Naunyn-Schmiedeberg's Arch Pharmacol (2000) 362: 299–309
DOI 10.1007/s002100000309

REVIEW ARTICLE

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Extracellular metabolism of ATP and other nucleotides

Published online: 18 August 2000
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Introduction

Within the past 10 years the field of signaling via extracellular nucleotides has witnessed a breath-taking expansion. Molecular cloning of receptors and physiological and pharmacological analyses revealed that essentially every cell in a vertebrate organism carries surface-located receptors for ATP (Abbracchio and Burnstock 1998; Fischer 1999). In addition, other nucleotides have been recognized as signaling molecules. These include ADP, UTP, UDP and a variety of diadenosine polyphosphates such as Ap_4A to Ap_5A (diadenosine tetraphosphate to diadenosine pentaphosphate; Miras-Portugal et al. 1998). Intercellular signaling pathways generally require mechanisms of signal inactivation. Besides receptor desensitization and receptor downregulation effective mechanisms for removing or inactivating the extracellular signaling molecule have evolved. These include cellular reuptake as in the case of catecholamines or amino acid transmitters or extracellular hydrolysis and salvage of hydrolysis products as in the case of peptides, acetylcholine or nucleotides. Nucleotides are hydrolyzed by an extracellular hydrolysis cascade that results in the formation of the respective nucleoside and free phosphate. The latter can be recycled by surrounding cells and reused for nucleotide resynthesis (Pastor-Anglada et al. 1998). In the case of adenine nucleotides the hydrolysis product adenosine can initiate additional receptor-mediated functions (Fredholm et al. 1996).

The molecular cloning and functional characterization of novel families of ectoenzymes capable of hydrolyzing ATP and other nucleotides has considerably revised our notion of extracellular nucleotide hydrolysis. Originally it was assumed that single and defined enzymes exist for the hydrolysis of either ATP (ecto-ATPase), ADP (ecto-ADPase), or also ATP and ADP (ecto-ATP diphosphohydrolase, ecto-apyrase). This type of nomenclature is therefore still prevailing in the current literature. This simplistic view had to be revised after the molecular and functional characterization of several novel enzyme families with overlapping substrate specificities and tissue distributions.

The currently known ectonucleotidases include members of the E-NTPDase family (ecto-nucleoside triphosphate diphosphohydrolase family), E-NPP family (ecto-nucleotide pyrophosphatase/phosphodiesterase family), alkaline phosphatases, and ecto-5'-nucleotidase which all have a broad tissue distribution. Ecto-enzymes hydrolyzing diadenosine polyphosphates, NAD^+ as well as nucleotide-converting enzymes such as ecto-nucleoside diphosphokinase or myokinase have also been described (Zimmermann 1992, 1999a; Ziganshin et al. 1994; Plesner 1995; Beaudoin et al. 1996; Pintor et al. 1997; Goding et al. 1998; Resta et al. 1998; Zimmermann and Braun 1999). In addition, ATPase activity has been found in association with the neural cell adhesion molecule (NCAM; Dzhandzhugazyan and Bock 1993, 1997) and with the muscle-associated alpha-sarcoglycan (Betto et al. 1999). The catalytic site of ectonucleotidases faces the extracellular medium. They are membrane-bound. But cleaved and soluble extracellular isoforms exist and may then be referred to as exonucleotidases. Maximal catalytic activity is adapted to the extracellular environment and requires the presence of divalent cations such as calcium or magnesium and an alkaline pH. In most cases K_m-values are in the lower micromolar range. Termination of nucleotide signaling accompanied by extracellular purine salvage is a major functional role of these enzymes. In tissues facing considerable amounts of extracellular nucleotides such as the intestinal brush border surface or in bile canaliculi purine salvage may be the dominating function. Furthermore, there is evidence that several of the enzymes may be multifunctional proteins. In addition to their catalytic activity they may act in cell adhesion or in transmembrane

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receptor functions. In the following major molecular and functional characteristics of the main enzyme families will briefly be reviewed.

The E-NTPDase family

The use in the current literature of different names for the enzyme family (ecto-apyrase, NTPase, or E-ATPase) and also for its members required the development of a unifying nomenclature (Zimmermann et al. 2000) that is applied here. Members of E-NTPDase family can hydrolyze nucleoside 5'-triphosphates and nucleoside 5'-diphosphates albeit with varying preference for the individual type of nucleotide. Therefore they represent ecto-nucleoside 5'-triphosphate diphosphohydrolases (E-NTPDases) The gene family has members not only within vertebrates but also in invertebrates, plants, yeast and protozoans (references in Handa and Guidotti 1996; Vasconcelos et al. 1996; Smith et al. 1997; Zimmermann 1999a; Zimmermann and Braun 1999). All members of the family share five highly conserved sequence domains ("apyrase conserved regions"; Handa and Guidotti 1996; Schulte am Esch et al. 1999) that presumably are of major relevance for their catalytic activity. All nucleotidases belonging to the E-NTPDase family contain the actin-hsp 70-hexokinase β- and γ-phosphate binding motif (A[IL]DLGG[TS]; Asai et al. 1995; Handa and Guidotti 1996; Kegel et al. 1997). Site-directed mutagenesis of conserved amino acid residues in the apyrase conserved regions I and IV severely disrupt nucleotidase activity (Smith and Kirley 1999a). It is therefore likely that members of the E-NTPDase family and actin/HSP70/sugar kinases are derived from common ancestors.

