



Free amino acids accelerate the time-dependent inactivation of rat liver nucleotide pyrophosphatase/phosphodiesterase Enpp3 elicited by EDTA

Ana Romero¹ · Guadalupe Cumplido-Laso² · Ascensión Fernández¹ · Javier Moreno³ · José Canales¹ · Rui Ferreira⁴ · Juan López-Gómez⁵ · João Meireles Ribeiro¹ · María Jesús Costas¹ · José Carlos Cameselle¹

Received: 3 July 2024 / Accepted: 24 November 2024
© The Author(s) 2024

Abstract

Nucleotide-pyrophosphatases/phosphodiesterases (NPP/PDE) are membrane or secreted Zn^{2+} -metallohydrolases of nucleoside-5'-monophosphate derivatives. They hydrolyze, for instance, ATP and 4-nitrophenyl-dTMP, and belong to the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) family that contains seven members (ENPP1-ENPP7). Earlier we had shown that an NPP/PDE activity solubilized and partially purified from rat liver membranes is inactivated by EDTA in a time-dependent fashion, an effect enhanced by glycine and blocked by the 4-nitrophenyl-dTMP. Here, we extended this observation to other free amino acids. Activity assays started after different incubation lengths with EDTA provided first-order, apparent inactivation constants ($k_{i(ap)}$). With the exception of cysteine (a strong inhibitor) and histidine (itself evoking a time-dependent inactivation), free amino acids themselves did not affect activity but increased $k_{i(ap)}$. The results are compatible with a conformational change of NPP/PDE evoked by interaction with free amino acids. The enzyme preparation was analyzed to identify what ENPP family members were present. First, the hydrolytic activity on 2',3'-cGAMP was assayed because until very recently ENPP1 was the only mammalian enzyme known to display it. 2',3'-cGAMP hydrolase activity was clearly detected, but mass spectrometry data obtained by LC-MS/MS gave evidence that only rat Enpp3, Enpp4 and Enpp5 were present with low abundance. This finding coincided in time with a recent publication claiming that mouse Enpp3 hydrolyzes 2',3'-cGAMP, and that Enpp1 and Enpp3 account for all the 2',3'-cGAMP hydrolase activity in mice. So, our results are confirmatory of Enpp3 activity towards 2',3'-cGAMP. Finally, the effect of amino acids could be relevant to NPP/PDE actions dependent on protein-protein interactions, like the known insulin-related effects of ENPP1 and possibly ENPP3.

Keywords Nucleotide pyrophosphatase · Phosphodiesterase · Conformational change · Free amino acid · Rat Enpp3 · ENPP family · 2',3'-cyclic-GMP-AMP · cGAMP

Introduction

Nucleotide pyrophosphatases/phosphodiesterases (NPP/PDE) are membrane or secreted metalloenzymes that hydrolyze almost all sort of phosphoanhydride and phosphodiester derivatives of nucleoside 5'-monophosphates

Handling editor: F. Polticelli.

✉ João Meireles Ribeiro
jribeiro@unex.es

¹ Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Medicina y Ciencias de la Salud, Universidad de Extremadura, 06006 Badajoz, Spain

² Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, 06006 Badajoz, Spain

³ Colegio María Inmaculada Ríos Rosas, c/Ríos Rosas 35, 28003 Madrid, Spain

⁴ Departamento de Química e Bioquímica, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554 Evora, Portugal

⁵ Servicio de Análisis Clínicos, Hospital Universitario de Badajoz, Servicio Extremeño de Salud, 06006 Badajoz, Spain

(NMP), yielding NMP products. Their active site contains two Zn^{2+} ions which are coordinated by seven residues, and are essential for catalytic activity (Borza et al. 2021; Stefan et al. 2005). The NPP/PDE preparation studied here was solubilized and partially purified from rat liver membranes (RLNPP/PDE). It is known to be active for example on ATP and 4-nitrophenyl-dTMP, and to be a Zn^{2+} ecto-metalloenzyme which is inactivated by EDTA (Bischoff et al. 1975; Cameselle et al. 1984; Corder and Lowry 1969; López-Gómez et al. 1998; Ribeiro et al. 2000; Stefan et al. 1997). The inactivation by EDTA or other metal chelators is reversed by Zn^{2+} or, less efficiently, by Ca^{2+} or Mn^{2+} (Bischoff et al. 1975; Cameselle et al. 1984; Corder and Lowry 1969). While studying RLNPP/PDE inhibition by a culture-grade preparation of acidic fibroblast growth factor (FGF-1), it was found that the inhibition is due to contamination by EDTA (López-Gómez et al. 1998; Stefan et al. 1997). The inhibition is time-dependent, blocked by the substrate 4-nitrophenyl-dTMP, and accelerated by glycine used as buffer instead of Tris. Since glycine itself does not alter RLNPP/PDE activity in the absence of EDTA, the acceleration of the inactivation by EDTA was interpreted as evidence for a conformational change evoked on RLNPP/PDE by the amino acid (López-Gómez et al. 1998). Such change of conformation per se would not affect enzyme activity, but would favor the removal of Zn^{2+} by EDTA leading to enzyme inactivation. This finding has not been extended, except for an unpublished PhD thesis (Romero 2003).

The first aim of the current study was to investigate whether other common amino acids present effects similar to glycine, and what could be the mechanism for this effect. This was studied with all the common amino acids that do not affect RLNPP/PDE activity by themselves, i.e. all except cysteine and histidine. The time-dependent RLNPP/PDE inactivation is treated as a first-order reaction with respect to active enzyme, considering [EDTA] invariant, enabling quantification in terms of apparent inactivation constants ($k_{i(ap)}$). The acceleration factors showed, with some conspicuous exceptions, good correlations with the stability constant of the Zn^{2+} -amino acid complexes (positive correlation) and with amino acid size (negative correlation). The results are compatible with a conformational change evoked by direct interaction of free amino acids with RLNPP/PDE.

