Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8

F. Kukulski1, S.A. Levesque1, É.G. Lavoie1, J. Lecka1, F. Bigonnesse1, A.F. Knowles2, S.C. Robson3, T.L. Kirley4 & J. Sévigny1

1Centre de recherche en Rhumatologie et Immunologie, Centre hospitalier de l’Université Laval, Ste-Foy, Québec, Canada; 2Department of Chemistry and Biochemistry, San Diego State University, San Diego, California, USA; 3Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA; 4Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati, Cincinnati, Ohio, USA

Received 17 September 2004; accepted in revised form 19 October 2004

Key words: ATP, ATPase, ectonucleotidase, extracellular nucleotide, P1 receptor, P2 receptor

Abstract

Nucleoside triphosphate diphosphohydrolases 1, 2, 3 and 8 (NTPDases 1, 2, 3 and 8) are the dominant ectonucleotidases and thereby expected to play important roles in nucleotide signaling. Distinct biochemical characteristics of individual NTPDases should allow them to regulate P2 receptor activation differentially. Therefore, the biochemical and kinetic properties of these enzymes were compared. NTPDases 1, 2, 3 and 8 efficiently hydrolyzed ATP and UTP with \( K_m \) values in the micromolar range, indicating that they should terminate the effects exerted by these nucleotide agonists at P2X1–7 and P2Y2,4,11 receptors. Since NTPDase1 does not allow accumulation of ADP, it should terminate the activation of P2Y1,12,13 receptors far more efficiently than the other NTPDases. In contrast, NTPDases 2, 3 and 8 are expected to promote the activation of ADP specific receptors, because in the presence of ATP they produce a sustained (NTPDase2) or transient (NTPDases 3 and 8) accumulation of ADP. Interestingly, all plasma membrane NTPDases dephosphorylate UTP with a significant accumulation of UDP, favoring P2Y6 receptor activation. NTPDases differ in divalent cation and pH dependence, although all are active in the pH range of 7.0–8.5. Various NTPDases may also distinctly affect formation of extracellular adenosine and therefore adenosine receptor-mediated responses, since they generate different amounts of the substrate (AMP) and inhibitor (ADP) of ecto-5’-nucleotidase, the rate limiting enzyme in the production of adenosine. Taken together, these data indicate that plasma membrane NTPDases hydrolyze nucleotides in a distinctive manner and may therefore differentially regulate P2 and adenosine receptor signaling.

Abbreviations: E-NTPDase – ecto-nucleoside triphosphate diphosphohydrolase; HPLC – high-performance liquid chromatography; PCR – polymerase chain reaction; RT – reverse transcription

Introduction

Ectonucleotidases control the level of nucleotides at the cell surface by breaking down these molecules [1]. The biologically most relevant substrates of these enzymes, ATP, ADP, UTP and UDP, initiate a number of cellular responses via the selective activation of ionotropic P2X and metabotropic P2Y receptors [2, 3]. Therefore, ectonucleotidases may dictate a number of physiological responses by regulating P2 receptor activation.

Members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family are dominant ectonucleotidases [1]. Four out of the eight members of this family, namely NTPDase1, NTPDase2, NTPDase3 and NTPDase8, appear relevant to the control of P2 receptor signaling since they are located at the surface of the plasma membrane and hydrolyze nucleotides in the range of concentration that activates P2 receptors [4–8]. NTPDases 1, 2, 3 and 8 have two plasma membrane spanning domains with an active site facing the extracellular milieu [1, 8]. In contrast, NTPDases 4–7 are anchored to the membranes of intracellular organelles by one (NTPDases 5 and 6) or two (NTPDases 4 and 7) transmembrane domains and their catalytic site faces the lumen of intracellular compartments such as the Golgi apparatus and the endoplasmatic reticulum [9–16]. Even though NTPDases 5 and 6 can be present at the surface of the plasma membrane and secreted as soluble enzymes following a proteolytic cleavage, their high \( K_m \) values and low specific activities [11, 12, 14, 15] make it unlikely that these enzymes regulate P2 receptor signaling.

