cytoplasm of both prokaryotic and eukaryotic organisms as a trimeric complex of ~90–100 kDa, but can also be found in the mitochondria [140,174]. The major role of mammalian cytosolic PNP is to phosphorylate (deoxy)guanosine and (deoxy)inosine to guanine and hypoxanthine respectively, which are then metabolised via xanthine to the stable end-reaction product uric acid. Interestingly, incubation of human nasal and bronchial epithelial cells [161] and leukemic cell lines [83,88] with [<sup>3</sup>H]adenosine and subsequent chromatographic imaging of the reaction products elicited significant conversion of the generated [<sup>3</sup>H]inosine further to [<sup>3</sup>H]hypoxanthine. The HPLC chromatograms shown in Fig. 4B further confirm that lymphocytes are able of converting inosine to hypoxanthine, without significant nucleoside uptake by the cells. Together, these data suggest that PNP can also be expressed on the cell surface where it regulates, in conjunction with ADA, local nucleoside levels.

Mutations that interfere with the production or function of PNP in humans disturb intracellular nucleotide/nucleoside homeostasis and result in severe T cell immunodeficiency, immune and neurological dysfunctions. These immune abnormalities in turn, contribute to the early death of PNP-deficient patients in the childhood from infections, autoimmunity or malignancy [175,176]. The PNP<sup>-/-</sup> mice display metabolic, immune and phenotypic abnormalities similar to those found in PNP-deficient patients, which are particularly manifested in markedly reduced mitogenic and allogenic responses, hypoplastic thymi, decreased numbers of maturing thymocytes and peripheral T cells, impaired thymocyte differentiation and abnormal T lymphocyte responses [177]. Recent data on the ability of PNP fused to a protein transduction domain (PTD–PNP) to normalize the abnormal function of PNP-deficient lymphocytes in vitro [174] and to increase the survival of PNP<sup>-/-</sup> mice in vivo [178] provide promise for the efficient delivery of PNP into various tissues, including the brain, and preventing metabolic disorders in purine homeostasis. Notably, while not as efficient as PTD–PNP, non-fused PNP also improved the viability and function of PNP-deficient human lymphocytes [174], increased thymus weight and corrected the immune disorder in PNP<sup>-/-</sup> mice [178]. While the authors explained the beneficial effects of this cell-impermeable PNP by creating an extracellular purine concentration gradient which could remove toxic metabolites from the cells, an alternative interpretation for these findings, by ectoenzymatic regulation of purinergic signalling via shifting the local nucleoside levels is also possible.

## 6. ATP re-synthesis via backward ecto-phosphotransfer reactions

### 6.1. Adenylate kinase (AK)

AK catalyzes reversible phosphoryl transfers  $ATP + AMP \leftrightarrow 2ADP$  through an associative path with a pentacoordinate

transition state, prefers ATP at the NTP site, and is very specific with respect to phosphate acceptors NMP [78]. The enzyme isoforms are thought to be mainly localized in the cytosol (AK1), mitochondrial intermembrane space (AK2), mitochondrial matrix (AK3) and nucleus (AK6) where they play a critical role in energy transfer and distribution between mitochondria, cytosol and nucleus [78,179,180]. The cytosolic AK1 is considered one of the most prominent members of the AK family and is expressed in well-differentiated tissues with a high-energy demand, like brain, heart and skeletal muscle. In addition, a novel isoform of cytosolic enzyme, AK1 $\beta$ , was shown to be localized in the plasma membranes of various tissues [181,182]. This membrane AK1 $\beta$  differs from the cytoplasmic AK1 by 18 extra amino acids at the N-terminus containing a consensus signal for protein myristoylation and the fatty acid chain enables its binding to the lipid bilayer [181]. By using two-photon excitation fluorescence imaging, it has been shown that AK1 $\beta$  undergoes lateral diffusion within the plasma membrane, is continuously exchanged with cytosolic AK1 and is likely interacting with other membrane proteins [183].

AK1 knockout muscles display lower energetic deficiency and increased vulnerability to metabolic stress, associated with a compromised ability to maintain adenine nucleotide pools and inefficient signal communication to the nucleus and cytosolic metabolic sensors [179,184]. Recent study also demonstrated that along with defective myocardial energetic communication, AK1 deficiency blunts AMP signal generation and adenosine efflux in the vasculature and further abrogates coronary reflow after ischemia–reperfusion [185]. AK-mediated phosphotransfer also provides an efficient way to couple transport to the cellular metabolic state and facilitates delivery of intracellular signals to the membrane environment, which might occur via association of cytosolic AK1 [186] and/or membrane-associated AK1 $\beta$  [182] with the ATP-sensitive potassium ( $K_{ATP}$ ) channels. Interestingly, ABC transporter CFTR can also function either as an ATPase or as an AK, with the latter phosphotransfer reaction being directly implicated in gating of this anion channel in vivo [187,188]. Likewise, other member of ABC family Rad50 that associates in a tight complex with an exo-/endonuclease Mre11, also catalyze both ATP hydrolysis and reversible AK reaction and in this way, plays an important role in DNA double-strand break repair [189].

