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Sesiones

Ecto-nucleotidases, molecular properties and functional impact

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ABSTRACT

Ecto-nucleotidases hydrolyze extracellular nucleotides. Nucleotides are amongst the most ubiquitous messenger substances in the vertebrate body. Receptors for nucleotides are expressed on the surface of essentially every cell and many cells carry several types of nucleotide receptors. Several families of ecto-nucleotidases have been identified that differ in tissue distribution and functional properties. They modulate ligand availability at nucleotide and adenosine receptors. Ecto-nucleotidases were first identified in the 1940ies. Work of the past two decades has unraveled molecular identities and important functional properties. Using targeted gene deletion clear examples highlighting the importance of ecto-nucleotidases in

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Abbreviations: Ap_nA, dinucleoside polyphosphate; ART, ADP-ribosyltransferase; NADase, NAD glycohydrolases; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; IAP, intestinal alkaline phosphatase; PC-1, plasma cell differentiation antigen-1; SVZ, subventricular zone; TNAP, tissue nonspecific form of alkaline phosphatase.

nucleotide and adenosine signaling have been elaborated. These reach from the control of blood flow and angiogenesis to the modulation of immune functions and neural development. Specific ecto-nucleotidases are associated with stem cells in the adult mammalian brain, implicating a role of nucleotides and nucleosides in the control of adult neurogenesis. Ecto-nucleotidases represent important therapeutic targets to interfere with P2 or P1 receptor-mediated receptor signaling pathways. The development of high throughput assays promises a considerable acceleration in the development of subtype-specific ecto-nucleotidase inhibitors.

Key words: Alkaline phosphatase.—ATP.—Ecto-ATPase.—Neurogenesis.—5'-nucleotidase.

RESUMEN

Ecto-nucleotidasas, propiedades moleculares e impacto funcional

Las ecto-nucleotidasas hidrolizan los nucleótidos extracelulares. Los nucleótidos se encuentran entre las sustancias mensajeras más ubicuas en vertebrados. Los receptores de nucleótidos se expresan en la superficie de prácticamente todas las células y muchas células expresan varios tipos de estos receptores. Se han identificado varias familias de ecto-nucleotidasas, las cuales difieren en su distribución tisular y en sus propiedades funcionales. Modulan la disponibilidad del ligando en los receptores de nucleótidos y de adenosina. Las ecto-nucleotidasas fueron identificadas por primera vez en la década de 1940. Los trabajos de las dos últimas décadas han mostrado sus características moleculares así como importantes propiedades funcionales. Utilizando delecciones génicas dirigidas se han mostrado claros ejemplos destacables de la importancia de las ecto-nucleotidasas en la señalización por nucléotidos y adenosina. Estos ejemplos abarcan desde el control del flujo sanguíneo y la angiogénesis a la modulación de las funciones inmunes y el desarrollo nervioso. Ecto-nucleotidasas específicas están asociadas con células madre en el cerebro adulto de mamífero, implicando un papel de los nucleótidos y nucleósidos en el control de la neurogénesis adulta. Las ecto-nucleotidasas representan importantes dianas terapéuticas para interferir en las vías de señalización mediadas por receptores P2 o P1. El desarrollo de ensayos de alto rendimiento promete una considerable aceleración en el desarrollo de inhibidores de subtipos específicos de ecto-nucleotidasas.

Palabras clave: Fosfatasa alcalina.—ATP.—Ecto-ATPasa.—Neurogénesis.—5´-nucleotidasa.

INTRODUCTION

Nucleotides are amongst the most ubiquitous messenger substances in the vertebrate body. Receptors for nucleotides are expressed on the surface of essentially every cell and many cells carry several types of nucleotide receptors (1). The concept of purinergic signaling as we understand it today reaches back into the 1960ies with a number of basic discoveries made by Geoffrey Burnstock in Melbourne (2). ATP was the first nucleotide whose signaling function was discovered, but it was later shown that also other nucleotides evoke cellular responses. These include ADP, UTP, UDP, nucleotide sugars and NAD^+ as well as a variety of dinucleoside polyphosphates (3, 4). The concept of nucleotide receptors was initially developed on pharmacological and physiological grounds and subsequently corroborated by the molecular cloning and heterologous expression of nucleotide receptors. Nucleotide receptors (P2 receptors) function either as cation channels (P2X receptors) or are G-protein-coupled (P2Y receptors) (1). ATP can be released from cells via constitutive or regulated pathways (5). Extracellular nucleotides are inactivated by hydrolysis via ecto-nucleotidases with the respective nucleoside as the final hydrolysis product that can be salvaged via specific transporters (6). In the case of adenosine, additional cellular functions can be mediated by P1 receptors. Diadenosine polyphosphates act on a variety of receptors, including P1, P2X and P2Y receptors (7) and endogenous dinucleotide receptors (8).