NTPDases dephosphorylate a variety of nucleoside triphosphates (e.g. ATP and UTP) and diphosphates (e.g.,
ADP and UDP) with different abilities and exclusively in the presence of divalent cations (Ca\(^{2+}\) or Mg\(^{2+}\)). Among the plasma membrane bound NTPDases (NTPDases 1, 2, 3 and 8), NTPDase1 (also named CD39, ATPDase, ecto-apyrase, ecto-ADPase) [17–20] hydrolyzes ATP and ADP equally, NTPDase2 (ecto-ATPase, CD39L1) [6, 21–23] is a preferential triphosphonucleosidase whereas NTPDase3 (CD39L3, HB6) [24] and NTPDase8 [8] are functional intermediates between NTPDases 1 and 2.

So far, the comparison of the biochemical properties of plasma membrane NTPDases is difficult as these enzymes were in general partially characterized, came from different species and were studied in different laboratories with different conditions and techniques. To better understand the potential roles of these enzymes in the control of P2 receptor signaling, and the different expression of various NTPDases in mammals, we have compared the biochemical properties of plasma membrane bound NTPDases from human and mouse. Our results indicate that these enzymes have the capacity to collaborate distinctly with P2 receptors either by terminating the stimulation of some of them or by providing the diphosphonucleoside ligands for other P2 receptors. These data also suggest that plasma membrane NTPDases may influence differently the formation of adenosine, and therefore adenosine receptor activation.

**Materials and methods**

**Materials**

Agarose, aprotinin, ethylene glycol-bis(2-aminoethyl-ether)-N-N’-N’-tetraacetic acid (EGTA), nucleotides, phenylmethanesulfonfyl fluoride (PMSF), sodium acetate, and tetrabutylammonium hydrogen sulphate (TBA) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Bis(2-hydroxyethyl)amino-tris(hydroxymethyl) methane) (Bis–Tris), ethylenediaminetetraacetic acid (EDTA), and tris(hydroxymethyl)aminomethane (Tris) were provided by EMD Chemicals (Gibbstown, New Jersey, USA). All cell culture media were obtained from Invitrogen (Burlington, Ontario, Canada).

**Methods**

**Plasmids**

The plasmids encoding human NTPDase1 (GenBank accession no. U87967) [18], human NTPDase2 (NM_001246) [23], human NTPDase3 (AF034840) [24], mouse NTPDase1 (NM_009848) [25], mouse NTPDase3 (AY376710) [7] and mouse NTPDase8 (AY364442) [8] have all been described in published reports. The plasmid encoding rat NTPDase8 corresponds to GenBank accession no. AY536920 (manuscript in preparation).

Mouse NTPDase2 was cloned as follows. Total RNA was isolated from mouse heart with Trizol reagent (Invitrogen). The complementary DNA was synthesized with Superscript II (Invitrogen) from 500 ng of total RNA with oligo(dT)$_{18}$ as the primer, in accordance with manufacturer’s instructions (Invitrogen). For amplification, 10% of the reverse transcription (RT) reaction was used as template in a final volume of 50 μl reaction mixture containing 0.6 μM primer, 400 μM dNTP and 3.5 U Expand High Fidelity PCR System (Roche, Laval, Canada). The following sets of primers were designed based on the 5’ and 3’ ends of mouse NTPDase2 published sequences (AK002553 and NM_009849): Set 1 forward 5’GGG-GTC-CCT-GCT-GTG-TTC3’ and reverse 5’CCG-AGG-GCA-TCT-CTG-ACC3’; set 2 forward 5’TCC-CTG-CTG-TGT-TCT-CCC-G3’ and reverse 5’TGA-AGC-AGC-CTG-GAC-GGT-C3’. Amplification was started by an incubation of 2 min at 94 °C which was followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C and 2 min primer extension at 72 °C and ending with 7 min incubation at 72 °C. The PCR product of approximately 1.8 kb was purified on agarose gel using the QIAEX II gel extraction kit (Qiagen, Mississauga, Canada) and ligated into the expression vector pcDNA3.1/V5-His (Invitrogen). Plasmid DNA was purified with QIaprep Spin Miniprep kit (Qiagen) and orientation of the insert was verified by restriction mapping. One clone obtained with each set of primers was amplified and fully sequenced in one direction. Both sequences were identical. The corresponding and combined sequence is given in accession number AY37674. The clone obtained with the first set of primers was used for transfection and activity assays.