Recent identifications of ecto-AK activities on human vascular endothelial cells [76], lymphocytes and leukemic cell lines [30], hepatocytes and hepatic cell lines [46,190], airway epithelia [75,191], keratinocytes [47], rat hippocampus [192] and other cell types suggest a novel role for this enzyme in the control of extracellular nucleotide levels and propagation of purinergic signalling. Notwithstanding, only moderate ADP-to-ATP conversion has been detected on the cultured HUVEC by using colorimetric tetrazolium reduction assay, and on this basis the authors concluded that external AK activity is “an artefact primarily reflecting a minor leakage of intracellular enzyme from living cells and/or a few dying cells” [193]. Such an interpretation needs critical reassessment though as the revealed phenomenon of little ATP formation from ADP simply reflects the fact that endothelial NTPDase/ADPase exceeds ecto-AK

activity by  $\sim$ 5-times [76] and hence, a nucleotide-hydrolysing pathway prevails under these experimental settings. In contrast, joint addition of [ $^3$ H]AMP and non-labelled ATP (which serves as a donor of  $\gamma$ -phosphoryl groups and concurrently inhibits ecto-5'-nucleotidase) to the HUVEC and subsequent TLC analysis of the generated high-energy  $^3$ H-phosphoryls ADP/ATP provides a more reliable tool for measuring the (ecto)phosphotransfer reactions [76]. Further comparative studies with viable cells and their lysates unequivocally confirmed that a significant portion of AK is indeed expressed as an ectoenzyme on surface of endothelial cells [30].

## 6.2. NDP kinase

NDP kinase is a ubiquitous enzyme which catalyzes transfer of  $\gamma$ -phosphate from nucleoside 5'-triphosphates to nucleoside 5'-diphosphates via a phosphoenzyme intermediate, requires  $Mg^{2+}$  or other divalent cations, and has a broad substrate specificity towards different ribo- and deoxyribonucleotides of purines and pyrimidines [194]. All NDP kinases are oligomeric proteins made of 17–20-kDa subunits with highly conserved sequences, which are thought to be mainly localized in the mitochondria, cytosol and nucleus where they link ATP-based energetics with the cellular NTP pool and facilitate channelling of nucleoside triphosphates into protein synthesis and DNA replication complexes [195,196]. Apart from its role in maintaining the balanced levels of nucleoside triphosphates in the cell, NDP kinase can have additional regulatory functions for growth and developmental control, signal transduction and tumor metastasis suppression [195–197]. Specifically, the nm23 tumor metastasis suppressor gene was found to encode a protein identical to NDP kinase and to date eight different genes (nm23-H1 to nm23-H8) have been identified in humans [196]. Mice lacking the Nm23-M1 gene (the murine homologue of Nm23-H1) have been generated recently, and these knockout animals are characterised by a high rate of neonatal mortality, the impaired mammary gland development and growth retardation [198].

Although it has become obvious that NDP kinase is an important multifunctional protein, little is known about the molecular mechanisms underlying its antimetastatic effects and role in cell biology. Interestingly, NDP kinases/Nm23 was shown to form stable complexes with a number of other cellular proteins, including molecular chaperones of the Hsp70 family and cytoskeletal components vimentin and tubulin [195], several enzymes involved in energy metabolism [179], AMP-activated protein kinase [199] and serine–threonine kinase receptor-associated protein [200]. Probably, some of these proteins could provide a physical link between NDP kinase/Nm23, cellular bioenergetics and transmembrane signalling cascades.

Renewed interest in this enzyme emerged from recent data showing that NDP kinase participates in outside-in and inside-out signalling either via recruitment onto the plasma membrane or constitutive expression as a cell-surface ectoenzyme. Particularly during cell migration or early stages of cell spreading, Nm23-H2 is recruited in the vicinity of integrins and co-localized with the negative regulator of  $\beta$ 1-integrin-mediated cell adhesion, ICAP-1 $\alpha$ , thereby interfering with the cell/extracellular matrix

machinery and markedly reducing cell motility [201]. Furthermore, it has been shown that the effects of ADP-Rybosylation-Factor-6 (ARF6), a member of the ARF family of proteins functioning as a molecular switch by cycling between active GTP-bound and inactive GDP-bound conformations, are dependent on the recruitment of Nm23-H1 to cell–cell contacts which in turn, induces a decrease in the cellular levels of a small GTPase protein Rac1 [202]. These studies provide a molecular basis for dynamin-mediated endocytosis during adherens junction disassembly in polarized epithelia. More recent studies further demonstrated that Nm23-H1 might regulate Rac1-induced cell migration and focal adhesion functions via dynamic association with a human prune (h-prune), which displays high cAMP-specific phosphodiesterase activity and has additionally been identified as a glycogen synthase kinase-3 binding protein [203].