Members of the E-NTPDase family may be separated into two groups according to their presumptive membrane topography (Fig. 1). To facilitate comparison with earlier literature, alternative nomenclature will be given in parentheses. Members of the first group include E-NTPDase1 to -4 and are predicted to have a transmembrane domain at the N- and at the C-terminus. Of the second group including NTPDase5 and putative NTPDase6, only NTPDase5 has been expressed and characterized. NTPDase5 lacks the C-terminal hydrophobic domain. Its N-terminal hydrophobic leader sequence is cleaved, resulting in a soluble and secreted form of the enzyme. The enzymes hydrolyze not only ATP or ADP but have in common a broad substrate specificity towards purine and pyrimidine nucleotides. They differ, however, regarding their preference for nucleotide 5'-tri- and nucleotide 5'-diphosphates.

NTPDase1 to NTPDase4

After heterologous expression NTPDase1 (CD39, ecto-apyrase, ecto-ATP diphosphohydrolase) hydrolyzes ATP and ADP at a molecular ratio of about 1:0.5 to 1:0.9 (Kaczmarek et al. 1996; Wang and Guidotti 1996; Heine et al. 1999). This corresponds to the values obtained for the enzymes purified from human placenta (1:0.9; Christoforidis et al. 1995), bovine aorta (1:1; Picher et al. 1996) or pig pancreas (1:0.9; LeBel et al. 1980). In contrast, NTPDase2 (CD39L1, ecto-ATPase) has a strong preference for ATP with molecular ratios of ATP:ADP of 1:0.03 or less (Kegel et al. 1997; Kirley 1997; Mateo et al. 1999). NTPDase3 (HB6) is a functional intermediate and reveals a molecular ratio of ATP:ADP of approximately 1:0.3 (Smith and Kirley 1998, 1999b) after heterologous expression. This corresponds to the ratio of 1:0.23 determined for the NTPDase3 purified from chicken oviduct (Strobel et al. 1996). Mutation of only two amino acid residues (D218E/W459A) of NTPDase3 (HB6) considerably increases its preference for nucleoside 5'-triphosphates (Smith et al. 1999). The activity of all three types of ecto-nucleotidases depends on millimolar concentrations of divalent cations such as Ca^{2+} or Mg^{2+} (Christoforidis et al. 1995; Kaczmarek et al. 1996; Strobel et al.

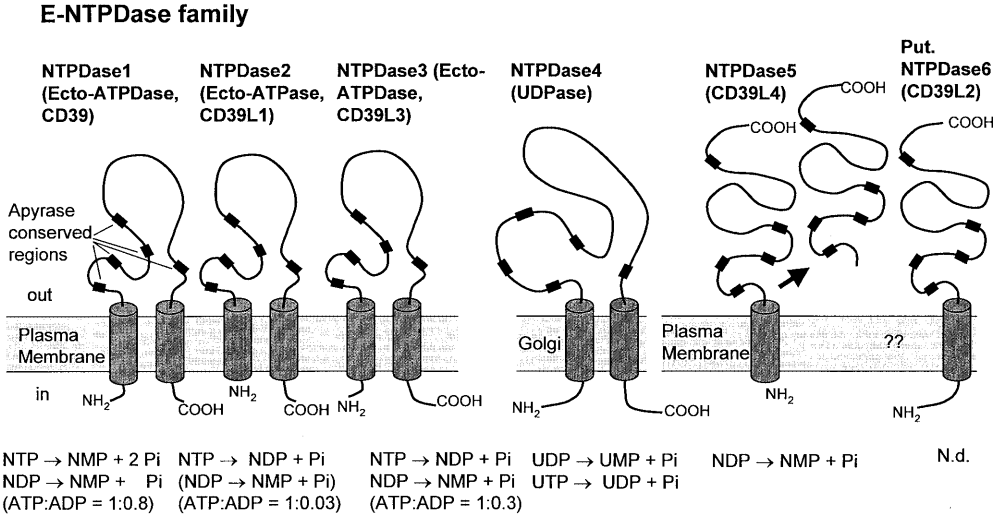

Fig. 1 Predicted membrane topography and catalytic properties of members of the E-NTPDase family. Enzymes may occur as homomultimers. NTPDase5 occurs as a soluble protein (*arrow*). Of putative NTPDase6 only the primary structure is known. Previously used names are given *in parentheses* (modified from Zimmermann 1996a)

