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Article *in* Immunology Letters · November 2018

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Extracellular nucleotides and nucleosides as signalling molecules

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ARTICLE INFO

Keywords:
Extracellular ATP
Extracellular adenosine
Purinergic receptors
Ecto-nucleotidases
P2X7

ABSTRACT

Extracellular nucleotides, mainly ATP, but also ADP, UTP, UDP and UDP-sugars, adenosine, and adenine base participate in the "purinergic signalling" pathway, an ubiquitous system of cell-to-cell communication. Fundamental pathophysiological processes such as tissue homeostasis, wound healing, neurodegeneration, immunity, inflammation and cancer are modulated by purinergic signalling. Nucleotides can be released from cells via unspecific or specific mechanisms. A non-regulated nucleotide release can occur from damaged or dying cells, whereas exocytotic granules, plasma membrane-derived microvesicles, membrane channels (connexins, pannexins, calcium homeostasis modulator (CALHM) channels and P2X7 receptor) or specific ATP binding cassette (ABC) transporters are involved in the controlled release. Four families of specific receptors, i.e. nucleotide P2X and P2Y receptors, adenosine P1 receptors, and the adenine-selective P0 receptor, and several ecto-nucleotidases are essential components of the "purinergic signalling" pathway. Thanks to the activity of ecto-nucleotidases, ATP (and possibly other nucleotides) are degraded into additional messenger molecules with specific action. The final biological effects depend on the type and amount of released nucleotides, their modification by ecto-nucleotidases, and their possible cellular re-uptake. Overall, these processes confer a remarkable level of selectivity and plasticity to purinergic signalling that makes this network one of the most relevant extracellular messenger systems in higher organisms.

1. Role of extracellular nucleotides and nucleosides

Purines and pyrimidines are basic elements of all living organisms. They are the basic constituents, together with a carbohydrate residue, either ribose or deoxyribose, of nucleosides that, with the addition of phosphate residues, make up the nucleotides (Fig. 1). Both nucleotides and nucleosides are ubiquitous molecules participating in a multiplicity of cellular processes, mainly as building blocks of nucleic acids, but also as energy intermediates, coenzymes, allosteric modulators, as well as intracellular and extracellular messengers.

It is well known that intracellular molecules can be released into the extracellular space and serve as stimulatory signals for receptors and sensors in different tissues and organs. Likewise, intracellular nucleotides and nucleosides can be released from cells in different conditions [1,2].

The first evidence of a role for nucleotides (ATP, ADP, UTP, UDP, UDP-sugars) and for nucleosides (adenosine) in the complex scenario of cell-to-cell signalling was provided by Geoffrey Burnstock who first coined the term "purinergic signalling" in 1970s [3]. After the initial scepticism regarding the odd possibility that ATP was released into the extracellular milieu by "healthy" cells, the hypothesis that ATP might serve as extracellular messenger gained momentum, and now the field of purinergic signalling has greatly expanded. Firstly identified as a neurotransmitter, ATP and its degradation products have been later found to serve as signalling molecules in many different cellular processes. The definitive sanction of the purinergic hypothesis was provided by ATP receptor cloning, initially the prototypical metabotropic and ionotropic nucleotide receptors named P2Y1 [4] and P2X1 [5] followed by the other members of the family [6]. Since then, an overall number of fifteen P2 receptors and four P1 receptors with various functions and wide distribution in different organs and tissues have been identified, powering an exponential growth of this research field with an unusual complexity and intriguing pathophysiological implications [7–10].

Virtually all cells release nucleotides and express plasma membrane receptors for nucleotides (P2 receptors) and nucleosides (P1 receptors) [11]. The majority of cells express several P2 or P1 receptor subtypes, with different ligand selectivity and/or affinity, thus enabling a wide range of cellular responses in both physiological and pathological conditions [12]. Physiological responses include: neurotransmission and neuromodulation [13], glial-neuron interactions [14], hormone secretion [15], sensory transmission [16], specialized functions of

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https://doi.org/10.1016/j.imlet.2018.11.006
Received 8 August 2018; Accepted 9 November 2018

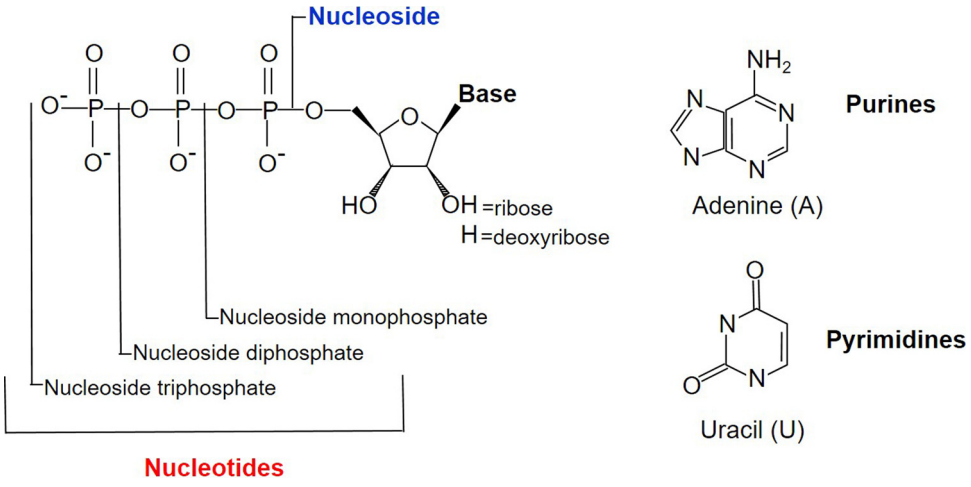

Fig. 1. Biochemical structure of nucleosides and nucleotides.