**Cell transfection and protein preparation**

COS-7 cells were transfected in 10 cm plates using Lipofectamine (Invitrogen), as previously described [18]. Briefly, 80%–90% confluent cells were incubated for 5 h at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) in the absence of fetal bovine serum (FBS) with 6 μg of plasmid DNA and 24 μl of Lipofectamine reagent. The reaction was stopped by the addition of an equal volume of DMEM containing 20% FBS and the cells were harvested 40–72 h later. For the preparation of protein extracts, transfected cells were washed three times with Tris-saline buffer at 4 °C collected by scraping in the harvesting buffer (95 mM NaCl, 0.1 mM PMSF and 45 mM Tris at pH 7.5) and washed twice by 300 × g centrifugation for 10 min at 4 °C. Cells were resuspended in the harvesting buffer containing 10 μg/ml aprotinin and sonicated. Nucleus and cellular debris were discarded by centrifugation at 300 × g for 10 min at 4 °C and the supernatant (crude protein extract), was aliquoted and stored at −80 °C until used for activity assays. Protein concentration was estimated by the Bradford microplate assay using bovine serum albumin as a standard [26].

**NTPDase activity measurement**

Enzyme activity of protein extracts was determined as previously described [19]. NTPDase activity was measured at 37 °C in 0.5 ml of the following incubation medium: 5 mM CaCl$_2$ and 80 mM Tris, pH 7.4 or as indicated. Protein extracts were added to the incubation mixture and pre-incubated at 37 °C for 3 min. The reaction was initiated by the addition of 25 μl of substrate to give a final
concentration of 0.5 mM nucleotide, and stopped with 0.125 ml of malachite green reagent. The liberated inorganic phosphate (P_i) was measured according to Baykov et al. [27]. In the kinetic determinations, less than 10% of substrate was hydrolyzed over the course of the reaction to ensure the conditions of initial velocity and to minimize ADP and UDP hydrolysis in experiments with ATP and UTP as substrates. Kinetic parameters (K_m and V_max) were calculated using GraphPad Prism software (GraphPad Software, San Diego, California, USA).

NTPDase activity at the surface of intact COS-7 cells transfected with expression vectors containing NTPDases 1, 2, 3 or 8 was measured in 24-well plate in the presence of 5 mM CaCl_2, 145 mM NaCl, 0.5 mM nucleotides, and 100 mM Tris at pH 7.4. The activity obtained with the control cells was subtracted from the one obtained with NTPDase transfected cells.

Optimum pH was determined in the presence of 2 mM CaCl_2 in the following buffer: 50 mM Bis–Tris, 50 mM Tris, 50 mM glycine (pH 4.0–11.0). In parallel, it was also measured in the following individual buffers: 100 mM acetate for pH 4.0–5.5, 100 mM MES for pH 5.5–7.0, 100 mM Tris for pH 7.0–9.0 and 100 mM glycine for pH 9.0–11.0 as described previously [7, 8]. In some experiments 1 mM CaCl_2 was substituted by 1 mM MgCl_2. In control assays, 1 mM EDTA plus 1 mM EGTA replaced CaCl_2 and MgCl_2 to remove traces of endogenous divalent cations from the medium. One unit of enzyme activity corresponds to the release of 1 μmol P_i/min at 37 °C. All experiments were performed in triplicate with controls.

**Figure 1.** Substrate specificity of plasma membrane bound NTPDases. The assays for the enzymatic activity were carried out with protein extracts from transiently transfected COS-7 cells in the presence of 0.5 mM adenine or uracil nucleotide with either 1 mM CaCl_2 (open bars) or 1 mM MgCl_2 (solid bars), as described under Materials and methods. The average ± SEM of two to five experiments performed in triplicate is shown.
where the protein extract was added after the reaction had been stopped.

Separation and quantification of nucleotides by HPLC
For HPLC analysis, NTPDase activity assays were performed as described above with the following modifications. Aliquots of 40 μl of reaction mixture were taken at different time points and transferred to an equal volume of ice-cold 1 M perchloric acid. The samples were centrifuged for 5 min at 1,000 × g at 4 °C. Supernatants were neutralized with 1 M KOH (4 °C), centrifuged for a subsequent 5 min at 1,000 × g and lipids extracted with n-heptane (5:1, v/v). An aliquot of 20 μl of the resulting samples was used for nucleotide analysis by HPLC.