THE BEGINNING

ATP was first identified in muscle extracts in 1929 independently by Karl Lohmann at the Kaiser Wilhelm Institute for Biology in Berlin and by Cyrus Hartwell Fiske and Yellagaprada SubbaRow at Harvard University (9) (Figure 1). At that time, this discovery evoked rather little response and the role of ATP as a high energy compound and its role in carbohydrate breakdown were only realized during the years following. The same year witnessed the first publication of a biological activity of AMP and adenosine (on the heart and vasculature) (10). In 1934 J. H. Gillespie in Belfast demonstrated a

biological action of ATP on several mammalian tissues (11). Other support for a potential extracellular function of ATP came from the discovery of its storage inside secretory granules of a variety of cells such as dense granules of blood platelets (12, 13), chromaffin granules (14, 15) and later adrenergic (16) and cholinergic (17) synaptic vesicles (18). ATP was found to be costored with the neurotransmitters noradrenaline and acetylcholine. On nerve stimulation, ATP is depleted from cholinergic synaptic vesicles in parallel with acetylcholine and replenished together with acetylcholine during a subsequent period of rest. Adenosine taken up into cholinergic nerve terminals via a high affinity transporter is immediately phosphorylated and ends up again in synaptic vesicles in the form of ATP that is coreleased with acetylcholine (adenosine cycle) (19, 20).

The notion of cell surface-located ATPases reaches back into the 1940ies. To the best of knowledge, first cell surface-located hydrolysis of ATP was observed in carefully washed bull spermatozoa by T. Mann in Cambridge in 1945 (21). More detailed reports followed on the hydrolysis of ATP and ADP by intact yeast cells (22) and subsequently in human erythorcytes (23), ascites tumor cells (24) and nucleated avian erythrocytes (25, 26). First evidence for an association of ATPase activity with peripheral nerves was obtained by Abood and Gerard in 1954 (27). The pioneering methodological work of Wachstein and Meisel (1957) (28) paved the way for the enzyme cytochemical demonstration of ATP hydrolysis at the electron microscopic level that revealed the ubiquity of cell surface located ATPase activity. Essner et al. (1958) (29) were among the first to use the electron microscope to localize adenosine triphosphatase and 5'-nucleotidase activities at the plasma membrane. The term ecto-ATPase was coined in 1957 by W.A. Engelhardt from the Academy of Sciences in Moscow on the basis of his findings of surface-located ATPase activity on avian blood cells (25). He also introduced the terms ectoenzyme and ectoapyrase (26). Yet, the functional role of extracellular ATP hydrolysis remained a matter of speculation. As for the concept of nucleotide receptors, the concept of extracellular nucleotide hydrolysis was met by the scientific community with great skepticism.

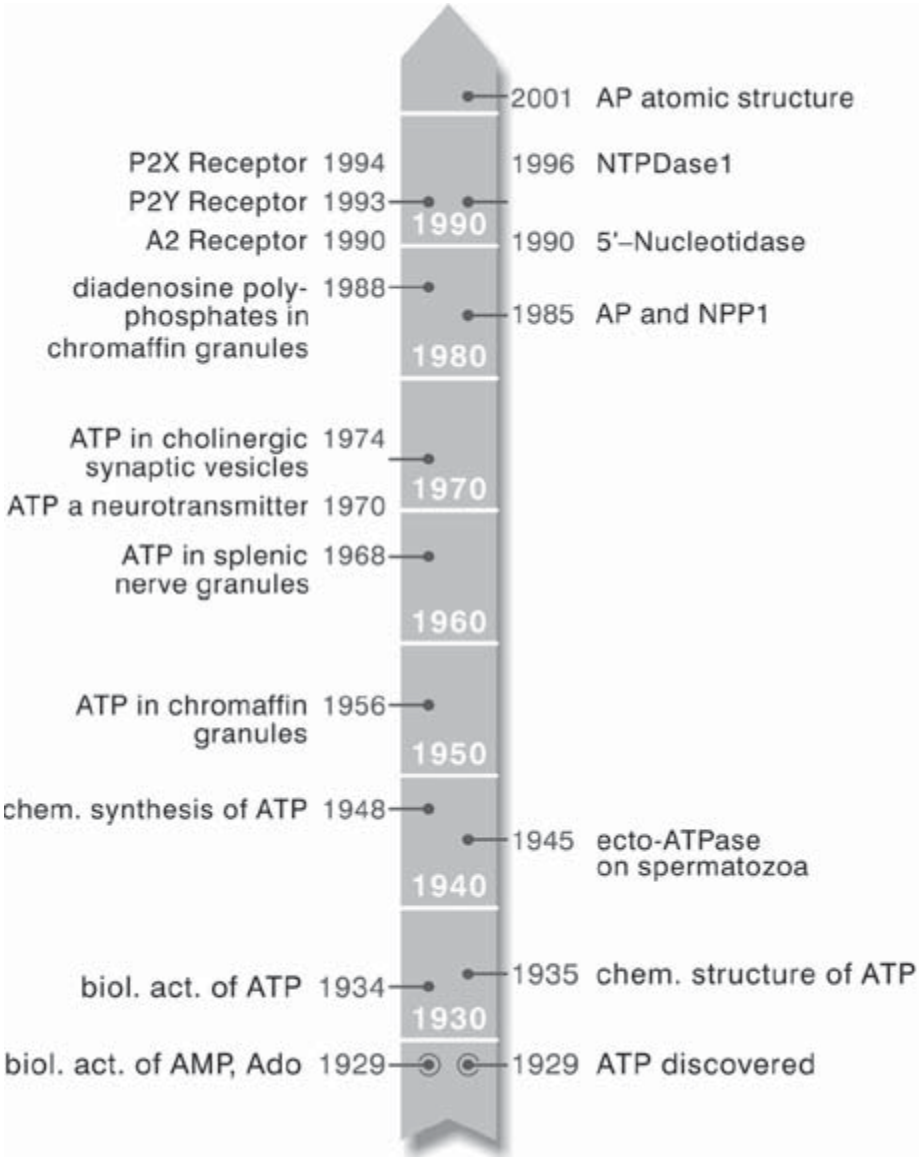

FIGURE 1. ***Hallmarks of nucleotide research with particular emphasis on signaling via extracellular nucleotides.*** *ATP was first discovered in 1929 and in the 1980ies and 1990ies the molecular and functional identity of prototype members of all ecto-nucleotidase families and of nucleoside and nucleotide receptor families was uncovered (for references see text). Ado, adenosine; A2 receptor, adenosine A2 receptor; AP, alkaline phosphatase (human placental).*