Our second aim was to establish the molecular identity of the enzyme susceptible to enhancement of EDTA inactivation by amino acids within the partially purified RLNPP/PDE preparation. The candidates were some of the members of the ENPP family known by their nucleotide pyrophosphatase and phosphodiesterase activities, by their location in membranes and by their ectoenzymatic character (Borza et al. 2021; Stefan et al. 2005). Within this protein family, rat Enpp1 was a prominent candidate due to the role of human ENPP1 as negative modulator of the insulin

receptor (Garup et al. 2006; Maddux et al. 2006; Maddux et al. 1995; Tassone et al. 2018; Zhou et al. 2009), and to the fact that free amino acids are relevant to insulin activity and resistance (Chen et al. 2022; Gar et al. 2018; Krebs et al. 2002; Lee et al. 2018; Patti et al. 1998; Saleem et al. 2019; Seibert et al. 2015; Tremblay et al. 2007). Our study included, among other things, the assay of hydrolytic activity on the STING (stimulator of interferon genes) agonist 2',3'-cGAMP (2',3'-cyclic-GMP-AMP), a feature of human ENPP1 (Borza et al. 2021; Kato et al. 2018; Li et al. 2014; Ritchie et al. 2022) and ENPP3 (Mardjuki et al. 2024). Data obtained by LC-MS/MS (liquid chromatography-tandem mass spectrometry) indicated that RLNPP/PDE did not contain Enpp1, but Enpp3, thus confirming the 2',3'-cGAMP hydrolase activity of the latter. The significance of the effect of amino acids on Enpp3 is discussed in the light of the known functions of human ENPP3 and orthologs.

Materials and methods

Chemicals, biochemicals and chromatography media

Ultrapure water, obtained with a Barnstead equipment composed of ROpure ST and NANOpure modules, was used throughout the work. The 20 common L-amino acids (collection LAA-21), p-aminobenzoic acid, γ -aminobutyric acid, ϵ -aminohexanoic acid, D-aspartate, n-butyric acid, glutaric acid, L-lactic acid, malonic acid, oxaloacetic acid, propionic acid, D-alanine, β -alanine, 2-aminobutane, aminoethane, formamide, trans-4-hydroxy-L-proline, 1-aminopropane, 2-aminopropane, ethylene glycol, glycylglycine, glycylglycylglycine, 4-nitrophenyl-dTMP sodium salt, 4-nitrophenyl-phosphorylcholine and Triton X-100 were from Sigma (now Merck Life Sciences, Madrid, Spain). Ammonium acetate and aminomethane were from Aldrich (now Merck Life Sciences, Madrid, Spain). L-Malic acid, succinic acid, ammonium chloride, urea, hydrochloric acid, sodium chloride, magnesium chloride, sodium acetate, monosodium phosphate, bisodium phosphate, EDTA bisodium salt (Titriplex-III) and sodium hydroxide were from Merck (now Merck Life Sciences, Madrid, Spain). α -Ketoglutarate, pyruvate, bovine liver catalase, bovine pancreas trypsin, soybean trypsin inhibitor, bovine serum albumin, sucrose and Tris were from Boehringer/Roche (now Merck Life Sciences, Madrid, Spain). 2',3'-cyclic-GMP-AMP was from Biolog Life Science Institute, Bremen, Germany. Sephacryl S-200 and Sephadex G-25 (PD-10 columns) were from Amersham-Pharmacia (Cytiva, now purchasable from Merck Life Sciences, Madrid, Spain). DEAE-cellulose DE52 was from Whatman (now purchasable from Merck Life Sciences, Madrid, Spain).

Preparation of crude rat liver membranes

Female Wistar rats of about 250 g, fed ad libitum with commercial pellet food and water, were used and euthanized. Fresh liver was homogenized in 0.25 M sucrose (3 mL per g of tissue) with a Potter-Elvehjem homogenizer (with ground glass pestle and tube) cooled on ice. The homogenate was centrifuged for 2 min at 600 g and the supernatant collected was centrifuged for 60 min at 100,000 g. The supernatant was discarded and the precipitate was resuspended in 20 mM Tris-HCl pH 8.7. This crude preparation was used to test the response of membrane RLNPP/PDE to incubation with EDTA in the absence and presence of glycine.

Solubilization of RLNPP/PDE

The enzyme was solubilized from crude rat liver membranes, except that the last precipitate was resuspended in 20 mM Tris-HCl, pH 8.7, supplemented with 5 mM MgCl₂ and 10 mg Triton X-100/mL. After standing 30 min at 4°C, the resuspended precipitate was centrifuged for 60 min at 100,000 g and the supernatant, containing 10–15 mg protein/mL, was treated by limited trypsinization at 37°C with a total of 31 µg of trypsin per mg of protein dosed as follows: 12.5 µg/mg were added immediately and 6.25 µg were added after 3.5, 6.5 and 7.5 h, respectively. The trypsinization was stopped 8.5 h after starting the treatment by addition of 62 µg of soybean trypsin inhibitor per mg of protein treated. The resulting sample was then dialyzed for 12 h at 4°C against 50 volumes of Tris-HCl, pH 8.7, with 5 mM MgCl₂, and afterwards centrifuged for 60 min at 100,000 g.

Partial purification of solubilized RLNPP/PDE

The solubilized preparation was adsorbed to a DEAE-cellulose column of 25 x 1.5 cm equilibrated in Tris-HCl, pH 8.7, with 5 mM MgCl₂. The activity assayed with 4-nitrophenyl-dTMP as substrate was recovered with a linear 0–400 mM NaCl gradient in the same buffer. A single, symmetrical activity peak was collected around 130 mM NaCl, concentrated by ultrafiltration through an Amicon PM30 membrane under nitrogen pressure, and applied to a Sephacryl S-200 column of 88 cm x 1.7 cm equilibrated in and eluted with 20 mM Tris-HCl, pH 8.7, with 5 mM MgCl₂. A single, symmetrical peak of activity was recovered with V_e 101 mL. It was concentrated again by ultrafiltration and was submitted to buffer exchange in a Sephadex G-25 column of 5 cm x 1.4 cm equilibrated in 5 mM sodium phosphate pH 8.25. It was supplemented with 1 mg bovine serum albumin/mL and divided in portions that were frozen at –20°C. Different preparations showed activities of ≈2–6 units/mL. The protein concentration, before the addition of albumin, was 140–200 mg/L. The same procedure was followed to obtain

the RLNPP/PDE preparation used in other studies (López-Gómez et al. 1998; Ribeiro et al. 2000).