Adenine nucleotides (ATP, ADP and AMP) were separated on a 15 cm × 4.6 mm, 3 μm SUPELCOSIL™ LC-18-T column (Supelco, Bellefonte, Pennsylvania, USA) with a mobile phase composed of 25 mM TBA, 5 mM EDTA, 100 mM KH₂PO₄/K₂HPO₄, pH 7.0 and 2% methanol (v/v), at a flow rate of 1 ml/min. Uracil nucleotides (UTP, UDP and UMP) were resolved using SUPELCOSIL™ LC-18-T column (25 cm × 4.6 mm, 5 μm, Supelco) as described above except the mobile phase did not contain methanol. Samples containing both adenine and uracil nucleotides were analyzed with the latter column with a mobile phase composed of 16.7 mM TBA, 3.3 mM EDTA, 66.7 mM KH₂PO₄/K₂HPO₄, pH 7.0 at the flow rate of 1 ml/min. The nucleotides were detected by UV absorption at 260 nm and identified and quantified by the comparison of the retention time with the appropriate standards.

Results

Biochemical characterization of plasma membrane bound NTPDases
The biochemical characteristics of human and murine plasma membrane bound NTPDases (members 1, 2, 3 and 8) were studied using protein extracts of COS-7 cells transiently transfected with an expression vector (pcDNA3) containing cDNA that encodes each enzyme. Time course analysis revealed that for all NTPDases tested, the reaction was linear for at least first 30 min with either ATP or ADP as a substrate (data not shown). To ensure the linearity of the reaction, the subsequent enzymatic assays were carried out for 10–20 min. Figure 1 shows that all plasma membrane bound NTPDases hydrolyze tri- and diphosphonucleosides that have capability to induce a P2 receptor response, namely ATP, ADP, UTP and UDP. NTPDases 1, 2, 3 and 8 all preferred tri- over diphosphonucleosides, although with different NTP/NDP ratios as determined by the measurement of P₁ release (Table 1). None of these enzymes hydrolyzed AMP (data not shown).

All human and murine NTPDases required Mg²⁺ or Ca²⁺ ions for their activity with optimal concentration between 1 and 5 mM (data not shown). Indeed, in the presence of 1 mM EDTA and 1 mM EGTA to chelate divalent cations, no activity could be detected (data not shown). Figure 1 shows that Ca²⁺ was preferred over Mg²⁺ by NTPDases 3 and 8, while there were less differences for NTPDases 1 and 2. Interestingly, all plasma membrane NTPDases hydrolyzed uracil nucleotides more efficiently in the presence of Ca²⁺. For few enzymes and substrates tested, the NTP/NDP ratio slightly changed when Ca²⁺ was replaced by Mg²⁺ (Figure 1 and Table 1).

Enzyme activity assays were carried out with protein extracts from transiently transfected COS-7 cells in 80 mM Tris, pH 7.4 with either 1 mM CaCl₂ or 1 mM MgCl₂. Reactions were started by the addition of 0.5 mM nucleotide as a substrate and P₁ release measured as indicated in Materials and methods. Results are expressed as the mean ± SD of at least two experiments performed in triplicate.

Table 1. NTP/NDP ratios of human and murine plasma membrane NTPDases.

| Enzyme          | Cation | Human or rat* | Mouse |
|-----------------|--------|---------------|-------|
| ATP/ADP ratio   |        |               |       |
| NTPDase 1       | Ca²⁺   | 1.8 ± 0.1     | 1.6 ± 0.2 |
|                 | Mg²⁺   | 1.9 ± 0.1     | 1.9 ± 0.1 |
| NTPDase 2       | Ca²⁺   | 7.2 ± 1.8     | 9.5 ± 1.8 |
|                 | Mg²⁺   | 3.5 ± 1.0     | 11.8 ± 2.4 |
| NTPDase 3       | Ca²⁺   | 4.3 ± 0.1     | 2.1 ± 0.4  |
|                 | Mg²⁺   | 2.9 ± 0.1     | 2.8 ± 0.3  |
| NTPDase 8       | Ca²⁺   | 2.2 ± 0.1a    | 1.4 ± 0.1  |
|                 | Mg²⁺   | 4.1 ± 0.7a    | 3.1 ± 1.2  |
| UTP/UDP ratio   |        |               |       |
| NTPDase 1       | Ca²⁺   | 1.7 ± 0.1     | 1.8 ± 0.7  |
|                 | Mg²⁺   | 1.9 ± 0.4     | 2.6 ± 0.5  |
| NTPDase 2       | Ca²⁺   | 9.3 ± 1.3     | 15.1 ± 4.9 |
|                 | Mg²⁺   | 12.8 ± 2.0    | 13.4 ± 4.5 |
| NTPDase 3       | Ca²⁺   | 6.2 ± 0.5     | 2.4 ± 0.8  |
|                 | Mg²⁺   | 2.8 ± 0.5     | 1.7 ± 0.3  |
| NTPDase 8       | Ca²⁺   | 4.8 ± 0.2a    | 1.9 ± 0.2  |
|                 | Mg²⁺   | 9.2 ± 2.4a    | 1.7 ± 0.4  |