This skepticism was eventually overcome by the molecular cloning, heterologous expression, analysis of membrane topology and functional characterization of the molecular players involved. The first ones were the nucleotide-hydrolyzing enzymes placental alkaline phosphatase (30), ecto-nucleotide pyrophosphatase/ phosphodiesterase-1 (31) and ecto-5'-nucleotidase (32), followed by the first adenosine (A2) receptor (33), the first P2Y receptors (34, 35) and P2X receptors (36, 37) and finally the molecular identification of the first mammalian member of the ecto-nucleoside triphosphate diphosphohydrolase family NTPDase1 (CD39) (38, 39).

MULTIPLICITY OF ECTO-NUCLEOTIDASES

We now know that extracellular nucleoside triphosphates and -diphosphates can be hydrolyzed by a variety of ecto-nucleotidases belonging to three different enzyme families with several members each (Figure 2, Table 1). These include the E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) (40, 41), the E-NPPs (ecto-nucleotide pyrophosphatase/phosphodiesterases) (42-44) and the alkaline phosphatases (45, 46). As for the adenosine and nucleotide receptors (47), the identification and molecular cloning of an increasing number of these enzymes required an adjustment of nomenclature. Scientists at the Second International Workshop on Ecto-ATPases proposed that all E-NTPDase family members be termed as NTPDase proteins and all members of the E-NPP-family as NPPs, classified in order of discovery and characterization (40, 48). These enzymes vary considerably regarding substrate preference and affinity, product formation, cation dependence and pH optimum. Recently, an ecto-form of the mitochondrial F_1F_o ATP synthase/F_1 ATPase has been implicated in extracellular ATP synthesis or also hydrolysis. The subunit arrangement of the ecto-form and its functional properties require further characterization (49).

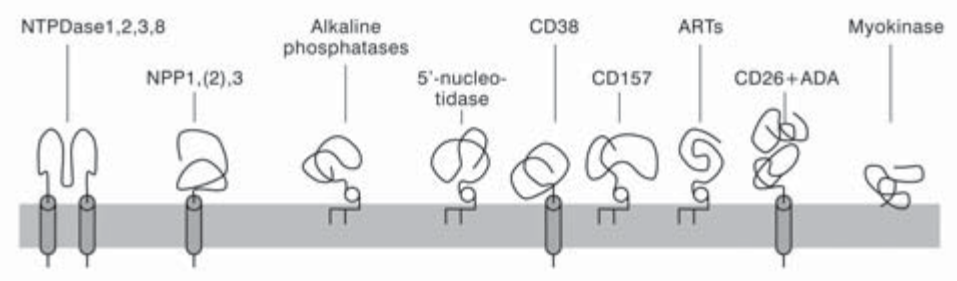

FIGURE 2. ***Membrane topology of ecto-nucleotidases and other nucleotide-metabolizing enzymes.*** *In contrast to NPP1 and NPP3 that are type 2 membrane proteins, NPP2 is a secreted protein. Soluble adenosine deaminase (ADA) is bound to the cell surface via the GPI-anchored protein CD26 (dipeptidyl peptidase IV). CD38 and CD157 are NAD glycohydrolases (NADases). ARTs are ADP-ribosyltransferases that (in part) also metabolize NAD^+. Substrates and products are given in Table 1. Not shown: Nucleoside diphosphate kinase and F_1F_o ATPsynthase/F_1 ATPase. Extracellular ATP can also serve as a co-substrate for ecto-protein kinases.*

Extracellular NAD^+ can be cleaved by E-NPPs but also by two closely related families of extracellular NAD^+ metabolizing enzymes, NAD glycohydrolases (NADases) (CD38, CD157) and mono(ADP-ribosyl)transferases (ARTs) (50) (Table 1). Ecto-5'-nucleotidase, of which only a single gene exists in the mammalian genome, hydrolyses only nucleoside monophosphates (51, 52). Additional surface-located enzymes catalyze nucleotide interconversion and thus the extracellular synthesis of nucleoside triphosphates and diphosphates. These include ecto-nucleoside diphosphokinase and the adenine nucleotide-specific ecto-ATP:AMP phosphotransferase (adenylate kinase, myokinase) (53, 54) (Figure 2). Obviously, ecto-nucleotidases are even more diverse than nucleotide receptors and we are only beginning to understand their specific function in the context of the modulation of ligand availability at nucleotide and nucleoside receptors. Some of the principal properties of ecto-nucleotidases are briefly summarized below.