1996; Marcus et al. 1997; Heine et al. 1999). Not all members of the E-NTPDase family are located at the cell surface. The two closely related forms of NTPDase4 were allocated to the Golgi apparatus (UDPase; Wang and Guidotti 1998) and to lysosomal/autophagic vacuoles (LALP70; Biederbick et al. 1999), respectively. The Golgi enzyme reveals highest activity with UDP as a substrate. It hydrolyzes also a number of other nucleoside 5'-di- and triphosphates but not ATP and ADP. It functions presumably in the import of nucleotide sugars into Golgi cisternae.

K_m-values of the purified enzymes for ATP and ADP are in the lower micromolar range. They tend to be lower than in many of the intact cellular systems where K_m-values for ATP or ADP were determined for undefined ecto-nucleotidases (references in Zimmermann 2000). The molecular masses of the enzymes as predicted from their primary structures and the deglycosylated forms (Christoforidis et al. 1996) are in the order of 55–60 kDa, those of the glycosylated proteins after heterologous expression in COS-7 or CHO cells are 70–80 kDa (Wang et al. 1997; Nagy et al. 1998; Smith and Kirley 1998; Heine et al. 1999). They may exist as homooligomers (dimers, trimers or even tetramers) and the state of oligomerization affects catalytic activity (Stout and Kirley 1996; Carl et al. 1998; Wang et al. 1998; Smith and Kirley 1999b).

NTPDase5 and putative NTPDase6

When expressed in COS-7 cells the secreted NTPDase5 (CD39L4) has a high preference for nucleoside 5'-diphosphates, especially for UDP (Mulero et al. 1999). Release of soluble nucleotidases with undefined molecular structure from nerve endings has previously been reported (Todorov et al. 1997). The sequence-related putative NTPDase6 has been cloned from a human cDNA library (CD39L2; Chadwick and Frischauf 1998) but has not yet been expressed or functionally characterized.

These examples demonstrate that some of the enzymes hydrolyze nucleoside 5'-triphosphates and nucleoside 5'-diphosphates about equally well whereas others have a high preference for either nucleoside 5'-triphosphates or nucleoside 5'-diphosphates. NTPDase1 and NTPDase3 effectively hydrolyze nucleoside 5'-diphosphates and would thus prevent ADP/UDP-receptor activation. In contrast, NTPDase2 with its high preference for nucleoside 5'-triphosphates would support this by generating ADP or UDP (Vigne et al. 1998; Heine et al. 1999). NTPDase5 would in turn preferentially inactivate any receptor functions of nucleoside 5'-diphosphates.

The E-NPP family

In the current literature the E-NPP family is also addressed as ecto-phosphodiesterase/pyrophosphatase, PC-1, or phosphodiesterase/nucleotide pyrophosphatase (PDNP) family (Goding et al. 1998; Stefan et al. 1999; Zimmermann 1999a). The nomenclature adopted here for the enzyme family and its members is that of Zimmermann et al. (2000). The E-NPP and the E-NTPDase families reveal no phylogenetic relationship. Members of the E-NPP (ecto-nucleotide pyrophosphatase/phosphodiesterase) family include the murine plasma cell differentiation antigen NPP1 (PC-1; van Driel et al. 1985; van Driel and Goding 1987), NPP2 (PD-Iα, autotaxin), and NPP3 (PD-Iβ, B10, gp130^{RB13-6}; van Driel et al. 1985; van Driel and Goding 1987; Buckley et al. 1990; Stracke et al. 1992; Narita et al. 1994; Deissler et al. 1995; Jin-Hua et al. 1997; Scott et al. 1997). Whereas PD-Iβ, B10 and gp130^{RB13-6} denominate an identical protein, human PD-Iα and autotaxin presumably represent splice variants of the same gene (Murata et al. 1994; Kawagoe et al. 1995). Additional, functionally as yet uncharacterized alternatively spliced mRNAs have been described (Fuss et al. 1997) and related sequences have been identified in plants, yeast and *C. elegans* (Zimmermann and Braun 1999).