different organs including liver [17], kidney [18,19], platelets [20] and cardiovascular apparatus [21], immune [22–24], musculoskeletal [25], respiratory [26] and gastrointestinal systems [27]. In addition, different pathological conditions foresee signalling via extracellular nucleotides. These include disorders of the central nervous system such as neuropathic pain, trauma, cerebral ischemia, multiple sclerosis, Parkinson's and Alzheimer's diseases [28]. In addition, a large number of pathophysiological alterations are included [29], such as liver [17] and cardiovascular diseases [30,31], infections [32], inflammation [33–35], pain [36] and cancer [37–39].

ATP and other nucleotides exhibit all the fundamental features of bona fide extracellular messengers. Firstly, they are present in very little amounts (nmol/l) in the extracellular space under physiological conditions, such as resting cells or healthy tissues. Secondly, they are stored intracellularly to very high amounts (from 5 to 10 mmol/l). Thirdly, they are water soluble, and finally they are rapidly hydrolysed by ubiquitous extracellular nucleotidases. Thanks to all these features, nucleotides may act as extracellular messengers characterized by: (a) high signal-to-noise ratio; (b) rapid diffusion through the liquid rich intercellular spaces; and (c) fast signal shut-off to avoid overstimulation or receptor desensitization.

2. Receptors for nucleotides and nucleosides

The specific and widely distributed receptors for extracellular nucleotides are named P2 receptors. Affinity of P2 receptors for extracellular nucleotides ranges from the high nanomlolar/low micromolar (100 nM to 20 μM), to the high micromolar/millimolar (300 μM to 1 mM) range. The P2 receptor family includes the P2X (P2XRs) and P2Y (P2YRs) receptor subfamilies. P2XRs are ATP-gated channels allowing Na^+, Ca^{2+} influx and K^+ efflux [40]. On the other hand, P2YRs are coupled to G proteins, triggering downstream effector signalling pathways ending with increased concentration of either intracellular Ca^{2+} or cyclic adenosine monophosphate (cAMP) [41]. P2YRs have in general higher affinity for their ligands (high nanomlolar/low micromolar), while affinity of P2XRs can widely vary, from the low micromolar to the millimolar level (e.g. the P2X7R). In addition, four receptors for adenosine have been identified and included in the P1 family, whereas receptors for adenine are included in the P0 family. Multiple receptors expressed and the wide range of affinities endowes purinergic signalling with an unusual plasticity.

Seven ionotropic receptors (P2XR1-P2XR7) have been identified and cloned so far in mammals, their physiological agonist being exclusively ATP [42]. The cation selective channels formed by P2XRs require the assembly of at least three subunits forming hetero- (e.g. P2XR2/3 and P2XR1/5) or homo-trimers (P2X7R) [42]. Each P2XR subunit has two membrane-spanning domains (TM1 and TM2), with intracellular N- and C-terminus, and most of the protein exposed in a large ectodomain. The functional receptor is a trimer showing three ATP-binding sites which all need to be occupied to trigger receptor/channel opening [43]. The P2X7R is a rather unusual receptor/channel since generates a non-selective plasma membrane pore (macropore) that allows transit of aqueous molecules of MW up to 900 Da. Despite previous claims that P2X7R needs recruitment of additional molecules (e.g. pannexin-1 or connexin-43) to generate the macropore, it is now clear that the macropore is intrinsic to the P2X7R [44–46]. P2X7R plays a key role in immunity and inflammation as a major activator of the NLRP3 inflammasome, and therefore a powerful trigger of IL-1β maturation and secretion [33,47–50].

Eight G protein-coupled metabotropic receptors (P2YR1, P2YR2, P2YR4, P2YR6, P2YR11, P2YR12, P2YR13, and P2YR14) have been so far identified and characterized in mammals, with an organ, tissue and cell specific distribution. Preferred agonists are ATP (P2YR11), ADP (P2YR1, P2YR12 e P2YR13), UTP (P2YR2 and P2YR4), UDP (P2YR6), UDP-glucose and UDP-galactose (P2YR14). P2YR1, P2YR2, P2YR4, and P2YR6 activate Gq and phospholipase C-β (PLC-β), generating inositol 1,4,5-trisphosphate (IP3), which increases intracellular Ca^{2+} via release from intracellular stores, and diacylglycerol (DAG), which in turn activates protein kinase C (PKC) [12]. P2YR12, P2YR13, and P2YR14 activate G_i, leading to inhibition of adenylyl cyclase (AC) and to reduction of intracellular cyclic adenosine monophosphate (cAMP) levels. P2YR11 stimulation triggers an increase of intracellular Ca^{2+} and cAMP via activation of both Gq and Gs. New intracellular signalling pathways activated by P2YRs have been very recently described, highlighting the great potential of nucleotide-stimulated intracellular signalling. These include the recruitment of the Gβγ subunit, leading to activation of phosphatidylinositol- 4,5-bisphosphate 3-kinase γ (PI3K-γ), phospholipase C-β2 and -β3, inward rectifying K^+ (GIRK) channels, G protein-coupled receptor (GPCR) kinases 2 and 3, Rho, and mitogen activated protein kinases (MAPKs) [51,52].