The first evidence for extracellular ATP synthesis via the ecto-NDP kinase reaction was provided over three decades ago in experiments with human erythrocytes [204] and cultured glioma and glia cell lines [205]. Since that time, cell-surface expression of NDP kinase has been documented in various tissues, including pig aortic endothelial and smooth-muscle cells [95], human astrocytoma cells [29], vascular endothelial cells [76], lymphocytes [30], hepatocytes [46], keratinocytes [47], perfused guinea-pig coronary arteries and cultured heart endothelial cells [44]. Interestingly, a role for spatially arranged near-equilibrium enzymatic networks (so-called “phosphoryl wires”), catalyzed by intracellular AK, NDP kinase, creatine kinase and glycolytic enzymes, in supporting high-energy phosphoryl transfer and communication between ATP-generating and ATP-consuming/ATP-sensing processes has emerged recently [179,180]. Data on the cell-surface expression of nucleotide kinases, together with the ability of the cells to retain substantial amounts of ATP in their vicinity (see Section 3), allow one to expand the above concept by hypothesizing the co-existence of spatially anchored intra- and extracellular phosphotransfer networks coordinating energetic homeostasis and metabolic signalling between cell compartments and along the interstitial space.

### 6.3. ATP synthase

ATP synthase (also called  $F_1F_0$  ATP synthase) is the universal enzyme found in the bacterial cytoplasmic membrane, the inner membrane of mitochondria and the thylakoid membrane of chloroplasts where it transforms energy from a transmembrane electrochemical gradient of protons into the phosphoric acid anhydride bond of ATP [206]. ATP synthase is a large protein complex (~500 kDa) composed of a central membrane-embedded portion ( $F_0$ ) and a water-soluble “headpiece” complex ( $F_1$ ). The complete complex harnesses the energy released by proton-motive force (which is the sum of the pH gradient and the membrane potential) to drive ATP synthesis and this process of oxidative phosphorylation needs NADH or  $FADH_2$ , oxygen, ADP and inorganic phosphate. The  $F_1$  portion of ATP synthase (known as  $F_1$ -ATPase or  $H^+$ -ATPase) contains five types of polypeptides in the ratio  $(\alpha\beta)_3\gamma\delta\epsilon$  and alone, without coupling to electron transport, it behaves as a rotatory engine mediating backward ATP hydrolysis via rotation of subunit  $\gamma$  relative to

$(\alpha\beta)_3$  [206,207]. Interestingly, the mammalian ATP synthase in its native state was shown to be localized in a cristae-like subfraction of the mitochondrial inner membrane, where it forms an “ATP synthasome” supercomplex with two required transporters, the protein carrier and the adenine nucleotide carrier [208].

Although ATP synthase is generally believed to be expressed in mitochondria, reports are accumulating that at least certain components of this enzyme complex also exist on the outer face of plasma membrane. Das et al. [209] first demonstrated the expression of a 51.5-kDa protein on the plasma membrane of several tumor cell lines, that has homology to the  $\beta$ -subunit of mitochondrial  $H^+$ -ATPase and is involved in an interaction with cytotoxic lymphocytes. More recently, Moser et al. [210] reported that both  $\alpha$ - and  $\beta$ -subunits of ATP synthase co-localize on the surface of cultured HUVEC and can be involved in the regulation of angiogenesis via direct binding of angiostatin, a potent antiangiogenic and antitumorigenic agent. Subsequent studies confirmed the ectopic localization of ATP synthase components on human vascular endothelial cells [211], adipocytes [212], keratinocytes [47] and various tumor cell lines [213], where they might participate in diverse processes such as regulation of lipid metabolism, immune responses, tumor metastasis and regression, as well as in control of cell proliferation and differentiation. It has also been shown recently that an entity related to the mitochondrial  $F_1$ -ATPase is expressed on the tumor cell surface where it binds a delipidated form of apolipoprotein A-I, and promotes tumor recognition by  $V\gamma 9V\delta 2$  T cells, a major  $\gamma\delta$  T lymphocyte subset in humans [214].