TABLE 1. *Ecto-nucleotidases and other enzymes metabolizing extracellular nucleotides*

Family	*Surface-located forms*	*Substrates*	*Final product*	*Inhibitors*
Ecto-nucleoside triphosphate diphosphohydrolases	NTPDase1, NTPDase2, NTPdase3, NTPDase8	NTP, NDP	NMP	ARL 67156, 8-BuS-ATP, polyoxometalates, P2 receptor antagonists
Ecto-nucleotide pyrophosphatase/ phosphodiesterases	NPP1, NPP3, NPP2, secreted	NTP (NDP)	NMP + PPi, (Pi)	P2 receptor antagonists, heparin, heparan sulphates
		dinucleoside polyphosphates	NMP + Np_{n-1}	
		NAD^+	AMP + nicotinamide mononucleotide	
		UDP-glucose	UMP + Gluose-6-phosphate	
Alkaline phosphatases	Kidney/bone/liver [= tissue non-specific form, TNAP],	NTP, NDP, NMP	Nucleoside	Levamisole, L-homoraginine
	Placental, Intestinal, Germ-cell			L-phenylalanine
Ecto-5'-nucleotidase	Ecto-5'-nucleotidase	NMP	Nucleoside	AOPCP
Adenylate kinase	AK1-6, not identified	ADP or AMP + ATP	AMP + ATP or ADP	Ap_5A
NAD^+ glycohydrolases (NADases)	CD38, CD157	NAD^+	ADP-ribose and nicotinamide, (cyclic ADP-ribose)	NAD^+ analogues
Mono(ADP-ribosyl) transferases	ART1-ART5	NAD^+	ADP-ribosylated protein	
	ART2	NAD^+	ADP-ribose and nicotinamide	
Nucleoside diphosphate kinase	nm23-M1-6, not identified	N_1TP, N_2DP	N_1DP, N_2TP	
ATP-synthases	Ecto-F_1Fo ATPsynthase/ F_1 ATPase	ADP	ATP	Oligomycin

Ap_5A, P^1P^5-di (adenosine-5') pentaphosphate; ARL 67156, 6-N,N-diethyl-D-β,γ -dibromomethylene ATP; BuS-ATP, 8-thiobutyladenosine 5'-triphosphate; AOPCP, α,β-methylene adenosine 5'-diphosphate; both ATP synthesis and ATP-hydrolysis has been reported for ecto-F_1F_o ATPase. The subtype of surface-located adenylate kinase and nucleoside diphosphate kinase has not been identified.

E-NTPDases

This enzyme family comprises eight different members of which only NTPDase1-3 and 8 are typical cell surface-located enzymes, with two transmembrane helices and a large extracellular domain carrying the catalytic site (40, 41, 55) (Figure 2). Of the intracellular enzymes (NTPDase4-7) that hydrolyze nucleoside diphosphates and/or triphosphates but not ATP, NTPDase6 and NTPDas6 can be released in soluble form. All enzymes are members of the actin/HSP70/sugar kinase superfamily with whom they share common sequence motifs as well as a conserved secondary structure (56, 57). Structural predictions have been derived from mutagenic analyses and computational modeling based on homology with a bacterial exopolyphosphatase (58). The ectoenzymes form homooligomeric complexes. All members of the NTPase gene family are also expressed in *Xenopus* (59).

The surface-located NTPDases1-3 and 8 reveal a partially overlapping tissue distribution but apparently are expressed by different cells (55, 60). NTPDase1 corresponds to the lymphoid cell activation antigen CD39. It is expressed by cells of the immune system, the quiescent vascular endothelium and a variety of other cells including the microglia of the brain (41, 61, 62). NTPDase2 is associated amongst others with the adventitial surfaces of the muscularized vessels, microvascular pericytes, portal fibroblasts from the liver, within taste papillae, the inner ear, with immature and nonmyelinating Schwann cells of the peripheral nervous system (63) and neural progenitor cells of the adult rodent brain (41, 64, 65). NTPDase3 is expressed in brain and in a variety of other tissues (56, 66, 67) whereas expression of NTPDase8 in the brain is very low or absent (55).

Importantly, the hydrolysis rates for nucleoside diphosphates vary considerably between the four enzymes (60, 68). NTPDase2 stands out for its high preference for nucleotide triphosphates and thus is also referred to as an ecto-ATPase. In addition the enzymes differ regarding product formation. Whereas NTPDase1 hydrolyzes ATP directly to AMP with the production of minor amounts of free ADP, ADP accumulates extracellularly on ATP hydrolysis by NTPDase2. NTPDase3 and NTPDase8 reveal intermediate patterns of product

formation. These differences in catalytic properties differentially affect the scavenging or the production of agonists for P2 receptors by individual enzymes or combinations of these.