* Rat NTPDase8.

Figure 2 shows the effect of pH on both ATPase and ADPase activities of plasma membrane NTPDases. In parallel, we determined the effect of pH using individual buffers and we obtained similar patterns to those depicted in Figure 2 (data not shown). All NTPDases were active (>60% maximal activity) in physiological to slightly basic pH range (7.0–8.5) except rat NTPDase8 with ADP as a substrate. Nevertheless, there were some important differences. By considering the activity greater than 50% of the maximal activity, NTPDase1 was active in neutral to alkaline pHs [7–10]. NTPDases 2 and 8 were active at more acidic pHs from 4.5 to 8.5, while NTPDase3 hydrolyzed substrates over a broad range of pH spanning from 4.5 to 11. Among the NTPDases analyzed, NTPDase8 hydrolyzed nucleotides with the highest velocity at acidic pHs (pH 4–6) while NTPDase3 was the most efficient at alkaline pHs (pH 9–11). Figure 2 also shows some relatively minor variations between human and murine NTPDases. The most significant difference was observed with NTPDase2 where the optimal pH of activity for ATP hydrolysis was pH 6.0 for human NTPDase2 and pH 8.0 for mouse NTPDase2. Another peculiarity was the peak of activity at pH 5.0, that was observed with mouse NTPDase3 but not with the human (Figure 2) and rat (data not shown) NTPDase3.
Kinetics of nucleotide hydrolysis by plasma membrane NTPDases

All NTPDases analyzed exhibited Michaelis–Menten kinetics for the hydrolysis of ATP, ADP, UTP, and UDP (data not shown). Apparent kinetic constants were calculated according to Woolf–Augustinsson–Hofstee plot and are summarized in Table 2.

Although human and mouse NTPDase1 had similar $K_m$ values for P2 receptor agonists, all human NTPDases tested had higher $K_m$ values than the ones corresponding to their mouse orthologue. The hydrolysis pattern and intermediate products of ATP and UTP dephosphorylation were also analyzed for each of these enzymes by HPLC. This technique, in contrast to Pi determination by calorimetric assays, distinguishes between the di- and monophosphonucleoside products of ATP and UTP degradation and also enables one to follow the simultaneous hydrolysis of ATP and UTP. HPLC analysis of adenine and uracil nucleotide hydrolysis was performed with either intact COS-7 cells transfected with mouse NTPDases 1, 2, 3 and 8 or protein extracts of these cells (Figure 3 and data not shown for intact cells). As the pattern of hydrolysis was similar in both cases, we conducted all other experiments with protein extracts of transfected COS-7 cells.

Of physiological interest, human and mouse NTPDase1 hydrolyzed ATP to AMP with a very modest appearance of ADP (Figure 3a). UTP was also hydrolyzed by both enzymes with a major difference that UDP accumulated in the medium and was hydrolyzed only when UTP levels...
Reactions were carried out for 10 min in the presence of 5 mM CaCl$_2$ and 80 mM Tris buffer pH 7.4 or 100 mM MES pH 6.4 for mouse NTPDase8. In separate experiments, each performed in triplicate.

Five mM by regression analysis of Woolf all assays, less than 10% of the substrate was hydrolyzed.

NTPDase1s, which was in agreement with the better substrates than uracil nucleotides for both and UTP hydrolysis showed that adenine nucleotides are significantly decreased (Figure 3b).