Molecular mass by analytical ultracentrifugation

A sample of 0.1 mL of RLNPP/PDE was supplemented with catalase (Roche) and was applied onto a linear gradient of 50–200 g/L sucrose in 20 mM Tris-HCl, pH 9. After standing at 4°C for 1 h, the tubes were centrifuged for 14.5 h at 38,500 rpm in a Beckman SW-41Ti rotor at 4°C. After centrifugation, the gradients were collected from the bottom in fractions of 0.4 mL. The sedimentation profiles were obtained by activity assays of RLNPP/PDE and catalase (by ultraviolet spectrophotometry; (Chance and Maehly 1955). RLNPP/PDE was recovered as a single, symmetrical peak, with a molecular mass of ≈73 kDa calculated, using catalase as a reference, as described (Martin and Ames 1961).

RLNPP/PDE activity assay

The standard RLNPP/PDE activity assay was run discontinuously measuring the formation of nitrophenol from 1 mM 4-nitrophenyl-dTMP in 50 mM Tris-HCl, pH 9.0, at 37°C. After different incubation lengths, the reaction was finished by adding 1 mL of 0.2 M NaOH over 0.2-mL reaction mixtures or over 0.2-mL aliquots taken from larger reaction mixtures. The amount of nitrophenol was determined from A₄₀₅ measurements ($\epsilon = 18,500 \text{ M}^{-1}\text{cm}^{-1}$) which correspond to the absorbance produced by anion nitrophenolate, since nitrophenol has a pK_a 7.14. To study the activity of RLNPP/PDE over 2',3'-cGAMP, reaction mixtures at 37°C contained in 100 µL 50 mM Tris-HCl, pH 9, 10 µM 2',3'-cGAMP and 1 or 10 µL of enzyme. Under assay conditions, the activity was proportional to the amount of RLNPP/PDE and linear with incubation length.

HPLC analysis of RLNPP/PDE action on 2',3'-cGAMP

HPLC was performed essentially as described (Ribeiro et al. 2023) on a Tracer Excel 120 column (150 x 4 mm) protected by a pre-column (10 x 4 mm) of the same material (octadecylsilica; Teknokroma, San Cugat del Vallés, Barcelona). An HP1100 system was used adjusted to measure A₂₆₀. Samples of 20 µl were injected and the elution was performed at 0.5 mL/min with two buffers: A, 5 mM Na-phosphate, pH 7.0, 5 mM tetrabutylammonium, 20% methanol (by vol.); B, 100 mM Na-phosphate, pH 7.0, 5 mM tetrabutylammonium, 20% methanol. The initial mobile phase was 100% A, and a linear gradient was applied up to 50% A and 50% B in 4 min, followed by another linear gradient up to 100% B in 1 min and isocratic elution with 100% B for 5 min. Retention times (min) were: Guo (3.9), Ado and GMP (5.2), AMP (5.9) and 2',3'-cGAMP (7.3).

RLNPP/PDE inactivation by EDTA

Enzyme inactivation by EDTA was studied in the absence of added bivalent cations. Under standard conditions, RLNPP/PDE treatment was performed in 100- μ L assay mixtures containing 50 mM Tris, pH 9.0, 1 μ L RLNPP/PDE, 5 μ M EDTA and one of the products tested as inactivation modifiers (amino acids or analogs). Controls were carried out without any modifier. These mixtures were pre-incubated for 10 min at 37°C, and the experiments were initiated by the addition of RLNPP/PDE and terminated by addition of 1 mM 4-nitrophenyl-dTMP. This last addition served a twofold purpose: it blocked the inactivation and started the assay of residual RLNPP/PDE activity. Usually, to study the kinetics of RLNPP/PDE inactivation, increasing periods of inactivation were carried out in a single mixture from which 196 μ L aliquots (or multiples) were removed at defined times and added over tubes containing 4 μ L (or the respective multiples) of 50 mM 4-nitrophenyl-dTMP. This started the assay of residual RLNPP/PDE activity.

The kinetics of inactivation of RLNPP/PDE by EDTA can be described according to the binary reaction $E-Zn + EDTA \rightarrow E + EDTA-Zn$ (where E represents the native rat protein), either by direct trapping of enzyme-bound metal or after slow metal release followed by rapid chelation. The apparent second-order equation for the whole process is

$$v_i = k_i[E - Zn][EDTA] \quad (1)$$

which can be simplified to an apparent first-order equation assuming that, under the experimental conditions, $[EDTA] \gg [E-Zn]$. Actually, from the composition of assay mixtures and the molecular weight of the enzyme, it can be inferred that $[E-Zn]$ is well below 30 nM at the beginning of the inactivation experiments, versus the standard 5 μ M concentration of EDTA or higher. Therefore $[EDTA]$ remains approximately constant during the inactivation allowing to define an apparent k_i as

$$k_{i(ap)} = k_i[EDTA] \quad (2)$$

$$v_i = k_{i(ap)}[E - Zn] \quad (3)$$

The time courses of inactivation by several EDTA concentrations are represented as direct plots of residual RLNPP/PDE activity versus time and as semilogarithmic transformations of the same data. The slope of linear semilogarithmic plots equals $-k_{i(ap)}$. The $k_{i(ap)}$ value increases linearly with EDTA concentration. If desired, $k_{i(ap)}$ can be divided by $[EDTA]$ to obtain an EDTA-independent k_i value. However, we have preferred to use $k_{i(ap)}$ values obtained at indicated EDTA concentrations (most frequently 5 μ M EDTA).