The P1 receptor family includes four adenosine receptors (A1, A2A, A2B, and A3) [53–55]. Their activation triggers stimulation or inhibition of AC, depending on the given receptor subtype, thus modifying intracellular cAMP levels [56]. A1 and A3 receptors are coupled to G proteins of the Gi, Gq, and Go family, thus leading to calcium release from intracellular stores. On the other side, A2A and A2B receptors are coupled to Gs or Gq proteins and to AC or PLC activation. Moreover, all P1 receptors stimulate MAPK pathways, extracellular signal regulated kinase 1 (ERK1), ERK2, Jun N-terminal kinase (JNK), and p38-MAPK included. In addition, extracellular adenosine can be taken up by the cells and stimulate different intracellular pathways such as AMP-activated protein kinase (AMPK), adenosine kinase (AK), and S-adenosyl

homocysteine hydrolase pathways [57]. The end result is the activation of anti-inflammatory and immune suppressive responses, aimed at promoting healing and finally restoring tissue homeostasis [57]. Adenosine acts as an immunosuppressant by inhibiting functions of several immune cell populations such as T and B lymphocytes, NK cells, dendritic cells, monocytes, and macrophages [58–60].

Finally, G-protein-coupled receptors (GPCRs) named P0, activated by adenine base but not adenosine, have been discovered more recently. The first receptor activated by adenine was discovered in rats [61]. Later, adenine receptors from other species, all belonging to the family of GPCRs, have been cloned and pharmacologically characterized [62,63]. Based on the nomenclature for other purinergic receptor families (P1 for adenosine and P2 for nucleotide receptors), adenine receptors were designated P0 receptors [64]. G protein-coupled P0 receptors in humans have been pharmacologically identified but not cloned so far [65].

3. Mechanisms of extracellular nucleotide release

Extracellular nucleotide release pathways include both specific and non-specific mechanisms [1]. Intracellular nucleotides can be released in a non-regulated fashion in response to various cell stress- and cell death-inducing conditions, such as shear stress following mechanical strain, stress induced by cytotoxic agents, hypoxia or plasma membrane damage [66,67]. During inflammation cell membrane disruption leads to non-specific release of large amounts of nucleotides due to the very large intracellular/extracellular nucleotide gradient. Nucleotides released from stressed or injured cells act as “danger” and “find-me” signals that drive phagocyte migration to damage sites [68,69] and mediate clearance of cell debris [70]. Release of ATP and UTP by necrotic cells is sensed by the P2Y2R expressed by neutrophils and macrophages and is relevant for detection of necrosis and clearance of dead cells [70,71]. On the other hand, nucleotides may also be released via specific mechanisms, including vesicular exocytosis, microvesicles and different types of channels and transporters [1,72]. Non-lytic, specific, nucleotide release pathways play a key role in the local modulation of inflammation and immunity.

A main mechanism for ATP release from intact cells is regulated exocytosis (Fig. 2). Cytosolic granules containing nucleotides can rapidly release their content via fusion with the plasma membrane [73,74]. A vesicular nucleotide transporter (VNUT) is responsible for storage of ATP. VNUT is localized to intracellular vesicles where it accumulates ATP into the vesicle lumen exploiting the proton-based electrochemical gradient (positive inside) established by the vacuolar-ATPase (v-ATPase) [75–77]. Exocytosis of ATP-containing granules then occurs via the well-known soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated route [78,79].

The specific transport mechanisms include connexins or pannexin hemichannels, ATP binding cassette (ABC) transporters, calcium homeostasis modulator (CALMH) channels or the P2X7R itself [8,72,80–84] (Fig. 2).

Connexin or pannexin family members involved in ATP release are mainly connexin-43 and pannexin-1 [71]. Although connexins and pannexins do not share sequence homology, the two families of proteins show similar structural features, with N- and C-terminal domains on the cytoplasmic side of the plasma membrane, four membrane-spanning segments and both intracellular and extracellular loop domains [85,86]. The main functional difference between these two channel-forming molecules, is that connexins can form both gap junctions and hemichannels, while pannexin proteins only form hemichannels [87]. Connexins and pannexins assemble to form hexameric membrane structures called connexons and pannexons, respectively, that mediate extracellular release of small molecules with MW below 1–2 kDa, ATP [88,89], glutamate, and prostaglandins included, besides influx of small cations such as Na^+ and Ca^{2+} [45,90]. Connexins forming gap junctions or unopposed hemichannels are involved in intercellular communications in various pathophysiological settings. Connexins are classified according to the MW of their subunits, and 21 connexin isoforms have been currently identified in humans [91]. Connexin-based gap-junctions allow direct communication between the cytoplasm of adjacent cells, while undocked connexin hemichannels mediate release of low MW cytoplasmic components into the extracellular milieu [92–94]. In resting cells, connexin hemichannels are very likely kept in the closed state to avoid dissipation of ion, metabolites and high-energy intermediates [95]. It is thought that cell perturbation by different agents triggers connexin hemichannel opening [96]. The main isoforms