The sequence of all members of the E-NPP family predicts type II membrane proteins that carry a single transmembrane domain and an intracellular N-terminus (Fig. 2). Members of this gene family possess a surprisingly broad substrate specificity. They reveal alkaline phosphodiesterase as well as nucleotide pyrophosphatase activity, which are properties of the same enzyme molecule (Goding et al. 1998). The enzymes are thus capable of hydrolyzing 3',5'-cAMP to AMP, ATP to AMP and PP_i, ADP to AMP and P_i, or NAD^+ to AMP and nicotinamide mononucleotide. Both purine and pyrimidine nucleotides serve as substrates. Furthermore they can hydrolyze phosphodiester bonds of nucleic acids and the pyrophosphate linkages of nucleotide sugars. The simultaneous presence of phosphodiesterase and nucleotide pyrophosphatase activity on the same enzyme molecule has been confirmed

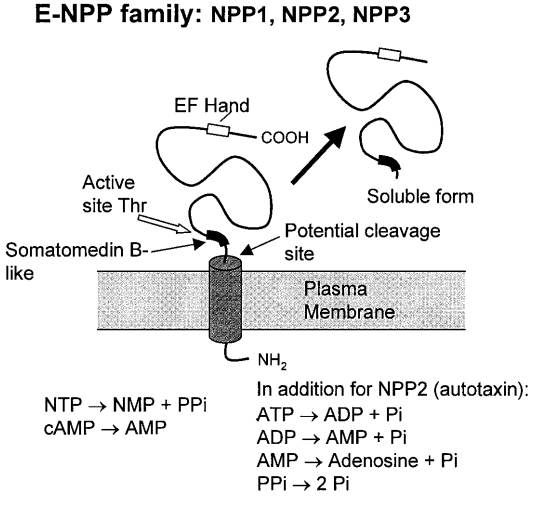

Fig. 2 Predicted membrane topography and catalytic properties of members of the E-NPP family. The enzymes may occur as dimers and may become transformed into soluble proteins by proteolytic cleavage. Specific protein domains are indicated (modified from Zimmermann 1999a)

by heterologous expression in COS-7 cells (Rebbe et al. 1993).

Soluble forms of NPP1 (PC-1) and NPP2 (autotaxin) exist, presumably resulting from proteolytic cleavage above a cysteine-rich stalk near the transmembrane domain (Belli et al. 1993; Clair et al. 1997). The mammalian members of the E-NPP family have apparent molecular weights in the order of 110–125 kDa. Murine NPP1 (PC-1) contains 905 amino acid residues, has an apparent molecular weight of 115 kDa, and occurs as a dimer of two identical 115-kDa subunits (van Driel et al. 1985; van Driel and Goding 1987). Catalytic activity of the E-NPP family members depends on divalent cations (Rebbe et al. 1991; Belli et al. 1994; Deissler et al. 1995; Grobben et al. 1999; Hosoda et al. 1999). They reveal an alkaline pH optimum and K_m-values for ATP between 20 μM and 50 μM (Grobben et al. 1999; Hosoda et al. 1999). Apparent K_m-values are thus similar to those of E-NTPDase family members.

Members of the E-NPP family contain the somatomedin B-like domain of vitronectin, the function of which is not known. The domain may act as a stable "stalk" that holds the catalytic domain away from the membrane. Furthermore the enzymes contain the consensus sequence of an EF-hand putative calcium-binding region (DXDXDGXXDXXE; X for an amino acid). This sequence is essential for enzymatic activity and is present in its complete form in NPP1 (PC-1) and with minor alterations also in the other mammalian E-NPPs. In the case of NPP1 (PC-1), this motif has been implicated in the stabilization of the protein by divalent cations. A monoclonal antibody that is specific for the unfolded form of this region only binds to the protein in the absence of divalent cations or when the protein is denatured (Belli et al. 1994; Kawagoe et al. 1995; Goding et al. 1998). Another unique sequence is that of the RGD-tripeptide which is potentially recognizable by several integrins. The sequence is contained in the NPP2 and NPP3 but not in the NPP1 sequence. The functional role of these motifs is not yet understood but they imply a functional Ca^{2+}-dependency and a potential interaction with cell surface receptors.

Alkaline phosphatases

Alkaline phosphatases represent a protein family of nonspecific ecto-phosphomonoesterases with a broad substrate specificity (references in Zimmermann 1996a; Fig. 3). They release inorganic phosphate from a variety of organic compounds including the degradation of nucleoside 5'-tri-, -di-, and -monophosphates. They also hydrolyze PPi (Fernley 1971; Coleman 1992). One single enzyme could thus catalyze the entire hydrolysis chain from a nucleoside-5'-triphosphate to the respective nucleoside. In contrast to members of the E-NTPDase and E-NPP families, K_m-values of alkaline phosphatases for a variety of substrates are in the low millimolar range. Alkaline phosphatases are glycosylphosphatidyl inositol (GPI)-anchored and also occur in serum. To date, the role of alkaline phosphatases in purinergic signaling and extracellular nucleotide metabolism has received little attention.