Combined ATP and UTP hydrolysis showed that adenine nucleotides are better substrates than uracil nucleotides for both NTPDases1, which was in agreement with the enzyme preferred adenine nucleotides over uracil nucleotides, as expected from the profile of substrate specificity depicted in Figure 1. In contrast, mouse NTPDase2 had similar $K_m$ and $V_{max}$ values for ATP and UTP and hydrolyzed these nucleotides with no preferences (Table 2, Figure 3c).

Human and mouse NTPDase3, as well as murine NTPDase8, hydrolyzed ATP and UTP efficiently to the corresponding diphosphonucleoside (Figure 3). A small accumulation of AMP was also observed for the hydrolysis of ATP that was a little higher with the human enzyme (Figure 3a). This and other slight differences between human and murine NTPDases may be attributable to some variations in the substrate preferences between enzymes from different species or may have also resulted from differences in the amount of activity used for the HPLC determination of the hydrolysis pattern. Compared to human NTPDases 1, 2, 3 and rat NTPDase8, half as many units of activity were used for mouse NTPDases with a doubled reaction time. The simultaneous hydrolysis of ATP and UTP by human NTPDase2 showed that this enzyme preferred adenine nucleotides over uracil nucleotides, as expected from the apparent $K_m$ and $V_{max}$ values (Table 2, Figure 3). This could not have been predicted by considering only the profile of substrate specificity depicted in Figure 1. In contrast, mouse NTPDase2 had similar $K_m$ and $V_{max}$ for ATP and UTP and hydrolyzed these nucleotides with no preferences (Table 2).

Human and mouse NTPDase3, as well as murine NTPDase8, hydrolyzed ATP and UTP with an important but transient formation of the respective diphosphonucleoside (Figure 3). Among these four enzymes, only mouse NTPDase3 hydrolyzed adenine and uracil nucleotides without any preferences (Figure 3), as expected from the apparent $K_m$ and $V_{max}$ values. In contrast, human NTPDase3 and murine NTPDase8 dephosphorylated ADP to AMP much faster than UDP to UMP, leading to an important and sustained accumulation of UDP (Figures 3b, c). Interestingly, human NTPDase3 had lower $K_m$ for ADP than for ATP but the former was a much poorer substrate, as suggested by its low $V_{max}$. The pattern of hydrolysis of 500 mM ATP (Figure 3a) and 25 mM ATP (data not shown) was similar in both cases.

### Discussion

This work reports the comparison of the biochemical properties and kinetics of hydrolysis of P2 receptor agonists by human and murine plasma membrane bound NTPDases. As the presence of detergents changes the biochemical properties of NTPDases, we used membrane preparations of transiently transfected COS-7 cells. The protein extracts from these cells have only background levels of nucleotidase activity and allow the analysis of NTPDases in their native membrane bound forms. For all assays performed, the results obtained with protein extracts were similar to those obtained with intact transfected cells. Although with different abilities, all NTPDases tested hydrolyzed tri- and diphosphonucleosides relevant to P2 receptor signaling. In general, human and murine NTPDases had similar substrate specificity (Figure 1). Only human NTPDase2 hydrolyzed ADP slightly more than its mouse counterpart. For comparison, it has been previously reported that mammalian NTPDase2 hydrolyses triphosphonucleosides preferentially by 10- to 40-fold while NTPDases 1, 3, and 8 hydrolyze tri- and diphosphonucleosides efficiently with ATP:ADP ratios of ~1:2:1, ~3:4:1 and ~2:1, respectively (for references, see Table 3).

It is well known that divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ are essential for nucleotide hydrolysis by NTPDases [1]. Our results showed that NTPDases 1 and 2 had no clear preferences with respect to Ca$^{2+}$ or Mg$^{2+}$ ions, whereas NTPDase 3 [28] and 8 preferred Ca$^{2+}$ for the hydrolysis of both tri- and diphosphonucleosides. Interestingly, uracil nucleotides were hydrolyzed more efficiently in the presence of Ca$^{2+}$ by all plasma membrane NTPDases.