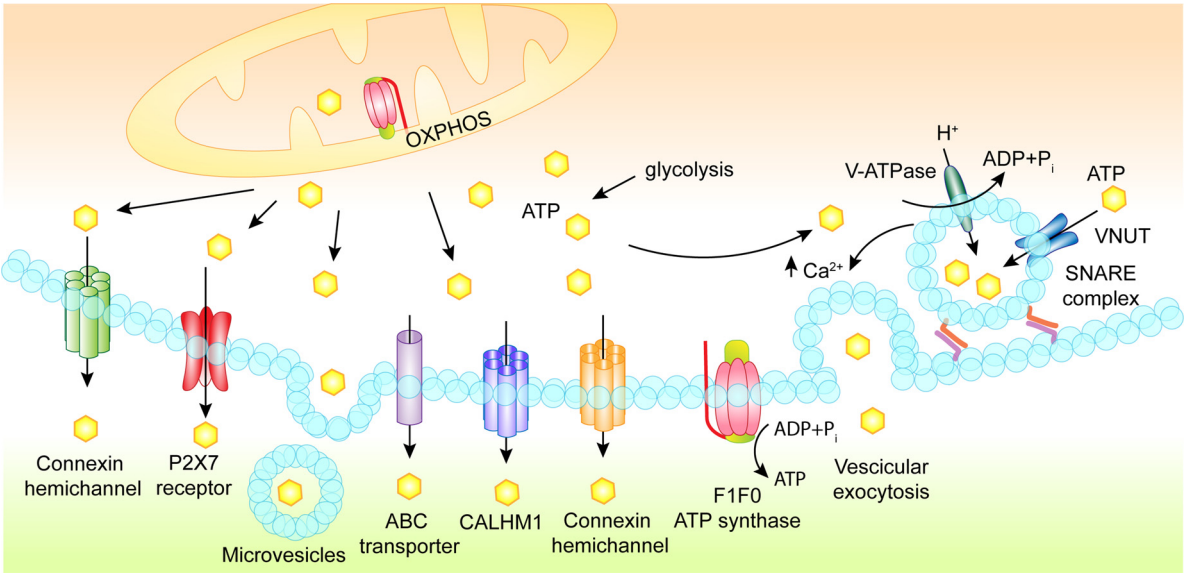

Fig. 2. Different pathways for regulated nucleotide release. ATP generated inside the cell by glycolysis and oxidative phosphorylation can be released through vesicular exocytosis, plasma membrane-derived microvesicles, connexin or pannexin channels, specific ATP binding cassette (ABC) transporters, calcium homeostasis modulators (CALHM) channels or the P2X7R. ATP is accumulated into secretory vesicles thanks to the concerted action of V-ATPase that generates a proton gradient (positive inside) across the vesicle membrane, and the vesicular nucleotide transporter (VNUT) that exploits the electrochemical gradient to transport ATP into the vesicles. Then, exocytosis occurs via the canonical Ca^{2+}-activated *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) complex. A plasma membrane F1F0 ATP synthase has also been suggested to contribute to ATP increase in the pericellular space.

shown to mediate ATP release are connexin-43, -37, -36 and -26 [97]. Connexin-43 activation may be driven by changes in the intracellular calcium concentrations, cell membrane depolarization, reactive oxygen species (ROS) and nitric oxide (NO). In monocytes and macrophages, connexin-43 activation is triggered by stimulation of TLR2 and TLR4 by the chemotactic factor N-formyl Met-Leu-Phe (fMLP) and LPS, respectively [98,99].

The human pannexin family numbers three members: pannexin-1, -2 and -3 [97]. Pannexin-1 and -3 are expressed in multiple tissues while pannexin-2 is almost exclusively expressed in the brain [90]. Under basal homeostatic conditions, pannexin channels, like connexin channels, are in a closed state. It is thought that the C-terminal tail plugs up the pore from the intracellular side thus keeping the channel closed [95]. Cleavage of the C-terminal tail by caspase-3 and 7 or caspase-11 relieves inhibition and allows pannexin-1 channel opening. [100,101]. Once pannexon is formed, cleavage of C-terminal tails in additional pannexin-1 subunits drives a progressive increase in size of the channel leading to increased permeability to both ions and larger molecules, nucleotides included [102]. Opening and activation of pannexin-1 channels is mediated by multiple intracellular events, including intracellular calcium increase, plasma membrane depolarization [103], activation of the P2X7R [104,105], redox potential changes [106], and mechanical stress [89]. ATP released through pannexin-1 channels and feeding back on the P2X7R can promote internalization of the pannexin-1 channel itself in an autocrine negative feedback loop [107]. Pannexin-1 channels may be responsible for controlled nucleotide release from apoptotic cells [108]. Both ATP and UTP released during apoptosis attract monocytes to remove cellular debris [109,110], while only ATP supports secretion of IL-1β by monocyte/macrophages via activation of the NLRP3 inflammasome [108].