The Pearson coefficients and P values for the correlation between the increase of $k_{i(ap)}$ and different parameters were calculated with the Pearson Correlation Coefficient Calculator in the web page Social Science Statistics (<https://www.socscistatistics.com/>, accessed on 27 October, 2024).

Preparation of modifiers of the inactivation of RLNPP/PDE by EDTA

Amino acids and analogs were dissolved individually at 100 mM concentration and adjusted at pH 9.0 by addition of HCl or NaOH as needed. Due to solubility limits, cysteine was dissolved at 20 mM and tyrosine at 6.25 mM. The latter was dissolved at 45°C immediately before addition to reaction mixtures.

Amino acid mixtures were prepared from a stock solution at pH 9.0 in which each amino acid was at a concentration 20-fold that prevailing under fasting conditions in rat portal blood (Patti et al. 1998) (see Table 1).

Proteomic analysis by LC-MS/MS

In summary, a 0.4-mL concentrated sample of RLNPP/PDE from the Sephacryl S-200 step containing 0.15 mg protein/mL was concentrated tenfold by speed-vac. The proteins were precipitated in chloroform/methanol at 4°C, resuspended in 50 mM ammonium bicarbonate, disulfide linkages were reduced with 10 mM dithiothreitol and blocked with 22.5 mM iodoacetamide. Proteolysis was run overnight at 37°C with 1:50 (w/w) recombinant trypsin (Roche). The peptides obtained were fractionated in a 44-min chromatographic run (Evosep One, Evosep) followed by electrospray ionization and analysis in a TIMS-TOF Pro 2 spectrometer (Bruker) set in DDA (data-dependent acquisition) mode. Precursor peptides were fragmented by high collision energy dissociation and the MS/MS spectra were collected and processed off-line with the MSFragger/FragPipe software (Kong et al. 2017) against the *Rattus norvegicus* proteome database (Uniprot/Swissprot). Sample treatment and analysis were performed in the Proteomic Unit (CAI Biological Techniques) of Complutense University (Madrid, Spain).

Results

Quantification and reversibility of the inactivation of RLNPP/PDE by EDTA

The course of the time-dependent inactivation of RLNPP/PDE at different EDTA concentrations is shown in Fig. 1a. In the absence of EDTA, the activity was stable over the assay period (60 min), whereas EDTA at 1 μ M, 5 μ M and 30 μ M evoked progressively faster inactivation. The corresponding

Table 1 Data for amino acids included in mixtures to be tested for their effect on the inactivation of RLNPP/PDE by EDTA. The acceleration factor (AF) equals the ratio of the $k_{i(ap)}$ obtained in the presence of the individual amino acid versus that in its absence (Fig. 3). The $k_{i(ap)}$ increment was calculated as $100 \times (AF - 1)$. Amino acid levels

Amino acid (5 mM)	$k_{i(ap)}$ (min^{-1}) ¹	Acceleration factor (AF)	$k_{i(ap)}$ increase ($100[AF-1]$) (%)	Amino acid concentration in rat portal blood under fasting (μM)	Theoretical $k_{i(ap)}$ increase produced by each amino acid in a 5 mM amino acid mixture (%)
None	0.054	1.0	0	–	–
Glycine	0.355	6.6	560	300	58.23
Tryptophan	0.333	6.2	520	70	12.62
Aspartate	0.331	5.3	430	30	4.47
Serine	0.230	4.4	340	200	23.57
Threonine	0.196	3.9	290	180	18.09
Asparagine	0.189	3.7	270	60	5.62
Alanine	0.180	3.7	270	400	37.44
Glutamate	0.172	3.4	240	100	8.32
Tyrosine	0.217	3.2	220	75	5.72
Phenylalanine	0.170	3.1	210	50	3.64
Methionine	0.151	2.7	170	40	2.36
Glutamine	0.130	2.7	170	350	20.62
Leucine	0.114	2.1	110	250	9.53
Arginine	0.102	2.0	100	100	3.47
Valine	0.115	2.0	100	180	6.24
Isoleucine	0.104	1.8	80	100	2.77
Lysine	0.093	1.7	70	300	7.28
Proline	0.089	1.6	60	100	2.08
Total				2945	232.06

¹The values of $k_{i(ap)}$ were obtained from the inactivation experiments shown in Fig. 3. The $k_{i(ap)}$ in the absence of amino acid is the mean obtained in all the experiments of Fig. 3