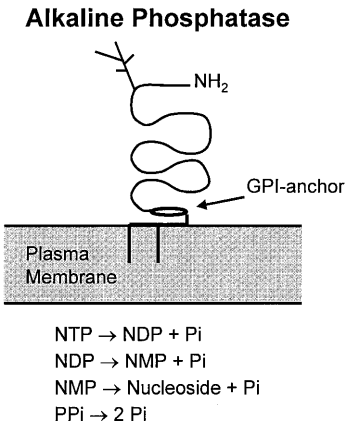

Fig. 3 Predicted membrane topography and nucleotide substrates of alkaline phosphatase (modified from Zimmermann 1996a)

Extracellular metabolism of diadenosine polyphosphates

The enzymes responsible for the extracellular hydrolysis of diadenosine polyphosphates are not yet clearly defined in molecular terms. E-NPPs carry the potential to hydrolyze the nucleotide pyrophosphate bonds of diadenosine polyphosphates. Plasma membrane fractions or cultured cells carrying NPP activity hydrolyze extracellular diadenosine tetraphosphate (Ap_4A), diguanosine tetraphosphate (Gp_4G) and NAD^+ and K_m-values in the low micromolar range have been reported (Gasmi et al. 1998; Grobben et al. 1999). Where investigated diadenosine polyphosphates are degraded extracellularly in an asymmetrical way, yielding AMP and Ap_{n-1} as products. In the case of Ap_4A the hydrolysis products would be ATP and AMP which can be further metabolized to adenosine by other members of the extracellular ecto-nucleotidase chain. Cultured bovine chromaffin cells or presynaptic plasma membranes from electric ray degrade nucleoside polyphosphates from Ap_3A to Ap_6N (or also Np_3N to Np_6N; Rodriguez-Pascual et al. 1992; Ramos et al. 1995; Mateo et al. 1997a, 1997b). The pH optimum is in the alkaline range (8.5–9.0) and K_m-values are in the order of 2–4 μM. Interestingly, the kinetic data differ for the corresponding ectoenzymes in cultured vascular endothelial cells (Ogilvie et al. 1989; Mateo et al. 1997a), implicating the existence of an additional enzyme. In endothelial cells K_m-values for Ap_3A to Ap_5A are very low (in the order of 0.4 μM) and catalytic activity is inhibited by Ca^{2+}-ions. Future experiments need to determine whether the catalytic activities investigated in intact chromaffin cells or endothelial cells relate to members of the E-NPP family or to a novel family of Np_nNases.

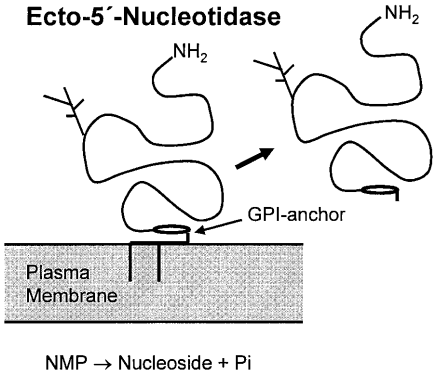

Fig. 4 Predicted membrane topography and catalytic properties of mammalian ecto-5'-nucleotidase. The enzyme occurs as a dimer and may be released from the membrane by endogenous GPI-specific phospholipase (modified from Zimmermann 1996a)

Ecto-5'-nucleotidase

The GPI-anchored ecto-5'-nucleotidase, also known as the lymphocyte surface protein CD73, represents a maturation marker for both T- and B-lymphocytes (Fig. 4). A soluble form cleaved from the GPI-anchor has been described. To date only a single gene has been identified in vertebrates. Related enzymes are found in arthropods and also in Archaea and Eubacteria. The enzyme catalyzes the final step of extracellular nucleotide degradation, the hydrolysis of nucleoside 5'-monophosphates to the respective nucleosides and Pi (Fig. 1). Ecto-5'-nucleotidase is the major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides. The protein occurs mainly as a dimer and the apparent molecular weight of the monomer ranges from 62 kDa to 74 kDa. As for members of the E-NTPDase and the ENPP families, the K_m-value for AMP is in the low micromolar range (Zimmermann 1992, 1996a, 1996b; Christensen 1997; Resta and Thompson 1997; Airas 1998; Resta et al. 1998; Zimmermann and Pearson 1998; Zimmermann and Braun 1999). Ecto-5'-nucleotidase is a zinc-binding metalloenzyme. The X-ray structure of the related *Escherichia coli* periplasmic 5'-nucleotidase has recently been determined (Knöfel and Sträter 1999). Regulatory elements within the ecto-5'-nucleotidase promotor that contribute to the transcriptional regulation and tissue-specific expression have been identified (Spychala et al. 1999).