While most of the above studies have defined this ecto-ATP synthase as a “catalytically active complex”, strictly speaking, no direct evidence supporting this statement had been provided so far. In fact, the assignment of cell-surface expression of ATP synthase is primarily based on immunofluorescence and electron microscopic observations, proteomic studies and structural and biochemical analyses of plasma membrane fractions after biotinylation of cell-surface proteins. As for the ability of this ectoprotein to generate ATP from ADP and orthophosphate using proton-motive force, this inference mainly rests on bioluminescent assays showing a significant increase in extracellular ATP levels within the first minute after addition of micromolar ADP concentrations, and on partial inhibition of this  $ADP \rightarrow ATP$  conversion by oligomycin and other inhibitors of ATP synthase.

However, such interpretation of data on extracellular ATP synthesis needs critical reassessment. Firstly, we [30,76] and others [46,47,193] also elicited ADP-to-ATP conversion on the surface of various cell types. However, marked inhibition of this reaction by a specific inhibitor of AK (diadenosine pentaphosphate,  $Ap_5A$ ) in these studies suggests the predominant contribution of ecto-AK, rather than ATP synthase, to extracellular ATP generation from ADP. Secondly, the abundant expression of yet another nucleotide-phosphorylating ectoenzyme, NDP kinase, does not exclude the possibility of immediate ADP transphosphorylation with endogenous ATP and other non-adenine nucleotides [44], which can be either acutely released upon mechanical cell stimulation (during addition of exogenous ADP) or constitutively retained in the cell vicinity at micromolar levels (see Section 3). Thirdly, dual-label TLC assays with [ $^3H$ ]

ADP and  $^{32}\text{P}_i$  have demonstrated that the  $[^3\text{H}]\text{ATP}$  generated from  $[^3\text{H}]\text{ADP}$  on surface of human hepatocytes was not  $^{32}\text{P}$ -labelled and thus, this catalytic reaction did not require  $^{32}\text{P}_i$  for ATP synthesis [46]. The inability of millimolar concentrations of non-labelled inorganic phosphate to increase the rate of  $[^3\text{H}]\text{ADP}$  conversion into  $[^3\text{H}]\text{ATP}$  was also confirmed in studies with human endothelial cells [76] and hepatocytes [46]. Lastly, the specificity of the inhibitors used to discriminate between the  $\text{F}_1/\text{F}_0$  contributions has not been tested on other purinergic ectoenzymes, which also might lead to misleading interpretation of the results. Collectively, while certain components of ATP synthase can be expressed on the cell surface and serve as receptor for angiotensin and other multiple ligands, further studies are required to elucidate the contribution of this ectoprotein into purinergic signalling via extracellular ATP synthesis.

## 7. Coordinated control of purinergic signalling via a network of purinergic ectoenzymes

### 7.1. Interplay between ATP-consuming and ATP-regenerating pathways

Current findings support the presence of an extensive network of ectoenzymes that are co-expressed to a variable extent among the mammalian tissues and share similarities in substrate specificity. In principle, the duration and magnitude of purinergic signalling can be coordinated via two opposite, ATP-consuming and ATP-regenerating, pathways, where sequential nucleotide breakdown to adenosine and further to inosine/hypoxanthine is counterbalanced by re-synthesis of high-energy phosphoryls through backward phosphotransfer reactions [30]. Under normal physiological conditions, the ecto-nucleotide kinase activities seem not to compete with ecto-nucleotidases for a limited pool of the released nucleotide substrate. Indeed, the nucleotide-binding centres of AK are assembled with large domain movements upon simultaneous binding of both substrates, whereas the existence of enzyme either in ATP- or AMP-bound forms does not cause sufficient conformational changes [78]. Therefore, the release of a particular nucleotide alone would cause its rapid inactivation through sequential ecto-

nucleotidase reactions (Fig. 2). However, in the settings of inflammation or traumatic shock, extracellular levels of nucleotides can be simultaneously increased up to  $10^{-5}$ – $10^{-4}$  mol/L thereby causing additive conformational changes of ecto-nucleotide kinases. Acute changes in the specific ratios of nucleoside mono-, di- and triphosphates, in conjunction with feed-forward inhibition of ecto-5'-nucleotidase activity by precursor ATP/ADP [76,96,215], should determine the directional shift from a nucleotide-inactivating pathway towards backward ATP regeneration (Fig. 5).