E-NPPs

The three nucleotide-hydrolyzing members of the E-NPP family, NPP1 to NPP3, hydrolyze 5'-monodiester bonds in nucleotides and their derivatives, resulting in the release of 5'-nucleoside monophosphates. E-NPPs are members of the alkaline phosphatase superfamily and their catalytic core structure reveals close similarity to the superfamily of phospho-/sulfo-coordinating metalloenzymes comprising alkaline phosphatases, phosphoglycerate mutases, and arylsulfatases (69, 70). All three enzymes share a typical modular structure. NPP1 and NPP3 are type II transmembrane glycoproteins that also occur in soluble form whereas NPP2 is secreted. The enzymes have a broad tissue distribution (42, 43). NPP1 was originally discovered at the surface of lymphocytes as plasma cell differentiation antigen (PC-1). Physiological substrates of these enzymes include ATP, NAD^+, nucleotide sugars and dinucleoside polyphosphates (42, 43, 71). ATP is an inhibitor of diadenosine polyphosphate hydrolysis (72). In several tissues investigated, diadenosine polyphosphates are hydrolyzed considerably more slowly than ATP, implicating a prolonged extracellular half-life of these nucleotides (73). Nucleotide hydrolysis by NPPs has a very alkaline pH optimum (8.5 - 9.0), comparable to that of alkaline phosphatases. In addition to its nucleotidase activity, the splice variant of NPP2, autotaxin, also exerts lysophospholipase D activity (74). The four additional and non-nucleotide-hydrolyzing members of the E-NPP family NPP4 to NPP7 are shortened at the N terminus, with presumptive inverse membrane topography. Of these, NPP6 and NPP7 exhibit lysophospholipase-C or choline glycerophosphodiester phosphodiesterase activity (43).

NPP1 to NPP3 hydrolyze ligands of nucleotide receptors. In the case of nucleoside triphosphates, hydrolysis typically proceeds directly to the nucleoside monophosphate with the formation of PPi (Table 1). This circumvents activation of receptors for nucleoside

diphosphates. NPP1 is a major source of extracellular PPi which is highly relevant in the control of bone mineralization (44). Hydrolysis of dinucleoside polyphosphates (Ap_nAs) by NPPs is asymmetric and involves the α, β-pyrophosphate bond, resulting in the production of AMP and Ap_{n-1} (71). Thus, hydrolysis of Ap_5A, Ap_4A, or Ap_3A yields adenosine tetraphosphate, ATP and ADP, respectively (72). This could in turn lead to the additional or also continued activation of P2 receptors, depending on available receptor subtype. NAD^+ is hydrolyzed by NPPs to AMP and nicotinamide mononucleotide.

Alkaline phosphatases

The four human isoforms of alkaline phosphatases (kidney/bone/liver [= tissue non-specific form, TNAP], placental, intestinal and germ-cell are dimers, share a glycosylphosphatidyl inositol (GPI) anchor and can give rise to soluble forms. The distribution of isoforms varies to some extent between humans and rodents (45, 46). Alkaline phosphatases are non-specific phosphomonoesterases that degrade nucleoside 5'-tri-, -di-, and -monophosphates, hydrolyze inorganic pyrophosphate (PPi) and also release inorganic phosphate from a large variety of organic compounds, including proteins. Thus, alkaline phosphatases are capable of directly producing the P1 receptor agonist adenosine from extracellular ATP. Their pH optimum is in the high alkaline range but they hydrolyze ATP also at a pH of 7.4. Alkaline phosphatases certainly play an important role in purine salvage but they can also modulate purinergic signaling. The crystal structures of bacterial, shrimp and human enzymes have been resolved (45, 75).

NUCLEOTIDE SYNTHESIS AND INTERCONVERSION

Ecto-nucleoside diphosphate kinase interconverts nucleoside 5'-di- and -triphosphates, leading to the formation of e.g. UTP and ADP from ATP and UDP or of ATP and UDP from UTP and ADP. This can result in the mutual activation or inactivation of receptors for ATP, ADP, UTP and UDP. Together with other ecto-nucleotidases, ecto-nucleoside diphosphate kinase may maintain low steady state

concentrations of nucleoside triphosphates at the cell surface, leading to tonic receptor activation. Ecto-ATP:AMP phosphotransferase (adenylate kinase, myokinase) catalyzes the extracellular formation of ATP and AMP from ADP and vice versa (53, 54, 76).

ECTO-5'-NUCLEOTIDASE

The GPI-anchored ecto-5'-nucleotidase (CD73) is a Zn^{2+}-binding dimeric protein that hydrolyzes nucleoside monophosphates to their respective nucleosides. It differs from intracellular 5'-nucleotidases (52). The crystal structure of the periplasmic 5'-nucleotidase from *Escherichia coli* (77) serves as a model for the structural analysis of the related mammalian enzyme (78). Like alkaline phosphatase, ecto-5'-nuceotidase can catalyze the formation of adenosine from extracellular AMP and the concomitant activation of P1 adenosine receptors. ATP and ADP inhibit AMP hydrolysis, resulting in feed forward inhibition of ecto-5'-nucleotidase (79). The enzyme is broadly distributed within various tissues and associated also with the vascular endothelium but apparently with an expression pattern different from alkaline phosphatase (TNAP) (80).

CD38 AND RELATED PROTEINS

NAD glycohydrolases (NADases) (CD38, CD157) and ADP-ribosyltransferases (ARTs) represent two closely related families of extracellular NAD^+ metabolizing enzymes that cleave NAD^+ at the adenosine diphosphoribosyl-nicotinamide linkage. NADases convert NAD^+ to ADP-ribose and nicotinamide and due to their additional ADP-ribosyl cyclase activity also to cyclic ADP-ribose. ARTs also transfer ADP ribose to acceptor proteins, including the $P2X_7$ receptor (81, 82). The extracellularly formed cyclic ADP-ribose is considered to be transported into the cell where it activates Ca^{2+} release from intracellular stores (50).