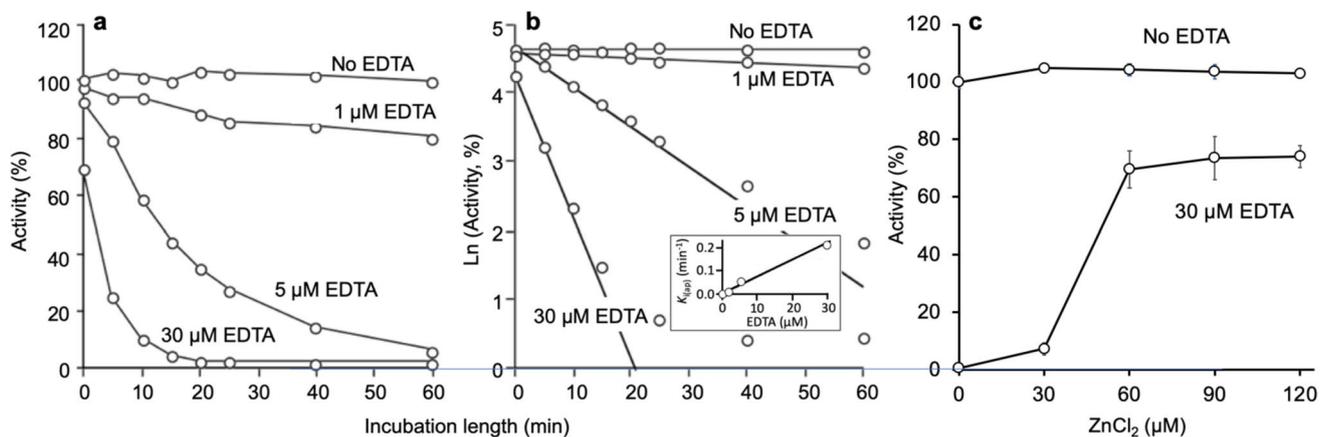


Fig. 1 RLNPP/PDE inactivation by EDTA. **a** Time dependence and **b** estimation of $k_{i(ap)}$ at different EDTA concentrations (the values of $k_{i(ap)}$ are plotted in the insert versus EDTA concentration). **c** Reactivation by excess Zn^{2+} of enzyme treated with $30 \mu\text{M}$ EDTA for 50

min, and then incubated with ZnCl_2 for 10 min before initiating the assay of activity (assays carried out in triplicate; error bars are standard deviations)

semilogarithmic plots are shown in Fig. 1b, from where $k_{i(\text{ap})}$ values were derived from the slopes of the linear parts of the plots ($k_{i(\text{ap})} = -\text{slope}$). The insert of Fig. 1b shows that $k_{i(\text{ap})}$ was directly proportional to EDTA concentration. The inactivation was reverted about 75% by the addition of excess Zn^{2+} (Fig. 1c), which supports that enzyme inactivation is due to chelation of enzyme-bound Zn^{2+} .

Effect of common amino acids on the inactivation of RLNPP/PDE by EDTA

The effect of glycine on the inactivation of RLNPP/PDE by EDTA was tested at various amino acid concentrations. In 5-min incubations with 6 μM EDTA, in the presence of 10–50 mM glycine, the inactivation of the enzyme was practically 100%, while in the presence of 1–5 mM glycine, 75–90% inactivation was observed (Fig. 2).

After the preliminary tests shown in Figs. 1 and 2, the selected conditions to study the effect of common amino acids on $k_{i(\text{ap})}$ were incubation with 5 μM EDTA and 5 mM amino acid. The twenty common amino acids plus hydroxyproline were studied: nineteen of them did not affect RLNPP/PDE activity in the absence of EDTA but accelerated enzyme inactivation by EDTA (Fig. 3). The numerical data are summarized in Table 1, including values of AF (acceleration factor) and of percent increase of $k_{i(\text{ap})}$.

In contrast to those amino acids, cysteine and histidine inhibited RLNPP/PDE strongly in the absence of EDTA, what made it difficult to quantify the acceleration evoked by these amino acids, since in the presence of EDTA and amino acid a synergistic effect was observed that led to a full inhibition (Fig. 4).

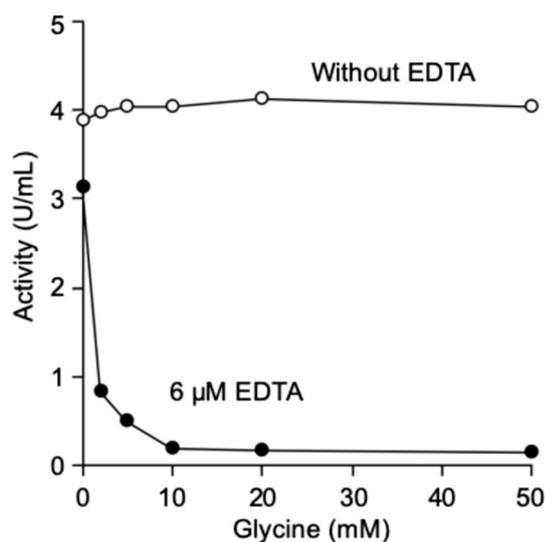


Fig. 2 Inactivation of RLNPP/PDE by 5-min incubation with EDTA in the presence of increasing glycine concentration

Effect of amino acid mixtures on the inactivation of RLNPP/PDE by EDTA

Common amino acid mixtures were prepared at different concentrations based on those prevailing in rat portal blood during fasting (Patti et al. 1998). Histidine and cysteine were omitted in these mixtures because their strong inhibitory effect in the absence of EDTA precludes a clear estimation of their effect on RLNPP/PDE inactivation by EDTA (Fig. 3). The fasting concentrations of individual amino acids range from 30–400 μM , yielding a total concentration of 2,945 μM (Table 1). Using the results obtained with the individual amino acids at 5 mM concentration (Fig. 3), acceleration factors were calculated and converted to percent $k_{i(\text{ap})}$ increases. Multiplying these values by the molar fraction of the corresponding amino acid in the fasting mix, one obtains the fraction of effect produced by each amino acid in a mix where the total concentration was 5 mM and the theoretical amino acid proportions were like in fasting conditions. The sum of these values provides a theoretical prediction of the effect expected for such a 5 mM mixture (Table 1). Figure 5 shows the effects produced by amino acid mixtures at different total concentrations, ranging from 0.25 to 4 fold the concentration under fasting. The effect of the 5 mM mixture of amino acids (1.7 fold the fasting concentration) was in good agreement with the theoretical prediction (see the insert of Fig. 5).