### 7.2. Peculiarities of purine metabolism in the vasculature at various (patho)physiological states

As stated above (see Section 4), specific ecto-nucleotidase activities can be spatially compartmentalized within different elements of the vasculature and also substantially vary among different subsets of lymphoid cells. In this scenario, the cells with high NTPDase and ecto-5'-nucleotidase activities, such as vascular endothelium, tend to rapidly inactivate the released ATP/ADP with respective generation of adenosine and in this way, represent the major effector system for termination of proinflammatory and prothrombotic responses in the cardiovascular system [12,22]. In contrast, the combination of relatively low ecto-nucleotidase and high ADA activities in the leukocytes and other hematopoietic cells would allow cells of the immune system either to generate less adenosine or to remove it more efficiently from the environment and consequently protect cells from its inhibitory effects [10,30]. Despite this general trend, the NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 activities can be selectively up-regulated on a certain population of activated  $\text{CD4}^+/\text{CD25}^+/\text{Foxp3}^+$  Treg cells, thereby contributing to the maintenance of peripheral tolerance and control of the inflammatory autoimmune diseases [108,123]. These differences in purine catabolism among the endothelial and lymphoid cells might be particularly pertinent firstly, in the context of clear-cut distinctions in the purinergic signalling pathways between these cell types, and secondly, because of divergent, often opposite, effects of ATP and its intermediate metabolite adenosine on endothelial function and hemostasis [10,11].

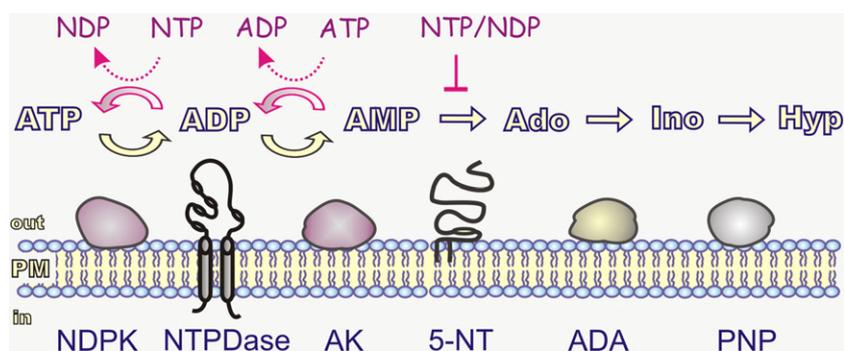


Fig. 5. Ectoenzymatic purine turnover in the vasculature. The elements of purine-inactivating chain include E-NTPDases, ecto-5'-nucleotidase (5-NT), adenosine deaminase (ADA), and purine nucleoside phosphorylase (PNP), whereas an opposite ATP-regenerating pathway is mediated via sequential adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK) reactions. Thin dashed arrows and vertical blunted line depict activatory and inhibitory mechanisms, respectively.

Local nucleotide and nucleoside concentrations can also be directionally modulated under certain physiological circumstances. For instance, in the promyelocytic HL60 cells, induced to differentiate along the macrophage lineage by phorbol esters, there were coordinated alterations in purinergic activities, accompanied by a switch from the adenosine-metabolising phenotype in undifferentiated cells toward the adenosine-producing phenotype in macrophages [32,147]. Coordinate regulation of purinergic activities occurring during cancer progression also allows solid tumors to escape cytostatic effects of ATP and at the same time, to maintain high concentrations of immunosuppressive and tumor-promoting adenosine [32].

Likewise, endothelial nucleotide-inactivating ectoenzymes NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 can be up-regulated during acute hypoxia and inflammation and in this way, increase the intravascular adenosine concentrations and dampen excessive inflammatory responses by affecting endothelial barrier function, adhesion and transmigration of lymphoid cells and expression of other molecules involved in the adhesion cascade [52,169,216]. On the other hand, other investigators have shown significant down-regulation of ecto-nucleotidase activities on vascular endothelium and other cell types during chronic hypoxia and oxidative stress, that was accompanied by concomitant perturbation of nucleotide-mediated signalling pathways and development of vascular disorders [45,105]. Along with increased adenosine production, adaptive responses to acute hypoxia might also include the diminished adenosine uptake into the cell via concurrent decreases in the expression levels of equilibrative nucleoside transporters [35], as well as increased adenosine signalling caused by transcriptional induction at the receptor level [48,139,150].

While most of the current studies have focused on the concerted actions of ecto-nucleotidase activities, recent findings on the co-expression of counteracting ATP-regenerating ectoenzymes suggest that regulation of purine metabolism might extend beyond the inactivating pathways. Specifically, along with low ecto-nucleotidase and high ADA activities, the lymphoid cells are characterised by relatively high ecto-AK and NDP kinase activities [30] and in addition, are capable of maintaining micromolar “ATP halo” in their immediate vicinity

[81,83]. This “ATP-regenerating/adenosine-eliminating” phenotype of lymphoid cells, in conjunction with leukocyte-governed suppression of endothelial ecto-5'-nucleotidase [11,88], could represent a novel form of cross-talk between adherent leukocytes and targeted endothelium which facilitates leukocyte transmigration from the blood into the tissues (Fig. 6).