The ABC transporters are a class of integral membrane proteins that use ATP hydrolysis to support transmembrane movement of different molecules, such as cholesterol, lipids and hydrophobic and hydrophylic drugs, across the cell plasma membrane [111]. Binding and hydrolysis of ATP take place at the level of two conserved intracellular ATP-binding domains present in each member of an ABC transporter. Among ABC transporters, the multiple drug resistance gene product P-glycoprotein is the best characterized for ATP release [112].

Channels belonging to the calcium homeostasis modulator (CALHM) family have been recently claimed as important contributors to extracellular ATP release [72]. To date six members of the family (CALHM1-6) have been identified. CALHM1 has been found to be expressed in taste buds [113], brain [114], airway epithelia [115], and bladder [116] and identified as a novel ATP permeable channel [117,118]. CALHM1 acts as a pore forming subunit of a plasma membrane voltage-gated ion channel that regulates Ca^{2+} permeability whose size is compatible with ATP molecules passage [114,117,119,120]. In addition, CALMH3 has been recently characterized as essential component of a CALMH1/CALMH3 hexameric fast voltage-gated ATP-release channel in type II taste bud cells necessary for GPCR-mediated taste [121].

An additional pathway for ATP release is the P2X7R itself, which is able to form a large membrane pore allowing passage of molecules up to 900 Da [110,122–124]. A long-standing debate concerned the identity of the P2X7R-associated macropore [125,126], but nowadays general consensus supports the view that the macropore is intrinsic to the P2X7R [46,124].

The amount of ATP in the extracellular environment can increase thanks to further mechanisms. Firstly the action of enzymes of the adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK/NME/NM23) families that are a focus of attention as they are responsible for the conversion of extracellular AMP and ADP to ATP [127], and secondly the activity of cell surface ATP synthase.

AK catalyzes reversible phosphoryl transfers according to the following reaction: ATP + AMP ↔ 2ADP. The different AK isoforms are mainly present in the cytosol (AK1), in the mitochondrial intermembrane space (AK2), in the mitochondrial matrix (AK3) and in the nucleus (AK6), with relevant role in energy transfer and exchange between mitochondria, cytosol and nucleus [128,129]. Recently, the discovery of an ecto-AK activity on different cell types, including keratinocytes [130], airway epithelia [131], hepatocytes [132], human vascular endothelial cells [133], and leukemic cell lines [134] suggests an additional role for this enzyme as a regulatory key element of extracellular nucleotide levels and propagation of purinergic signalling [135].

NDPK is an ubiquitously expressed enzyme that catalyzes transfer of γ-phosphate from nucleoside 5′-triphosphates to nucleoside 5′-diphosphates. NDPK exerts its catalytic activity on different ribo- and deoxyribonucleotides, belonging to both the purine and pyrimidine families. Attention has been recently paid to this enzymes since they are expressed on the plasma membrane as cell-surface ectoenzymes contributing to outside-in and inside-out nucleotide transfer. Extracellular ATP synthesis by an ecto-NDPK was initially found *in vitro* in human erythrocytes [136] and glioma cells [137]. Plasma membrane NDPK has been later identified in astrocytoma cells [138], vascular endothelial cells [133], lymphocytes [134], keratinocytes [130] and hepatocytes [132].

ATP synthase (also called F1F0 ATP synthase) is the ubiquitous enzyme found in bacterial plasma membranes, thylakoid membranes of chloroplasts as well as in the inner membrane of mitochondria necessary to convert the energy of a transmembrane electrochemical protons gradient into the phosphoric acid anhydride bond of ATP. Ectopic localization of ATP synthase components has been found on human keratinocytes [130], vascular endothelial cells [139], adipocytes [140,141], and tumour cell lines [142]. Nonetheless, the exact contribution of F1F0 ATP synthase to extracellular ATP synthesis is still matter of controversy.

4. Fate of the extracellular nucleotides

Cell surface enzymes that hydrolyze extracellular nucleotides (ecto-nucleotidases) were described some years after the discovery that signalling via extracellular nucleotides was a fundamental system for intercellular communication [143]. The four major families of ecto-nucleotidases include the ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases), ecto-5′-nucleotidase (5′-NT), ecto-nucleotide pyrophosphatase/phosphodiesterases (ENPPs), and alkaline phosphatases (APs) [144] (Fig. 3). Ecto-nucleotidases typically hydrolyze nucleoside tri-, di-, and monophosphates and dinucleoside polyphosphates and produce nucleoside diphosphates, nucleoside monophosphates, nucleosides, phosphate, and inorganic pyrophosphate (Fig. 4).