Effect of amino acid analogs on the inactivation of RLNPP/PDE by EDTA

All the common amino acids studied accelerated RLNPP/PDE inactivation by EDTA to different degrees (Fig. 3), with the only apparent exception of cysteine. In this case, the strong inhibition caused at 5 mM concentration in the absence of EDTA, precluded observation of any effect in the presence of EDTA. In the case of histidine, which in the absence of EDTA was itself a significant time-dependent inhibitor of RLNPP/PDE, with both inhibitors together (histidine and EDTA) the effect was immediate and time-dependency was not apparent (Fig. 4). With the rest of common amino acids, the increase of $k_{i(\text{ap})}$ ranged from 60% by proline to >500% by glycine and tryptophan (Table 1). This variation gives information about the role of the amino acid side chain in the interaction with RLNPP/PDE (see the Discussion), but not about the rest of the amino acid structure ($-\text{CHNH}_2-\text{COO}^-$). To investigate this question, analogs and structural variants of common amino acids were tested, including two D-amino acids (D-aspartate and D-alanine), analogs devoid of the α -amine group (acetate, propionate, malonate, butyrate, succinate and glutarate), α -oxoacids (pyruvate, oxaloacetate and α -oxoglutarate), α -hydroxyacids (L-lactate and

Fig. 3 Acceleration of the time-dependent inactivation of RLNPP/PDE by EDTA: effects of nineteen common amino acids that do not affect enzyme activity in the absence of EDTA. The course of inactivation and semilogarithmic plots are shown for each amino acid. Activity assays were performed in triplicate. Error bars are standard deviations. A few $k_{i(ap)}$ values can be underestimations due to large immediate decreases of activity at time zero in the presence of amino acid

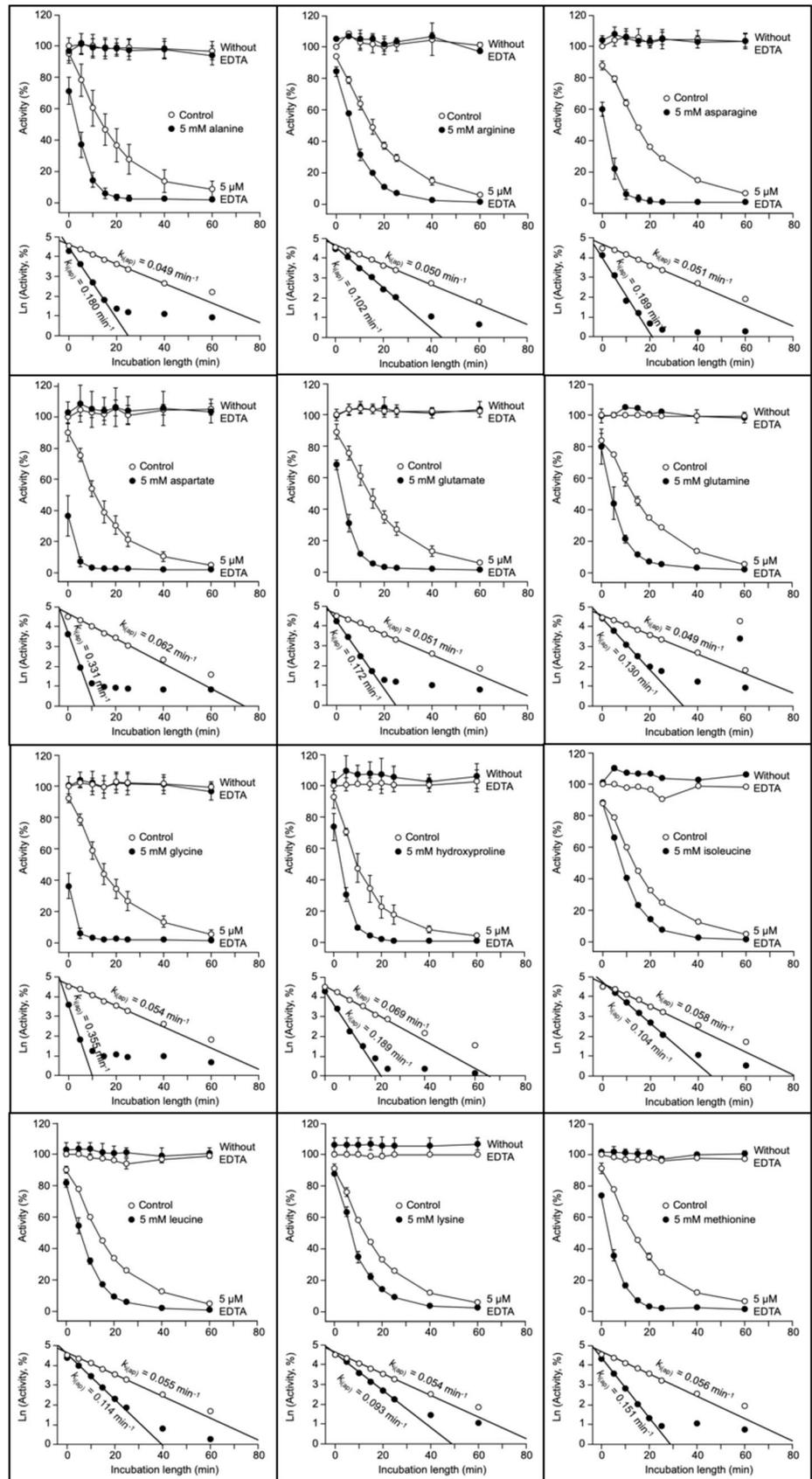
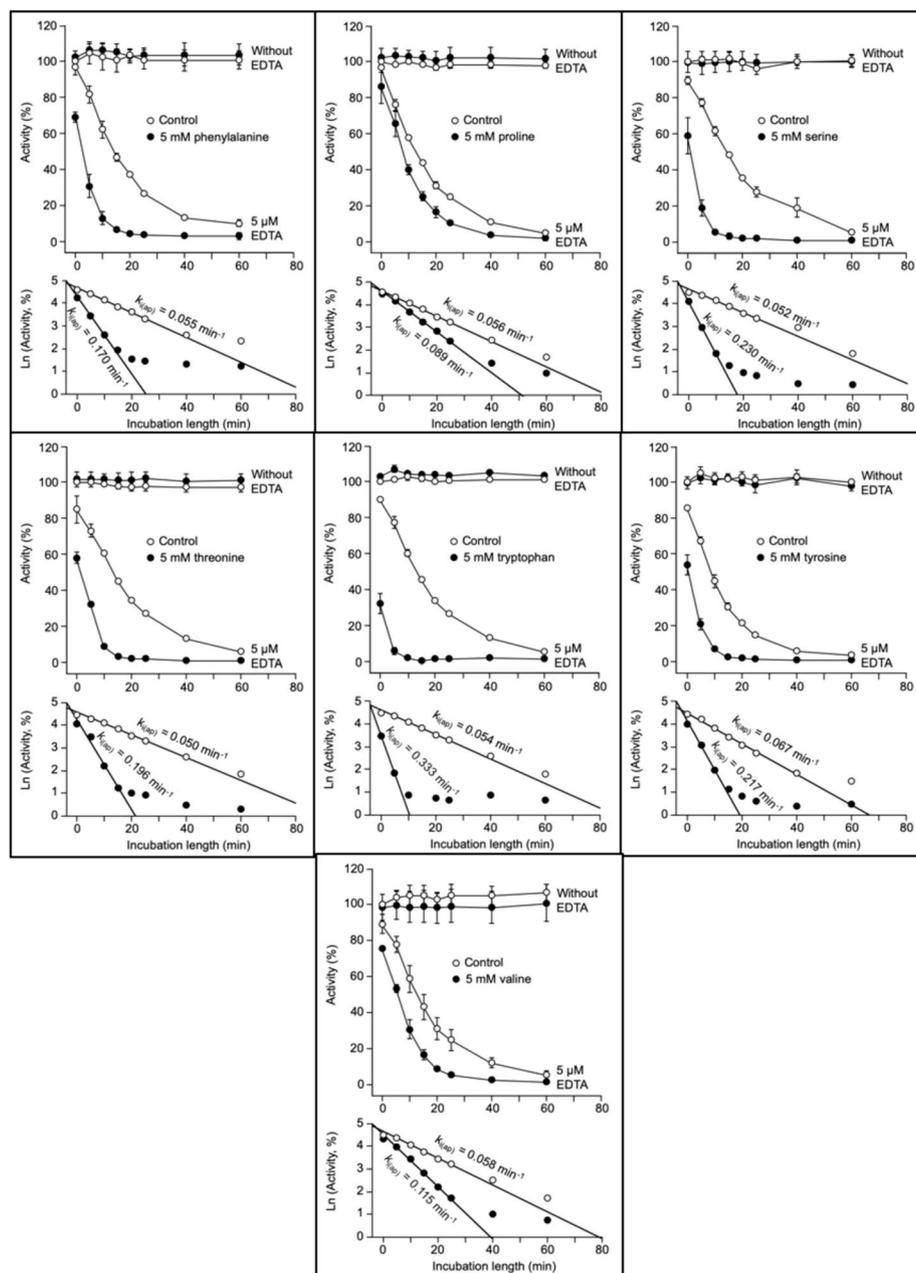