### 7.3. Membrane compartmentalization of ectoenzymes and other components of purinergic signalling cascade

Based on kinetic analysis of sequential nucleotide breakdown by vascular endothelial and smooth muscle cells attached to polystyrene beads, the “two-compartment” model has been proposed earlier [96,215]. According to this model, extracellular ATP and other nucleotide substrates are initially concentrated from the bulk phase on the cell surface followed by preferential “hand-to-hand” delivery of the generated product for the succeeding phosphatase reactions. This work nicely foreshadowed the ensuing observations which directly demonstrated the accumulation of relatively high ATP concentrations in the cell vicinity (see Section 3). Another important discovery from these kinetic studies of Slakey and colleagues is that the nucleotide-inactivating ectoenzymes are located in close proximity with each other, presumably within certain caveolae or pits. Membrane compartmentalization is an important feature which permits divergent cellular functions to take place at specific microdomains on the cell surface. These membrane-specialized assemblies are formed by high concentrations of cholesterol and sphingolipids immersed in a more fluid unsaturated glycerophospholipid environment, are enriched in GPI-anchored proteins, caveolin and numerous signalling proteins, and have been implicated in many cellular functions such as signal transduction, endocytosis and cholesterol transport [217].

Like other GPI-anchored proteins, the enzyme ecto-5'-nucleotidase/CD73 is mainly localized in these microdomains [25,141], remains relatively resistant to the solubilization by non-ionic detergents and can be cleaved from the cell membrane after treatment with phosphatidylinositol-specific phospholipase C [51,88,145]. Detailed localization studies based on enzymatic assays and immunochemistry have shown that the

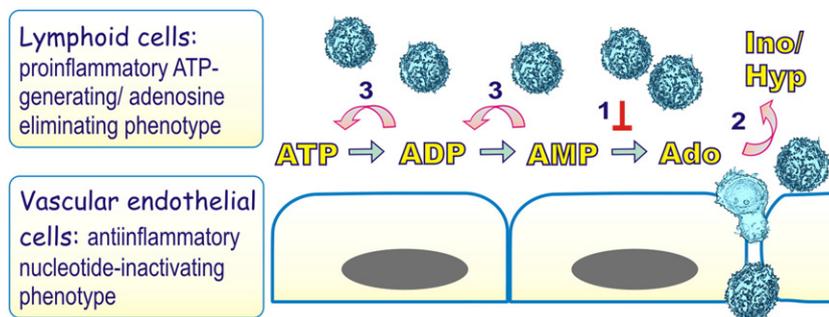


Fig. 6. Regulation of purine metabolism during leukocyte-endothelial adhesion. Under basal conditions, vascular endothelium efficiently inactivates extracellular ATP and ADP via AMP to adenosine through sequential NTPDase and ecto-5'-nucleotidase reactions. In contrast, due to the high ecto-nucleotide kinase and ADA and relatively low ecto-nucleotidase activities, the lymphocytes are generally characterised by ATP-regenerating/adenosine-eliminating phenotype. During inflammation, the adherent lymphoid cells (blue) could facilitate their transendothelial migration via (1) inhibition of endothelial ecto-5'-nucleotidase, (2) rapid deamination of the remaining adenosine and (3) maintenance of external levels of proinflammatory ATP at relatively high levels.

other nucleotidase NTPDase1/CD39 is also localized in characteristic flask-shaped invaginations of the endothelial plasma membrane, called caveolae [218]. Post-translational modification (e.g. palmitoylation or phosphorylation) which is known to influence association of proteins with rafts, can account for the dynamic association of CD39 with lipid assemblies [219]. Cholesterol depletion from human placental membranes by methyl- $\beta$ -cyclodextrin or cholesterol sequestration within the membrane by filipin or nystatin treatments was also shown to affect the conformation of the catalytic ecto-domain and strongly inhibit both the catalytic and antiplatelet activities of CD39 [220]. Other components of the purinergic signalling cascade could also be associated with lipid rafts and caveolae, including ATP permeation sites [91], releasable ATP stores [83], ATP synthase [211,212], adenosine A<sub>1</sub> receptors [221] and nucleotide-selective P2Y [222], P2X<sub>1</sub> [223] and P2X<sub>3</sub> [224] receptors.