ECTO-NUCLEOTIDASES AND PURINERGIC SIGNALING

It is obvious that the broad spectrum of ecto-nucleotidase enzyme species as well as the considerable variability of their catalytic properties allows for multiple modulations of purinergic signaling-depending on type and combination of enzymes as well as receptors. Ecto-nucleotidases could for example (1) inactivate P2 receptors sensitive to nucleoside triphosphates by directly hydrolyzing a nucleoside triphosphate to the inactive nucleoside monophosphate, (2) inactivate P2 receptors sensitive to nucleoside triphosphates and at the same time (by generating the nucleoside diphosphate) transiently or tonically produce agonists for nucleoside diphosphate receptors, (3) inactivate receptors for e.g. diadenosine polyphosphates and at the same time generate the physiologically active ligands Ap_4, ATP or ADP, (4) prevent P2 receptor desensitization, (5) generate the P1 receptor agonist adenosine from extracellular ATP. Following release of ATP, this allows for complex signaling mechanisms in cellular networks that can involve both, P2 and P1 receptor activation. Therefore a detailed analysis of nucleotide signaling pathways requires information on the identity and exact location of both ecto-nucleotidase and purinergic receptor subtypes for each individual cellular setting investigated.

In vitro studies using recombinant proteins, mimicking different combinations of receptors and enzymes underline this notion. (83). The data demonstrate that ecto-nucleotidases can selectively modulate the effective agonist concentration at $P2Y_1$ receptors on identical or neighboring cells, either by degrading ATP or by generating ADP from ATP. In addition, colocalized ecto-nucleotidases, by reducing levels of constitutively released nucleotide, reduce receptor desensitization. Preventing receptor desensitization following tonic or acute nucleotide release may be an important function of ecto-nucleotidases.

This close interaction between enzymes and ecto-nucleotidases is also of immediate relevance *in situ*. The interaction between the two ecto-nucleotidases NTPDases1 and NTPDase2 is of pivotal importance for the control of vascular platelet activation. NTPDase1 is expressed by endothelial cells, vascular smooth muscle cells and to a small extent by red blood cells and platelets. Due to its capacity

to hydrolyze ATP to AMP and ADP to AMP it blocks platelet aggregation in response to ADP thus supporting blood flow. Accordingly, NTPDase1 knockout mice reveal increased infarct volumes. Infusion of soluble derivatives of NTPDase1 or soluble plant apyrases improves blood flow and tissue reperfusion following ischemia and in tissue transplants (41, 84). NTPDase2 produces ADP from ATP and thus promotes platelet activation. The enzyme is located at adventitial surfaces of the muscularized vessels, microvascular pericytes of some tissues and organs as the heart and the stromal cells and would potentially favor ADP-induced platelet aggregation at sites of vessel injury and thus support hemostasis (85, 86).

In another setting, portal fibroblasts were suggested to regulate bile duct epithelial proliferation via expression of NTPDase2. NTPDase2 inhibits and knockdown of NTPDase2 by RNA interference increases the proliferation of epithelial cells, apparently by compromising their nucleotide scavenging effect. Loss of portal fibroblast NTPDase2 following bile duct ligation may thus mediate the bile ductular proliferation typical of obstructive choleostasis (87).

BROAD FUNCTIONAL IMPACT OF ECTO-NUCLEOTIDASES

As shown by a considerable number of immunocytochemical or enzyme histochemical studies defined ecto-nucleotidases are expressed in specific tissues or cellular systems. In many cases a resolution at the electron microscopic level would be desirable to identify their exact localization and site of action. This particularly applies to the nervous system. Yet, the demonstration of the expression of an enzyme in a defined cellular setting does not permit an immediate anticipation of its functional impact. This depends on the actual catalytic activity and the scenario of the surrounding purinergic receptors. But the identification of ecto-nucleotidases may serve as a first indicator of a functional involvement of nucleotide signaling pathways. Presently, the best tool for studying the functional impact of a particular ecto-nucleotidase in a defined purinergic signaling pathway is the use of specific high affinity inhibitors (if available), the knockdown by RNA interference or the

targeted deletion of the respective gene. The addition of the soluble ATP- and ADP hydrolyzing enzyme apyrase can corroborate the involvement of nucleotide signaling but does not provide immediate evidence for a participation of endogenous ecto-nucleotidases.

TARGETED DELETION OF ECTO-NUCLEOTIDASES

The targeted deletion of NTPDase1 has yielded important insight into the functional role of NTPDase1 in vascular control (88) and in the immune system (89). Inactivation of mouse intestinal alkaline phosphatase (IAP) revealed a functional involvement in fat absorption (90). Knockout mice for NPP1 have been characterized in addition to a natural mutant of NPP1 (tiptoe walking mouse). Deficiency of NPP1 is associated with pathological calcification of ligaments and joint capsules (42). Mutations in the TNAP gene in humans cause hypophosphatasia involving defective mineralization of hard tissue. Mice lacking TNAP can serve as a model for the infantile form of hypophosphatasia and also reveal deficiencies in nervous system development and a number of severe tissue abnormalities (91, 92). Knockouts of the embryonic form of alkaline phosphatase or double knockouts for TNAP and the embryonic form do not reveal additional phenotypes (91). Double knockouts for TNAP and NPP1 underline the opposing and reciprocal actions of NPP1 and TNAP in the production and hydrolysis of extracellular PPi and thus in the control of bone mineralization, respectively. They suggest that both TNAP and NPP1 are potential therapeutic targets for the treatment of mineralization disorders (44, 93, 94). These effects on mineralization appear to reflect the balance of extracellular PPi and Pi levels controlled by the two enzymes rather than P2 or P1 receptor-mediated mechanisms.