The ENTPDases (including CD39, CD39 L and CD39L3) are nucleotide-specific and hydrolyze nucleoside triphosphates and diphosphates producing nucleoside monophosphates as the final product of the hydrolytic process. The E-NTPDases are the most important nucleotide-hydrolyzing enzymes involved in purinergic signalling although they hydrolyze dinucleoside polyphosphates, ADP ribose, NAD^+, and AMP, with low efficiency. 5′-NT (also known as CD73) is the major enzyme producing extracellular adenosine from AMP. The other two groups of enzymes hydrolyze other substrates besides nucleotides. ENPPs hydrolyze nucleoside triphosphates and diphosphates, dinucleoside polyphosphates, ADP ribose, NAD^+, but not AMP, some members of this family being also able to hydrolize phospholipids. Lastly, APs hydrolyze nucleoside tri-, di-, and monophosphates, and pyrophosphates. These four ecto-nucleotidases families share structural similarities and their crystal structure has been resolved.

ENTPDases are expressed in virtually all tissues [135,145,146]. The E-NTPDases hydrolyze extracellular nucleotide tri- and diphosphates requiring both the presence of Ca^{2+} or Mg^{2+} at millimolar concentration, and an extracellular pH in the physiological range between 7 and 8. The final products of the reaction are nucleoside

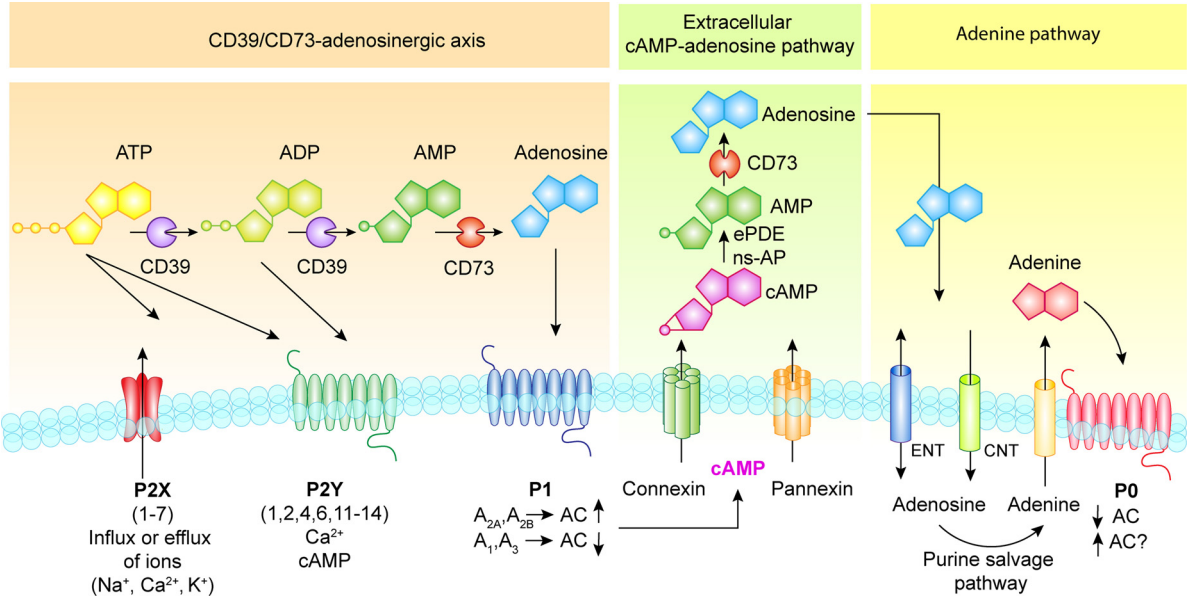

Fig. 3. Schematic rendition of the basic elements of the purinergic signalling. ATP activates different P2X ionotropic and/or P2Y metabotropic receptors, leading to changes in the intracellular ion and/or cAMP concentration. Ecto-nucleotidases (ENTPDase1/CD39, AP, ENPP and 5′-NT/CD73) hydrolyse ATP, generating ADP, AMP, and adenosine. Adenosine can also be released (or taken up) via the equilibrative transporter, and taken up via the concentrative nucleoside transporter, ENT and CNT, respectively. Extracellular adenosine stimulates P1 receptors coupled to increases in the cytosolic Ca^{2+} and/or cAMP concentration. cAMP can be released via the connexin hemichannels or pannexin channels. In the extracellular space, cAMP can be converted to AMP and adenosine via an ecto-phosphodiesterase (ePDE), or by tissue non-specific alkaline phosphatase (ns-AP). Finally, intracellular adenine generated from adenosine in the purine salvage pathway, is released via nucleoside transporters. Extracellular adenine ligates G-protein-coupled P0 receptors that in turn inhibit AC activity.