## 8. Regulation of local nucleotide and nucleoside levels by soluble enzymes

### 8.1. Soluble purine-inactivating and -phosphorylating activities circulate in blood plasma

The ability of human plasma to dephosphorylate micromolar concentrations of ATP and other adenine nucleotides into adenosine and further to inosine/hypoxanthine was first demonstrated over half century ago [225]. Subsequent radio-TLC and HPLC assays allowed the identification and kinetic characterisation of at least three soluble nucleotidase activities in the human blood, i.e. soluble ATPase, ADPase and AMPase (5'-nucleotidase) [226,227]. More recent chromatographic analyses of the pattern of [ $\gamma$ -<sup>32</sup>P]ATP breakdown by serum or plasma from humans and other species (sheep, mouse, rabbit) revealed that circulating ATP is primarily converted into AMP and PP<sub>i</sub> via soluble NPP activity [77,228]. While the initial step of ATP breakdown in human plasma is mainly attributable to NPP activity, recent studies elicited the co-existence of another soluble enzyme NTPDase which accounts for ~7–10% of the total soluble ATP-hydrolysing pool and in addition, efficiently utilises ADP as another preferred substrate [131]. Notably, additional ultracentrifugation of serum samples did not affect their nucleotide-hydrolysing capacity [77,131], indicating that both NPP and NTPDase presumably circulate in the human blood as “true” soluble enzymes. The resulting AMP is then degraded via adenosine to inosine through sequential 5'-nucleotidase and ADA reactions, which are also present in the bloodstream as soluble enzymes [77,122,227]. Furthermore, the co-existence of counteracting nucleotide-phosphorylating pathway has been demonstrated in human serum, with the elements of ATP-regenerating chain comprising two soluble enzymes, AK and NDP kinase [77,131]. Interestingly, the existence of soluble AK activity in human plasma and its contribution to intravascular ADP metabolism has been proposed four decades ago [229]. TNAP can also be found in serum in an anchor-depleted form, where it most likely regulates the intravascular PP<sub>i</sub> pool, while the contribution of this phosphatase to the metabolism of circulating ATP seems to be quite negligible [28].

### 8.2. Physiological relevance of soluble versus membrane-associated ectoenzymes

Understanding the physiological role of soluble enzymes within the larger framework of membrane-associated ectoenzymes is another important issue. Under basal conditions, vascular endothelial NTPDase1/CD39, in concert with ecto-5'-nucleotidase/CD73, efficiently regulate nucleotide homeostasis in the vasculature [12,105]. Among cellular elements of the blood, the leukocytes, but not erythrocytes and platelets, also contribute to the catabolism of circulating nucleotides, whereas the activities of soluble nucleotidases in plasma are far less than that on vascular endothelial and lymphoid cells [122,227]. However, vascular NTPDase activity can be diminished under conditions of inflammation, thereby causing profound shifts in vascular permeability and local procoagulant responses. The strategies to offset the reduced nucleotide-scavenging effectiveness of vascular endothelium include either NTPDase expression in the injured vessels or administration of soluble nucleotidases for antiplatelet therapy [105]. Indeed, soluble form of human NTPDase/CD39 is currently considered a promising aspirin-insensitive antithrombotic drug, which inhibits platelet reactivity under experimental prothrombotic conditions, including ischemia–reperfusion murine models of cerebral shock [124], cardiac [129], hepatic [105], pulmonary [127] and renal [128] injuries as well as various acute and chronic models of transplantation [105]. Likewise, treatment with soluble 5'-nucleotidase reduced infarct size and improved cardioprotection both in wild-type and CD73<sup>-/-</sup> mice during acute myocardial ischemia [61] and also attenuated tissue damage and improved survival during acute lung injury [127].

Taken together, the above data suggest that soluble nucleotidases could represent an important auxiliary effector system for local inactivation of acutely elevated nucleotides, especially at sites of injury and inflammation. Moreover, the identification of soluble AK and NDP kinase capable of rapidly transphosphorylating micromolar concentrations of nucleoside mono-, di- and triphosphates [77] might represent a novel and currently unappreciated route regulating the pattern of nucleotide receptor stimulation and/or desensitization in the blood. Further studies are required to understand the pertinence of soluble purinergic activities within the larger framework of membrane-bound ectoenzymes as well as to elucidate the origin and exact mechanisms underlying their appearance in the bloodstream at various (patho)physiological conditions.