Fig. 3 (continued)

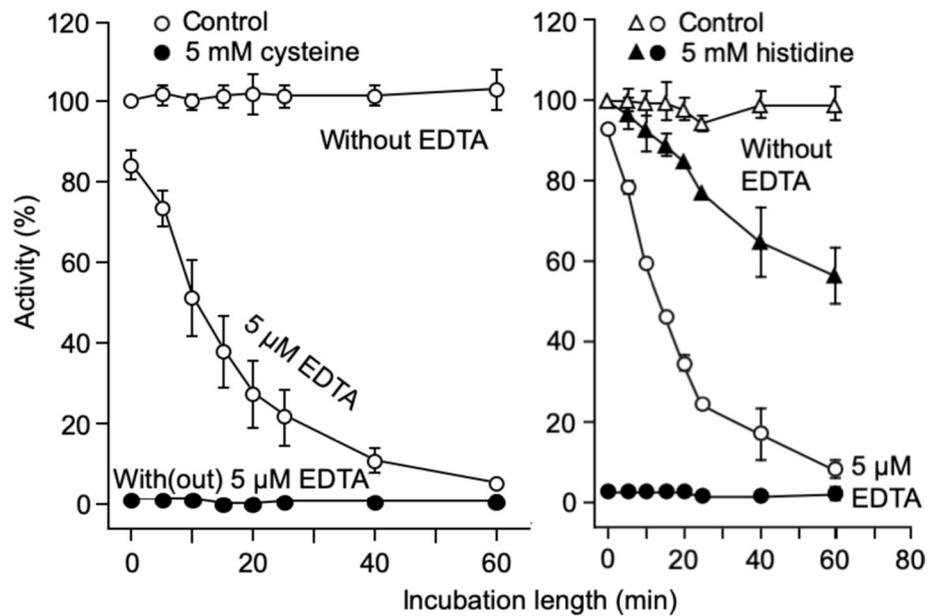


L-malate), analogs devoid of the α -carboxyl group (aminomethane, aminoethane, 1-aminopropane and 2-aminopropane, 2-aminobutane), non- α -amino acids with increasing distances between the carboxyl and the amine groups (β -alanine, γ -aminobutyrate and δ -aminohexanoate), glycine peptides which also increase the distance between the carboxyl and the amine groups (glycylglycine and glycylglycylglycine), and other miscellaneous compounds (ammonium chloride, ethylene glycol, formamide, urea, p-aminobenzoate). The results obtained with all these variants are shown in Table 2 and discussed later.

Effect of glycine on the inactivation of RLNPP/PDE by EDTA in crude membrane preparations

In previous sections, the effect of amino acids on RLNPP/PDE inactivation was studied with a solubilized enzyme preparation. However, in its natural location the enzyme is bound to membranes. Figure 6 shows the results of testing the effect of 5 mM glycine on the inactivation of the RLNPP/PDE activity by 10 μM EDTA in a crude preparation of rat liver membranes. The effect was tested on freshly obtained membranes and in membranes kept at 4°C for 2–6 days. The value of $k_{i(\text{ap})}$ in the absence of glycine did not change with

Fig. 4 Acceleration of the time-dependent inactivation of RLNPP/PDE by EDTA: effects of two common amino acids that are strong inhibitors of enzyme activity in the absence of EDTA



time elapsed. In contrast, the accelerating effect of glycine, that was clear in every case, became stronger in aged membranes, approaching the values observed with solubilized RLNPP/PDE. This result argues strongly against the formation of EDTA-Zn²⁺-amino acid complexes in solution as possible explanation for the pro-inactivating effect of amino acids (see the Discussion).