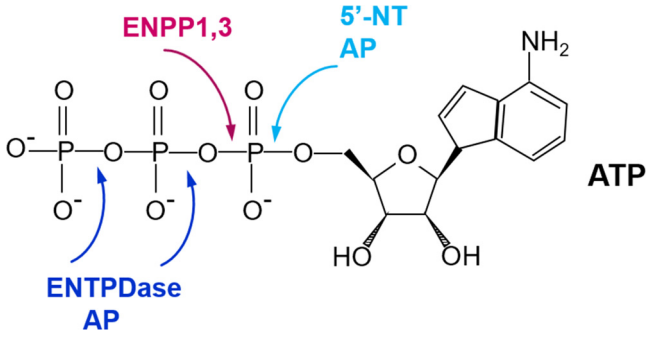

Fig. 4. Different cleavage sites of members of the four types of ectonucleotidases on extracellular ATP, ADP and AMP. Ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases), alkaline phosphatases (APs), and ecto-nucleotide pyrophosphatase/phosphodiesterases (ENPPs) 1 and 3 cleave ATP and ADP. ENPPs cleave the same bond in ATP and ADP whereas ENTPDases hydrolyze different bonds. Eventually ecto-5′-nucleotidase (5′-NT) and APs hydrolyze AMP.

monophosphates (AMP, UMP). Eight members of the family have been identified in mammals. Four of these (NTPDase1/CD39, NTPDase2/CD39 L, NTPDase3/CD39L3, and NTPDase8) are located on the cell surface. NTPDase4–7 are localized at level of intracellular organelles, the NTPDase5 and NTPDase6 also present in secreted forms [146,147]. While the NTPDase1, 2, 3, and 8 hydrolyze both nucleoside triphosphates and diphosphates, the other enzymes present a more restricted substrate range. NTPDase1/CD39 is the best characterized enzyme, initially described as a lymphocyte activation marker, found to be expressed on natural killer cells, monocytes, dendritic cells, and subsets of activated T cells [19,148,149].

5′-NT/CD73 hydrolyzes with high affinity both ribo- and deoxyribonucleoside 5′-monophosphates including AMP, and with lower affinity CMP, UMP, IMP, and GMP. Structurally, 5′-NT is a Zn^{2+}-binding glycosylphosphatidylinositol (GPI)-anchored, extracellularly-oriented, homodimeric protein made of two identical 70-kD subunits containing binding sites for catalytic ions at the N-terminal domain, and an AMP binding site at the C-terminal domain, respectively. 5′-NT is expressed by stromal cells, follicular dendritic cells, endothelial cells [150] and by subpopulations of human T and B lymphocytes [151,152]. 5′-NT is overexpressed by many tumours, and its expression and function are increased by hypoxia and by inflammatory mediators [150,153]. Since the main function of 5′-NT is production of adenosine from extracellular AMP this enzyme is considered a key control step in regulating extracellular adenosine concentration. ATP and ADP in the low micromolar range act as competitive inhibitors of 5′-NT since they bind to the catalytic site without being hydrolysed [154]. Extracellular ATP/ADP thus need to be hydrolysed by other nucleotidases until their extracellular levels are reduced to relieve inhibition of 5′-NT.

The family of ENPPs includes seven components, numbered ENPP1–7 according to their order of cloning, only three of which (ENPP1–3) hydrolyze nucleotides [155]. Indeed, ENPP1, 2 and 3 cleave pyrophosphate or phosphodiester bonds in a wide variety of substrates such as nucleoside triphosphates and diphosphates, NAD^+, FAD, UDP-sugars, and dinucleoside polyphosphates. NPP2 hydrolyzes also phospholipids in addition to nucleotides, whereas NPP6 and 7 hydrolyze phospholipids only and catalytic properties of NPP4 and 5 remain unknown.

Adenosine deaminase (ADA) is another important enzyme of the purine-inactivating chain, which catalyzes the irreversible deamination of adenosine to inosine. ADA is diffusely expressed in different tissues such as intestine, thymus, spleen and other lymphoid and non-lymphoid tissues [156,157]. In addition, ADA is expressed as an ectoenzyme on the surfaces of dendritic cells [158] and lymphocytes [159].

In conclusion, ATP released into the extracellular space is converted into a series of degradation products each of which has defined function and receptor specificity. ATP is sequentially hydrolysed to ADP and AMP by NTPDase1 (CD39) a key enzyme in the regulation of purinergic signalling. Alternatively, ATP can be directly hydrolysed to AMP by ENPPs. Extracellular AMP, besides being the substrate for CD73, has no clear-cut function by itself, and accordingly AMP-selective receptors have not been identified so far. The counteracting ATP-regenerating

pathway that comprises enzymes of the AK and NDPK families and ATP synthase are relevant elements in the balance between extracellular nucleotides and nucleosides [127].