NPP2 (autotaxin) knockouts die at embryonic age and investigations on heterozygous mice underline the notion that phospholipase D activity and thus the production of lysophosphatidic acid represents a major functional role of the enzyme (95). Mice in which the ecto-5'-nucleotidase gene is disrupted do not reveal an apparent phenotype when kept under normal conditions but detailed studies corroborated the physiological importance of the enzyme in

the formation of extracellular adenosine from extracellular nucleotide in several tissue functions (96). Deletion of CD38 underlined the role of the protein in regulating the humoral immune response (97) but also revealed its participation in osteoclast formation and bone resorption (98). Similarly, deletion of CD157 implicates a role in B cell development and antibody production (99).

From these and from many additional studies it has become evident that ecto-nucleotidases are involved in the control of a large variety of physiological and pathophysiological functions mediated by nucleotides (41-44, 50, 96, 100, 101). Examples include epithelial ion and fluid transport (5'-nucleotidase), tissue barrier functions such as the transmigration of leucocytes through the vascular endothelium (5'-nucleotidase; CD38), blood flow, including platelet aggregation and ischemia and reperfusion (5'-nucleotidase, NTPDase1), angiogenesis and vascular remodeling (NTPDase1), renal function (5'-nucleotidase), hypoxia (5'-nucleotidase, NTPDase1), the immune system (CD38, NPP1, NTPDase1, 5'-nucleotidase), the function of airway epithelia including chronic lung disease (NPPs, NTPDases, alkaline phosphatase, ecto-adenylate kinase, ecto-5'-nucleotidase) or bone and cartilage mineralization (NPP1, TNAP). In the nervous system, ecto-nucleotidases have been implicated in a considerable variety of functions, including synaptic signal transmission, microglial function and neurogenesis. There is increasing evidence for an involvement of ecto-nucleotidases in sensory transmission as for example for NTPDase1 to NTPDase3 in the inner ear (102).

INHIBITORS OF ECTO-NUCLEOTIDASES

Inhibition of ecto-nucleotidases represents an important mode to target nucleotide signaling pathways. Obviously, targeting ecto-nucleotidases will affect not only nucleotide signaling *per se* but also additional cooperative or opposing signaling pathways. Ideally, ecto-nucleotidase inhibitors should neither be P2 or P1 receptor agonists or antagonists nor substrates of ecto-nucleotidases (60, 103).

Inhibitors of NTPDases (Table 1) include non-hydrolyzable nucleotide analogues and P2 receptor antagonists. Among the

compounds that have been reported to inhibit hydrolysis of ATP without significantly acting on purinoceptors are the structural analogues of ATP, ARL 67156 (FPL 67156) (6-*N*,*N*-diethyl-D-β,γ-dibromomethylene ATP) (104-106) and 8-thiobutyladenosine 5'-triphosphate (8-BuS-ATP) (107) and 1-naphthol-3, 6-disulfonic acid (BG0136) (103). Recently, high throughput assay systems for ecto-nucleotidase analysis have been developed and polyoxometalates have been implicated as a new class of very potent NTPDase inhibitors (108, 109). These substances can be applied systemically and hold promise to finally provide a pharmacological access to this enzyme family. Importantly, the potency of these and other inhibitors can vary considerably between individual members of the E-NTPDase family (109-111). This opens the possibility of isoform-specific ecto-nucleotidase targeting.

Similarly, the hydrolysis of Ap_nAs by NPP1 to NPP3 is differentially inhibited by P2 receptor antagonists. Thus, the application of P2 receptor inhibitors will potentially obscure pharmacological experiments in which the effects of Ap_nAs on P2 receptors are investigated. Ap_nA hydrolysis and P2 receptors would be simultaneously blocked. In addition, submilligram quantities of heparin or heparan sulphate inhibit activity of NPPs in a variety of cellular systems (71). Inhibition of alkaline phosphatase equally varies between isoforms. TNAP is particularly sensitive to levamisole and L-homoraginine. The other isoforms are sensitive to L-phenylalanine (112). Ecto-5'-nucleotidase is inhibited by ATP and ADP and by the non-hydrolyzable ADP analogue α,β-methylene adenosine 5'-diphosphate (AOPCP) (51). Several NAD^+ analogues have been employed as inhibitors of CD38 (113) and P^1P^5-di (adenosine-5') pentaphosphate (Ap_5A) is a potent inhibitor of adenylate kinase, inclusive its surface-located form (76).

ECTO-NUCLEOTIDASES IN THE NERVOUS SYSTEM

Since both nucleotides and nucleosides contribute to signaling between neurons and also between glial cells and glia and neurons (114, 115) it is expected that ecto-nucleotidases play an important role in modulating and controlling these signaling pathways.