Other nucleotide-metabolizing enzymes

The cell surface can also contain catalytic activity for the interconversion of nucleotides. Ecto-nucleoside diphosphokinase can interconvert nucleoside 5'-di- and -triphosphates such as UTP and ADP to UDP and ATP, respectively. The parallel cellular release of nucleotides can thus result in the mutual activation or inactivation of receptors for e.g. ATP, ADP, UTP and UDP (Harden et al. 1997). If not carefully controlled, this interconversion pathway may lead to misinterpretation of the effects of nucleotides experimentally applied to the cell surface. The enzyme is inhibited by EDTA, and K_m-values for the nucleotides are in the order of 20–100 μM. Activity of ecto-nucleoside diphosphokinase has been identified on human astrocytoma cells and human airway epithelia but its molecular identity and overall tissue distribution is unknown (Harden et al. 1997; Lazarowski et al. 1997). In addition, the presence of an ecto-ATP:AMP phosphotransferase (myokinase) reaction needs to be considered. The enzyme can lead to the extracellular formation of ATP and AMP from ADP and vice versa. In the nervous system the presence of this catalytic activity has been suggested for rat brain synaptosomes (Nagy et al. 1989) and hippocampal mossy fiber synaptosomes (Terrian et al. 1989).

Additional nucleotide-converting reactions include ecto-protein phosphorylation by ecto-protein kinase with ATP as a cosubstrate (references in Ehrlich and Kornecki 1999; Redegeld et al. 1999) and ecto-ADP ribosylation using NAD^+ as a cosubstrate (references in Zimmermann 2000). Both pathways consume extracellular nucleotides and result in the posttranslational modification of surface-located proteins.

Overlapping tissue distribution

The broad functional diversity of the extracellular nucleotide-hydrolyzing enzymes is not matched by a selective tissue distribution that might be expected if there were tissue-specific requirements in the extracellular nucleotide hydrolysis pathways. Both Northern-blot and Western-blot analyses reveal that many of the enzymes show tissue colocalization.

Not only members of the E-NTPDase family can be co-expressed in the identical tissue. They can be coexpressed together with members of the E-NPP family or with alkaline phosphatases. Examples for coexpression of E-NTPDases and E-NPPs include brain, heart, kidney, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, testis and thymus. Soluble forms of ectonucleotidases can be found in serum (references in Zimmermann 2000). In spite of this, significant differences exist in the distribution between individual members of the families. Isoenzymes of alkaline phosphatase are present throughout the body and are abundant e.g. in hepatic, skeletal, intestinal and renal tissue. Detailed studies using antibodies that can differentiate between closely related enzyme species need to resolve the question as to what extent the cellular localization of the enzymes varies within a given tissue or to what extent their distribution may even differ between surface domains of an identical cell. First examples support these possibilities. NTPDase1 and NTPDase2 were found to be coexpressed by PC12 cells (Kegel et al. 1997). Hepatocytes reveal a surprising differential distribution of NPP1 (PC-1) and NPP3 (Scott et al. 1997; Meerson et al. 1998). NPP1 is exclusively located at the basolateral (sinusoidal) surface whereas NPP3 is ex-

clusively associated with the bile canalicular surface. Presently neither the functional role of the differential subcellular distribution of the two enzymes nor the cellular sorting mechanisms are understood.

The enzyme responsible for the final hydrolysis of nucleoside 5'-monophosphates to the respective nucleoside, ecto-5'-nucleotidase, occurs in essentially all tissues. Nevertheless its tissue expression (as revealed by immunocytochemistry) is generally restricted to defined cellular components. For example, in the mature brain it is mainly associated with astroglia and only exceptionally with nerve terminals (Zimmermann et al. 1993). In the rat heart the enzyme was immunodetected at interstitial cells identified as pericytes or fibroblasts but not at cardiomyocytes (Mlodzik et al. 1995). All these data suggest that careful biochemical analyses are required to define the enzyme species participating in extracellular nucleotide metabolism at the surface of a given cell or within defined tissue compartments.

Functional roles and pathological implications

The physiological function of nucleotides and of their metabolites displays great variance between individual tissues. Accordingly the functional implication of the ecto-nucleotidase pathway can be expected to vary with the physiological mechanism it is governing. A few examples have come to light which highlight the role of these enzymes in tissue function.

Platelet aggregation

Endothelial NTPDase1 (CD39), by converting pro-aggregatory ADP to anti-aggregatory adenosine, limits the extent of intravascular platelet aggregation (Kaczmarek et al. 1996; Marcus et al. 1997; Imai et al. 1999). Recombinant soluble and catalytically active CD39 blocks ADP-induced platelet aggregation in vitro, and inhibits collagen-induced platelet reactivity. Soluble forms of NTPDase1 (CD39) are therefore potential therapeutic agents for inhibition of platelet-mediated thrombotic diatheses (Gayle et al. 1998). Intravenous administration of soluble apyrase prolongs discordant xenograft survival in rats undergoing heterotopic cardiac xenografting from guinea pigs. This is presumably due to the systemic antiaggregatory effects of the administered enzyme. Indeed, histological analyses revealed that administration of apyrase abrogates local platelet aggregation and activation (Koyamada et al. 1996). The generation of NTPDase1-deficient mice ($cd39^{-/-}$) corroborated the role of NTPDase1 as a chief vascular ecto-nucleotidase and affirmed its importance in the control of purinergic signaling in both hemostasis and thromboregulation (Enjyoji et al. 1999; Zimmermann 1999b). The secreted ecto-nucleotidase NTPDase5 (CD39L4) has been allocated to macrophages and may in addition affect hemostasis and platelet aggregation (Mulero et al. 1999).