Even though all NTPDases tested were highly active at physiological pH, our studies indicated differences between these enzymes. NTPDase1 had the narrowest range...
of pH for > 50% maximal activity in the physiological to basic conditions (pH 7–9.5). In comparison, NTPDases 2 and 8 were efficient from acidic to slightly basic pHs (4.5–8.5) while NTPDase3 appeared to be fairly active in the entire pH range tested (4.5–11). Depending on the localization of NTPDases, these characteristics may have physiological importance. Before the nomenclature of NTPDases was established, pH optima between 7 and 8 for ATP hydrolysis and 7.5–9 for ADP hydrolysis were reported for enzymes assigned with different names, which
most likely correspond to NTPDase1 ([29] and references therein). More recently, pH optima in physiological range for synaptosomal NTPDase1 and NTPDase2 were reported [30] in agreement with what we observed for the recombinant human and mouse orthologues.

The comparison between \( K_m \) values of plasma membrane NTPDases and EC\(_{50} \) of nucleotide receptors is of physiological importance as it provides information on which of these enzymes can regulate a particular P2 receptor function. Our results show that all apparent \( K_m \) constants of human and murine NTPDases obtained for adenine nucleotides as substrates were in the low micromolar range with the exception of rat NTPDase8 with ADP as a substrate (265 \( \mu \)M). Higher \( K_m \) values were obtained for the hydrolysis of uracil nucleotides with human NTPDase1 (UDP) and NTPDase2 (UTP), rat NTPDase8 (both UTP and UDP) and mouse NTPDase1 and NTPDase8 (UDP). Micromolar \( K_m \) values were also previously reported for NTPDases (Table 3). However, these experiments were performed with enzymes from different species, purified to different extent and with different detergents, or from intact cells or protein extracts from

![Figure 3. Continued.](image-url)
different transfected cell lines. Therefore, the comparison of the kinetic properties of these ectonucleotidases is virtually impossible. Importantly, the apparent $K_m$ values of all human and murine NTPDases measured were consistent with the hydrolysis pattern of ATP and UTP, except for human NTPDase3. ADP and UDP had respectively higher and equal affinity for human NTPDase3 compared to ATP and UTP. In this case, however, the $K_m$ values are less important, because ADP and UDP are poor substrates of human NTPDase3, as reflected by the low $V_{\text{max}}$ values of their hydrolysis compared to $V_{\text{max}}$ of ATP and UTP hydrolysis, respectively. Accordingly, ADP and UDP accumulation was observed during ATP and UTP hydrolysis by human NTPDase3. The same explanation would also apply to the hydrolysis of ATP by porcine NTPDase2 (Table 3). It was also noteworthy that the profiles of nucleotide hydrolysis together with $K_m$ measurements provide valuable information about nucleotide hydrolysis compared to the simple profile of substrate specificity that may sometimes lead to erroneous conclusions. For example, the latter suggests that UTP is a better substrate than ATP for human NTPDase2 (Figure 1) while the HPLC pattern of hydrolysis showed that ATP is preferred over UTP (Figure 3c).
Table 3. Biochemical and kinetic parameters of recombinant and purified plasma membrane NTPDases.  

| Enzyme | Species | Source (detergent used) | Kinetic parameters | ATP/ADP Ratio<sup>b</sup> | Ion preference | Reference |
|--------|---------|-------------------------|-------------------|---------------------------|----------------|-----------|
|        |         |                         | $K_m$ (µM) | $V_{max}$ (U/mg prot.) | $K_m$ (µM) | $V_{max}$ (U/mg prot.) |              |                 |
| NTPDase1 | Mouse | Rec., extract of COS-7 cells | 1 | 1.4 | [35] |
|         | Rat | Rec., extract of COS-7 cells | 75 | 1.7 | [40] |
|         | Human | Pur., heart (Triton X-100) | 20 | 1.1 | Ca<sup>2+</sup> > Mg<sup>2+</sup> | [41] |
|         | Porcine | Pur., extract of COS-7 cells | 97 | 1.4 | Mg<sup>2+</sup>: ATP | [30] |
|         | Bovine | Pur., aorta (Triton X-100) | 12 | 1.1 | Ca<sup>2+</sup> ≠ Mg<sup>2+</sup> | [4] |
| NTPDase2 | Rat | Rec., extract of COS-7 cells | 20-30 | 40 | [35] |
|         | Human | Rec., extract of HeLa cells | 11 | 40 | [43] |
|         | Porcine | Pur., brain (Polydocanol) | 22 | 22 | [23] |
|         | Rabbit | Pur., muscle (Digitonin) | 6 | 9.4 | [6] |
| NTPDase3 | Human | Rec., extract of COS-1 cells | 18 | 18 | [4] |
| hATPase | Porcine | Pur., liver (Triton X-100) | 20 | 20 | Mg<sup>2+</sup>: ADP | [30] |

Rec.: recombinant enzyme expressed in cell line; Pur.: homogenous or highly purified enzyme with the estimated purity given in parentheses.