Synaptic transmission: Some of the best evidence for a modulatory role for ecto-nucleotidases in purinergic signal transmission stems from investigations of the peripheral nervous system. Stable analogues of ATP are up to a hundred times more potent than ATP in causing smooth muscle contraction. ARL 67156 potentiates contractions caused by nerve stimulation or application of ATP (116). This suggests that ecto-nucleotidases tonically attenuate ATP-mediated signal transmission, an effect relieved by the enzyme inhibitor. At hippocampal synapses, ATP is rapidly hydrolyzed to adenosine that in turn activates pre- or postsynaptic receptors, thereby modulating synaptic transmission (79, 117, 118). In accordance with these observations, the inhibitory synaptic effects of nucleotides or of adenosine are abrogated in mice lacking A_1 adenosine receptors (119).

Astrocyte signaling: ATP is a major extracellular mediator in the propagation of Ca^{2+}-waves between astrocytes in various brain regions and within the retina. Ca^{2+}-waves may be involved in the modulation of synaptic transmission and in neuron-glia bi-directional communication (120, 121). While inhibition of ecto-nucleotidase activity facilitates and addition of potato apyrase attenuates the physiological action of ATP in these experimental settings, sites and extent of endogenous hydrolysis and the type of endogenous ecto-nucleotidase involved need to be identified.

Microglia: Of all neuronal and glial cells, microglia stands out for its high surface-located nucleotidase activity that has been identified as NTPDase1 (61, 62). Ischemia enhances long-term microglial ecto-nucleotidase expression. ATP stimulates microglia to release various biologically active substances, induces chemotaxis of cultured microglia and can, at high doses, induce microglial death (122). In addition, spinal cord microglia is involved in inducing tactile allodynia caused by peripheral nerve injury which is gated by $P2X_4$ receptors (123). The functional role of the very high microglial ecto-nucleotidase activity needs to be elucidated. It may increase the threshold for ATP-mediated microglial activation and the subsequent release of signaling substances or may prevent receptor desensitization.

Cerebral vascular control: NTPDase1 knockout mice reveal increased cerebral infarct volumes and reduced postischemic perfusion following middle cerebral artery occlusion. Similarly, a

recombinant soluble and catalytically active form of NTPDase1 restores postischemic cerebral perfusion and rescues from cerebral injury (84, 124). The production of the antithrombotic metabolite adenosine by endothelial ecto-5'-nucleotidase provides an additional mechanism for down-regulating platelet aggregation. The functional importance of vascular ecto-5'-nucleotidase in the formation of extracellular adenosine from released adenine nucleotides has recently been corroborated by the analysis of ecto-5'-nucleotidase knockout mice (80, 125).

Adult neurogenesis: Ecto-nucleotidases are expressed on neural progenitors and their expression pattern varies during development of the nervous system (126). Recent work has established the presence of stem cells in the adult mammalian brain. In the adult murine brain, neurogenesis, the formation of new neurons from neural progenitor cells, continuously takes place in two actively proliferating zones, the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus of the hippocampus. These cells share properties of astrocytes and give rise to highly proliferating intermediate cell types that finally mature and form neurons. Interestingly, the ecto-ATPase NTPDase2 is highly and selectively expressed by the stem cells (type B cells) of the SVZ (64) and by the progenitor cells (residual radial glia) of the dentate gyrus (65). The enzyme is no longer expressed by the progenitor cell-derived neurons. Moreover, SVZ-derived stem cells cultured as neurospheres in the presence of the growth factors EGF and FGF-2 express NTPDase2. The enzyme becomes expressed in the neurogenic regions of the rodent brain only during late gestation and thus is not involved in embryonic neurogenesis. NTPDase2 represents a very useful and reliable marker for the identification of adult neural stem cells.

Additional investigations revealed the expression of the tissue non-specific form of alkaline phosphatase (TNAP) on all cell types of the neurogenic SVZ, including stem cells, transient amplifying cells and immature neuroblasts (D. Langer, unpublished). This imposes a challenging scenario to the regulation of nucleotide signaling to the densely interacting cellular elements of the neurogenic zone and implies the functional involvement of both nucleotide and nucleoside receptors. Indeed, neurosphere cells *in vitro* respond to P2 receptor agonists and to adenosine with an increase in cell proliferation.

Inhibition of the P2 receptors attenuates cell proliferation in spite of the presence of mitogenic growth factors (127). Similarly, nucleotides stimulate proliferation and dopaminergic differentiation of human fetal midbrain neural precursor cells (128). These data provided strong evidence that ecto-nucleotidases, nucleotides and nucleosides, in concert with other signaling substances, can play a role in controlling neurogenesis from resident stem cells in the adult mammalian brain.

SYNOPSIS

Nucleotides control major physiological and pathophysiological functions in every tissue and, due to the essentially ubiquitous distribution of nucleotide and adenosine receptors, initiate a large variety of cellular responses. They act as fast transmitters and, presumably in the majority of cases, as modulators, often in combination with other signaling molecules. Crosstalk between nucleotide or nucleoside receptors with receptors for e.g. growth factors has been demonstrated. This highlights the functional significance of interactive pathways between nucleotides and other cellular messengers. A major role of ecto-nucleotidases in these settings is in the modulation of ligand availability for nucleotide and nucleoside receptors. The understanding of the abundance of ecto-nucleotidases and of their varying catalytic properties remains a challenge but excellent examples for a cell or tissue-specific association and function of individual enzymes have been elaborated. Ecto-nucleotidases represent important therapeutic targets for interfering with P2 or P1 receptor-mediated cellular signaling pathways. The recent development of high throughput assays for the development of ecto-nucleotidase inhibitors is expected to considerably accelerate this long neglected aspect of nucleotide research.

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