Inflammation and reperfusion

Activation of endothelial cells as it accompanies inflammatory diseases results in a loss of ecto-ATP diphosphohydrolase activity and may thus be crucial for the progression of vascular injury (Robson et al. 1997). Activity of ecto-ATP diphosphohydrolase is highly sensitive to oxidative stress and greatly reduced in vivo with reperfusion injury (Kaczmarek et al. 1996). Similarly, glomeruli of transplanted kidneys reveal diminished immunostaining for ecto-ATP diphosphohydrolase, presumably reflecting the extent of ischemic tissue damage (van Son et al. 1997). Immunostaining of glomeruli is also reduced after perfusion with 100KF, a plasma serine protease, that induces plasma protein leakage (Cheung et al. 1996).

Ischemia

Transient global cerebral ischemia results in an upregulation of extracellular hydrolysis from ATP to adenosine (Braun et al. 1998a, 1998b; Schetinger et al. 1998). As revealed by Northern blotting, NTPDase1 (CD39) and ecto-5'-nucleotidase are upregulated during the days following transient forebrain ischemia in the rat (Braun et al. 1998a, 1998b). A comparison with markers for activated astrocytes and microglia suggests that the increased expression of ecto-nucleotidases in the regions of damaged nerve cells is associated with activated glia, mainly microglia. The upregulation of the ecto-nucleotidase chain is suggestive of an ischemia-induced increased and sustained cellular release of potentially cytotoxic ATP which may be counteracted by the elevated ecto-nucleotidase pathway.

Calcification

NPP1 (PC-1) plays an essential part in a mechanism that controls the balance between calcification and inhibition of calcification in skeletal tissues. It is highly expressed in chondrocytes and appears to regulate soft-tissue calcification and bone mineralization by producing PPi, a major inhibitor of calcification (Goding et al. 1998; Bächner et al. 1999). Mice carrying the naturally occurring mutation Ttw (tip-toe walking) that is accompanied by an ossification of the spinal ligaments, as well as abnormal ossification at many other sites, have a nonsense mutation in the NPP1 (PC-1) gene (Okawa et al. 1998). Soluble E-NPPs are contained in the synovial fluids and the serum levels are elevated in patients with degenerative arthritis (Cardenal et al. 1998). A functional role in bone mineralization is also discussed for alkaline phosphatase (Whyte 1994).

Additional functions

Ecto-nucleotidases may display functional properties that do not appear to be primarily related to their catalytic

function (references in Zimmermann 2000). For example, the soluble NPP2 (autotaxin) has the ability to promote tumor cell motility (Murata et al. 1994). Motility stimulation requires an intact 5'-nucleotide phosphodiesterase active site (Stracke et al. 1995; Lee et al. 1996). A potential role of NTPDase1 (CD39) in cell adhesion is implicated from the observation that treatment of NTPDase1-positive B-cell lines with subsets of anti-NTPDase1 monoclonal antibodies induce homotypic adhesion. The phenomenon involves tyrosine kinase activity but is unaffected by EDTA, suggesting that catalytic activity of NTPDase1 is not involved. In addition, studies with a variety of inhibitors suggest that the ecto-enzyme may play a role in controlling lymphocyte function including antigen recognition and/or the activation of effector activities of cytotoxic T-cells (Dombrowski et al. 1995). Ecto-5'-nucleotidase can bind to extracellular matrix proteins such as the laminin/nidogen complex and fibronectin (Stochaj et al. 1989, 1990; Stochaj and Mannherz 1992). The two matrix proteins can modulate the enzymatic activity of 5'-nucleotidase. In addition, the enzyme was shown to mediate lymphocyte adhesion to cultured endothelial cells (Airas et al. 1997). The mechanism by which the adhesion is accomplished is not known.