<sup>a</sup> In the presence of Ca<sup>2+</sup> ions, unless stated otherwise.

<sup>b</sup> Ratios with concentration of substrate of at least five times the $K_m$ of the indicated enzyme corresponding to concentrations of 100–2,500 µM.

<sup>c</sup> This enzyme was identified as NTPDase1 from the biochemical properties and partial amino acid internal sequences.

<sup>d</sup> Experiments carried out with 5 mM Mg<sup>2+</sup>.

<sup>e</sup> Activity per 1 million cells.

<sup>f</sup> This enzyme was identified as NTPDase2 from the biochemical properties and N-terminal sequence.

<sup>g</sup> Dr. T.L. Kirley (personal communication).

Some slight variations were found in the pattern of nucleotide hydrolysis between NTPDase orthologues. As it was previously shown for rat NTPDase1 [21], mouse and human NTPDase1 hydrolyze ATP to AMP with the liberation of two molecules of Pi. However, a modest and human NTPDase1 hydrolyze ATP with the was previously shown for rat NTPDase1 [21], mouse and nucleotide hydrolysis between NTPDase orthologues. As it may also favor the transient activation of P2Y<sub>6</sub> receptor or may also terminate it, depending on the magnitude and identity of nucleotide release. NTPDase2 was expected to terminate P2X<sub>1</sub>,7 and P2Y<sub>2,4,11</sub> receptor activation and simultaneously provide ligands for receptors stimulated by diphosphonucleosides (P2Y<sub>1,6,12,13</sub>). This potential function of NTPDase2 was corroborated by an in vitro aggregation assay where its activity facilitated platelet aggregation by generating ADP in the presence of ATP [35]. NTPDase3 and NTPDase8, which efficiently hydrolyze ATP and UTP, may attenuate and/or terminate the activation of receptors for these two nucleotides (P2X<sub>1,7</sub> and P2Y<sub>2,4,11</sub>), but also favor the activation of ADP and UDP specific receptors (P2Y<sub>1,6,12,13</sub>), as they produce a transient accumulation of diphosphonucleosides. NTPDases 1, 8 and human NTPDase3, which hydrolyze UDP poorly compared to ADP, would be expected to maintain a prolonged activation of P2Y<sub>6</sub> receptor. This could be of potential interest in inflammation, as for example UDP was shown to promote IL-8 secretion in human monocytic cells by the activation of P2Y<sub>6</sub> [36].

AMP, the end product of ATP hydrolysis by NTPDases 1, 3 and 8, is further dephosphorylated by ecto-5'-nucleotidase (CD73) to adenosine, a biologically active molecule that exerts a diversity of physiological responses via its own receptors [37–39]. Our data suggest that.
NTPDases may have a different impact on the formation of adenosine. NTPDase1 that hydrolyzes ATP to AMP without any significant accumulation of ADP should facilitate fast appearance of adenosine. In contrast, since ecto-5'-nucleotidase is inhibited by ADP [37], it is expected that the formation of adenosine would be prevented in the presence of NTPDase2 alone and delayed in the presence of NTPDases 3 or 8. Indeed, the latter enzymes would be expected to generate a time lapse between the activation of P2 receptors and the subsequent activation of adenosine receptors as they require more time to produce the substrate (AMP) of CD73 and remove its inhibitor (ADP).

In summary, NTPDases have the potential to exert a tight, distinct and sophisticated regulation of P2 and adenosine receptor signaling, and therefore affect a number of biological functions. The results presented in this work in conjunction with the localization of these enzymes will help define the functions of cell surface NTPDases.

Acknowledgements

We thank Mrs. Julie Pelletier for technical assistance. This work was supported by grants from the Canadian Institutes of Health Research (CIHR; MOP-49460), The Arthritis Society (TAS01/0078) and from the Fonds de la Recherche en Santé du Québec (FRSQ; 2822) to J.S. S.A.L. was a recipient of a scholarship from the “Fond Hydro-Québec”.

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