### 8.3. Secreted Ca<sup>2+</sup>-activated nucleotidases and other purinergic enzymes in other biological systems

The phenomenon of secreted purinergic enzymes has also been documented in other biological systems. Specifically, a certain portion of nucleotide-inactivating enzymes NTPDase and/or 5'-nucleotidase can be released from the nerve terminals of the guinea-pig vas deferens during electric stimulation [230], human lymphocytes during mAb triggering [145], cultured human endothelial cells in response to the application of shear force [43], nasal lavage after stimulation of submucosal gland

secretion [75] and rat pancreatic acini under resting conditions [231] and upon stimulation with cholecystokinin octapeptide-8 [106,119]. Exact cellular mechanisms underlying the releases of soluble enzymes remain largely unclear and might particularly involve selective shedding of ecto-5'-nucleotidase from the cell surface via directional cleavage of its GPI-anchor [43,145] or the presence of certain secretory pathways as it was suggested for microvesicle-wrapped ATP-diphosphohydrolase/CD39 [106,231]. In a variety of human tumor cell lines, the constitutive secretion of Nm23/NDP kinase protein can also be detected [197]. In addition, significant release of catalytically active AK and NDP kinase has been reported from human airway epithelial cells and nasal lavage after stimulating submucosal gland secretion [75].

Interestingly, the saliva of most blood-feeding arthropods (including fleas, ticks, biting midges, and mosquitoes) also contains potent soluble apyrases, designed to counteract a host's normal blood-clotting mechanisms. Secreted by the arthropod into the host's wound during blood-feeding, these soluble apyrases inhibit hemostasis via rapid hydrolysis of intravascular ADP into AMP and are strongly activated by  $\text{Ca}^{2+}$  [232]. Although analogous to the E-type NTPDases with regard to their enzymatic properties, these secreted enzymes from blood-sucking insects are not homologous to the apyrase family with regard to their amino acid sequences, contain invariant "nucleotidase conserved regions" [233] and in addition, have related homologs in humans, rodents and several invertebrates [234]. Recently, human [232] and rat [235] soluble  $\text{Ca}^{2+}$ -dependent apyrases related to two secreted apyrases of the bloodsucking insects, *Cimex lectularius* and *Phlebotomus papatasi*, have been cloned and characterised. However, in striking contrast to insect subfamily members, these mammalian apyrases preferred GDP and UDP as substrates. Using a combined structural biology approach, Dai et al. were able to redesign human apyrase and shifted its substrate specificity for ADP by more than 100-fold [236]. This engineered enzyme potentially inhibited ADP-induced platelet aggregation and may serve as the basis for development of a new class of antithrombotic agents for treatment of patients with heart and vascular diseases.

## 9. Concluding remarks

Extracellular nucleotides and adenosine are versatile signalling molecules known to be implicated in an array of cell-specific responses both in an autocrine and paracrine fashions. The integral response of the cell is determined by multiple factors, including the release of endogenous ATP and other agonists, expression of different types of receptors with respective diversities in the transduction pathways, as well as specific make-up of enzymes finely governing the duration and magnitude of purinergic signalling. The data that have been described in this review highlight the importance of nucleotide-releasing and inactivating mechanisms in the regulation of cell responses and on this basis provide a sufficient justification for re-examination of the current concepts of purinergic signalling cascade.

In particular, nanomolar ATP concentrations detected in bulk extracellular fluids by using conventional bioluminescent assays

are generally far below the micromolar (or even submillimolar)  $\text{EC}_{50}$  values for most P2 receptors. This apparent discrepancy could be reasonably resolved based on the innovative nucleotide-sensing approaches providing independent lines of evidence that the released ATP can be spatially confined in the pericellular space at high micromolar levels with only minor nucleotide portion being diffused and/or convected into the bulk medium. Recent data on the ability of various cells to release, along with ATP and ADP, other related compounds (adenosine, UTP, dinucleoside polyphosphates and nucleotide sugars) gain another important insight into our understanding of mechanisms initiating a signalling cascade.

Next, the co-existence of two opposite, purine-inactivating and ATP-regenerating, ectoenzymatic pathways represent a principally distinct route for appearance and inactivation of extracellular agonists on the cell surface, where sequential ATP and ADP breakdown to adenosine and further to inosine/hypoxanthine is counterbalanced by rapid interconversion of adenine and non-adenine nucleotides via ecto-phosphotransfer reactions (Fig. 5). The identification of a complex mixture of soluble enzymes freely circulating in the bloodstream adds another level of complexity to our understanding the regulatory mechanisms of purine homeostasis in the vasculature and in addition, may open up further research to assess the potential therapeutic and diagnostic applications of purinergic enzymes. Clearly, identifying the exact mechanisms by which the extensive network of membrane-associated and soluble enzymes regulates local nucleotide and nucleoside concentrations and co-ordinately governs the purinergic responses will be an area of intense interest in the future.

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