Inhibitors

An analysis of nucleotide release or of the potency of externally applied ATP or its analogues requires the availability of inhibitors of ecto-nucleotidases that should preferably have no or only a small effect on P2-receptor activation (Ziganshin et al. 1994). It has been demonstrated that stable analogues of ATP can elicit tissue contractions up to a hundred times more effectively than ATP (Ziganshin et al. 1994; Bailey and Hourani 1995). This suggests that the effects of exogenously applied ATP on P2-receptors is limited by its enzymatic degradation. Inhibitors of ecto-nucleotidases could thus serve as drugs that increase the life time of extracellular ATP in situ. Regarding the diversity of enzyme families involved in extracellular nucleotide metabolism and the multiplicity of enzyme members, the task of developing specific inhibitors is demanding. It requires the identification of ecto-nucleotidases contributing to nucleotide signaling at the specific cells or tissues in question and a detailed analysis of the potency of the inhibitor on the identified enzymes.

Ecto-nucleotidases are generally inhibited by chelators of divalent metal cations. Ecto-5'-nucleotidase can effectively be inhibited by the nucleotide analogue adenosine 5'-[α,β-methylene]diphosphate (AMPCP; references in Zimmermann 1992). In the past years a considerable number of compounds have been tested for their potency to inhibit the extracellular catabolism of ATP or ADP (references in Zimmermann 2000). Many of the compounds reveal only a mild inhibitory potency and in addition often affect receptor function. Due to their interaction with the active site, non-hydrolyzable nucleotide analogues may inhibit members of the NTPDase and E-NPP families as well as the extracellular hydrolysis of Ap_nAs (Clair et al. 1997). Of the compounds presently available, the structural analogue of ATP, ARL 67156 (FPL 67156; 6-*N*,*N*-diethyl-D-β,dibromomethylene ATP), has been shown to inhibit hydrolysis of ATP in a variety of tissues (albeit with moderate potency) without significantly acting on purinoceptors (Crack et al. 1995; Kennedy and Leff 1995; Kennedy et al. 1996; Westfall et al. 1997). The compound potentiates purinergic synaptic transmission, supporting the notion that endogenous ecto-nucleotidases reduce the effective concentrations of released ATP. The compound has not yet been tested on defined species of ecto-nucleotidases.

Inhibitors of P2-receptors such as suramin and PPDAS (pyridoxal phosphate-6-azophenly-2',4'-disulphonic acid) or a variety of textile and protein dyes also attenuate hydrolysis of ATP. These compounds inhibit members of different enzyme families albeit with different potency. Whereas PPADS has only a mild inhibitory effect on NTPDase1 and NTPDase2 (Heine et al. 1999), NPP1 (PC-1) is effectively inhibited by 20 μM PPADS (Chen et al. 1996; Wittenburg et al. 1996). PPADS is an inhibitor also of the extracellular hydrolysis of Ap_nAs and suramin is considerably more effective in inhibiting hydrolysis of Ap_nAs than of ATP (Mateo et al. 1996). The K_i-value of about 2 μM observed for suramin on the ecto-Ap_nAase from *Torpedo* synaptic membranes is considerably lower than that of its antagonistic effect on P2-receptors. Whereas suramin and PPADS have only a small inhibitory effect on ecto-5'-nucleotidase, non-hydrolyzable analogues of ATP inhibit ecto-5'-nucleotidase (Servos et al. 1998). The potential of laminin to stimulate activity of 5'-nucleotidase, of fibronectin to reduce enzyme activity of ecto-5'-nucleotidase (Dieckhoff et al. 1986; Olmo et al. 1992; Méhul et al. 1993), or of glycosaminoglycans such as heparin and heparan sulfate to inhibit catalytic activity of NPP1 suggests that the ecto-nucleotidases may be under the control of extracellular matrix proteins (Hosoda et al. 1999).

Summary and outlook

Extracellular nucleotides can be hydrolyzed by various enzyme families each containing several related enzyme species. The previously held view that single enzyme species may be responsible for the hydrolysis of ATP, ADP and AMP thus needs to be abandoned. One and the same nucleotide can be hydrolyzed by different enzyme species, depending on their tissue or cellular expression. This is further complicated by the existence of splice variants whose functional differentiation and tissue-specific distribution need to be further evaluated. It is likely that enzymes belonging to different enzyme families are colocalized on individual cell or tissue surfaces. The occurrence of soluble species of ecto-nucleotidases allows their diffusion within the interstitial medium and their transport by body fluids. Whereas in some tissues metabolism of

extracellular nucleotides governs the control of nucleotide receptor function, in others it may predominantly serve purine salvage or even – as in the intestine – nutrition. In some cases (E-NPPs and alkaline phosphatases) we may not even know the most relevant physiological substrates. The multiplicity of ecto-nucleotidase pathways also renders difficult the development of specific inhibitors. Its ubiquity may complicate any systemic application of inhibitors. Future research will have to focus on a further characterization of the biochemical and structural properties of the individual enzymes, their pattern of cellular and developmental expression and molecular control of expression, their tissue-related physiological functions and also on the development of specific inhibitors.

Acknowledgements Supported by grants from the Deutsche Forschungsgemeinschaft (SFB 269, A4) and the Fonds der Chemischen Industrie.

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