Adenosine signalling is ended to limit the duration of P1 receptors activation when adenosine is either degraded by ADA or removed from the extracellular environment. Since adenosine cannot permeate biological membranes, its transport across the phospholipid bilayer occurs via specific adenosine transporter proteins. Adenosine is taken up from the extracellular milieu to be reused for intracellular purine-nucleotide synthesis through two types of transporters, the equilibrative nucleoside transporters (ENTs) and the concentrative nucleoside transporters (CNTs) [160–162]. The ENT family is restricted to eukaryotes and includes four members, ENT1-4, the best characterized, being ENT1 and 2 that are widely distributed facilitated diffusion transporters. They have similar selectivity for purine and pyrimidine nucleosides [163] but differ in that ENT2 can also transport nucleobases. ENTs are potentially bidirectional transporters, the direction being determined by the nucleoside concentration gradient across the membrane. On the other hand, limited attention has been paid so far to CNTs in the context of purinergic signalling. Three members, CNT1, 2 and 3 have been identified CNT2 and 3 likely significantly contribute to purinergic signalling modulation thanks to their high affinity for adenosine and high concentrative capacity. CNTs are obligatory inward transporters exploiting sodium gradient to transfer nucleosides into the cells from the extracellular space. Nucleosides and sodium are co-transported with stoichiometry 1:1 (CNT1 and 2) and 1:2 (CNT3). Both ENTs and CNTs have been shown to be under regulation via P1 receptors [160], the latter data reinforcing the role of these transporters in purinergic signalling. Finally, CD73-generated adenosine can follow different pathways: it can be converted to inosine thanks to the activity of an ADA [164] or transformed in AMP by the intervention of AK [165].

The pathway that generates ATP from adenosine and sets the balance between P2 and P1 receptors signalling is known as the “CD39/CD73-adenosinergic axis”. Responses triggered by the P2 receptor stimulation by ATP and other nucleotides are often counteracted by P1 receptor stimulation by adenosine. Nucleotide degradation is essential for purinergic signalling since on one hand it prevents receptor desensitization, and on the other activates additional P2 or P1 receptors by the hydrolysis products generated [166]. Extracellular pyrophosphate from ATP is also involved in important extracellular pathophysiological events such as bone mineralization and vascular smooth muscle calcification [167,168].

Adenosine is also generated from extracellular cAMP through the so-called extracellular “cAMP-adenosine pathway” (Fig. 3). cAMP can be released from cells through the same transporters used for ATP and other nucleotide release. Once in the extracellular environment, cAMP is relatively stable both in the interstitial milieu and in blood so that it can reach, through the circulation, organs distal to the site of release. Extracellular cAMP can be converted to AMP, thus entering the purinergic signalling pathway, by an ecto-phosphodiesterase (ePDE) or by tissue non-specific alkaline phosphatase (ns-AP). The final destiny of AMP is the conversion to adenosine by CD73.

The CD39/CD73 axis is very important in terminating the pro-inflammatory activity of ATP and other nucleotides [23]. In support of this role, *cd39*$^{-/-}$ or *cd73*$^{-/-}$ mice develop spontaneous inflammatory bowel or lung tissue injury [169,170]. In humans, inflammatory bowel disease might be, at least in part, associated to CD39 defect since patients harbouring single nucleotide polymorphism associated with reduced CD39 expression show increased susceptibility to Crohn’s disease [169]. In addition, *cd73-/-* mice show unremitting lung injury following LPS injection due to lack of immunosuppressive activity by T_{reg} cells [171].

A role for adenosine in blunting inflammation is supported by several findings [172,173]. A2B receptor signalling reduces inflammation in acute lung injury and inflammatory bowel diseases [174] whereas *a2a-/-* mice show enhanced inflammatory response, with increased cytokine production and tissue damage, in response to the administration of sub-threshold doses of inflammatory agents [173]. Even absence or inhibition of the ENTs causes an increase in the extracellular adenosine concentrations that is followed by a milder inflammatory condition [170,175]. On the other hand, P1 receptors blockade can improve macrophage phagocytosis thus enhancing survival in a mice model of microbial sepsis [176]. Extracellular adenine does not originate from conversion of adenosine, since this is degraded to inosine by ADA, but rather is intracellularly generated in the purine salvage pathway and released through nucleoside transporters.

In conclusion, the “CD39-adenosinergic axis” and the “cAMP-adenosine pathway” synergize to the achievement of similar results in different contexts. While the former regulates purinergic signalling mainly locally, generating counteracting and balancing effects on the same organs and tissues where nucleotides are released, the latter may have a hormonal-like effect on target organs distal to the site of nucleotide secretion. Balance between diverse enzymes and receptors, belonging to the different families and sub families, is crucial for the final outcome of this signalling pathway.

5. Conclusions

Extracellular nucleotides and nucleosides participate in a wealth of different cellular responses, among which stimulation (or inhibition) of cell death, proliferation, migration, differentiation, secretion of growth factors and inflammatory mediators [23,33,57,177]. Fundamental pathophysiological processes such as tissue homeostasis, wound healing, neurodegeneration, immunity, inflammation and cancer are modulated by purinergic signalling.

The discovery that ATP and adenosine (and possibly other nucleotides) are fundamental biochemical constituents of the inflammatory and tumor microenvironment has substantially extended our understanding of the molecular mechanisms underlying the activation of the first steps of inflammation and the regulation of the immune response. These findings have opened exciting novel avenues for the design and development of anti-inflammatory and anti-cancer drugs.

Acknowledgements

FDV is supported by grants from the Italian Association for Cancer Research (n. IG 13025 and IG 18581), the Ministry of Health of Italy (n. RF-2011-02348435), and by institutional funds from the University of Ferrara. FDV also acknowledges networking support from COST Action BM-1406.

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