Experiments designed to find out the molecular identity of the enzyme responsible for RLNPP/PDE activities: assays of the hydrolysis of 2',3'-cGAMP, ATP and 4-nitrophenylphosphorylcholine, and proteomic analysis by LC-MS/MS

The hydrolysis of 2',3'-cGAMP is a well-known activity of human ENPP1 (Borza et al. 2021; Kato et al. 2018; Li et al. 2014; Ritchie et al. 2022). However, while this work was in progress, this activity was also described for human SMPDL3A (ASM3A_HUMAN, acid sphingomyelinase-like phosphodiesterase 3a) (Hou et al. 2023) and for human ENPP3 (Mardjuki et al. 2024). Like RLNPP/PDE, these three proteins are also active against ATP and 4-nitrophenyl-dTMP. Therefore, the 2',3'-cGAMP hydrolase activity of RLNPP/PDE was assayed by HPLC with positive results (Fig. 7), pointing to the possible presence of rat orthologs of any of those enzymes. As a complement to this study, the activity of RLNPP/PDE on ATP was also assayed by HPLC (Fig. 8), and that on 4-nitrophenyl-phosphorylcholine (typical of SMPDL3A) was assayed by A₄₀₅ measurements with detection of an activity much smaller (<1%) than that on 4-nitrophenyl-dTMP (Table 3).

The proteomic analysis of RLNPP/PDE by LC-MS/MS (Table S1) revealed the absence of four members of the

ENPP family (Enpp1, Enpp2, Enpp6 and Enpp7), and the presence of the other three (Enpp3, Enpp4, Enpp5). In addition, the presence of Smpdl3a was also detected (Table 4). Enpp4 and Enpp5 are orthologs of the murine and human proteins Enpp4/ENPP4 and Enpp5/ENPP5, respectively. Smpdl3a is the ortholog of murine and human proteins Smpdl3a/SMPDL3A. Anyhow, it should be considered that detection of peptides of a specific enzyme by LC-MS/MS does not necessarily mean that active protein was present in the sample. In the Discussion, all these enzyme-activity and mass-spectrometry data are used in support of the attribution of RLNPP/PDE activities to rat Enpp3.

Discussion

The evidence favors Enpp3 as responsible for the enzyme activities of RLNPP/PDE and for the acceleration of the EDTA-dependent inactivation by amino acids

For this discussion, one has to consider first all the enzyme activities shown by the RLNPP/PDE preparation in this study (Table 3) and in previous ones, i.e. phosphodiesterase activity on 4-nitrophenyl-dTMP (Table 3 and (López-Gómez et al. 1998; Ribeiro et al. 2000)) and 4-nitrophenyl-phosphorylcholine (Table 3), nucleotide pyrophosphatase activity on ATP (Fig. 8, Table 3 and (Ribeiro et al. 2000)) and 2',3'-cGAMP hydrolase (Fig. 7 and Table 3). On the other hand, these activities should be compared to those of the relevant candidates, namely Enpp3, Enpp4, Enpp5 and Smpdl3a (Table 4).

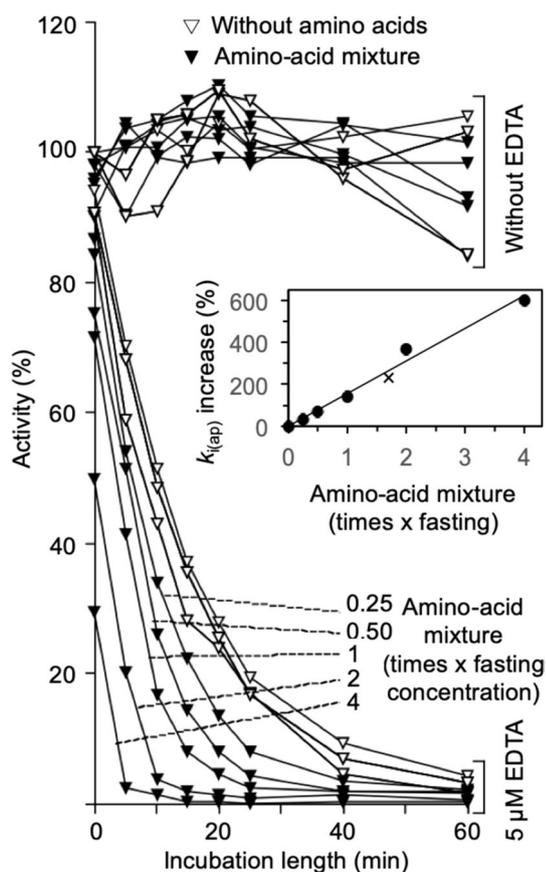


Fig. 5 Acceleration of the time-dependent inactivation of RLNPP/PDE by EDTA: amino acid mixtures of different concentration. The composition of the mixtures is shown in Table 1. The amino acid concentrations were 0.25–4 fold those prevailing under fasting conditions in rat portal blood. The insert shows the proportionality between the concentration of the mixture and the $k_{i(\text{ap})}$ increase; the X symbol marks the theoretical prediction made in Table 1 for a 5 mM mixture (1.7-fold the fasting concentration). The values of $k_{i(\text{ap})}$ increase at high amino acid concentration can be underestimations due to the large immediate decrease of activity in these cases

The comparisons, summarized in Table 5, indicate that the best candidate to define a molecular identity for RLNPP/PDE is Enpp3, which is fully compatible with the activities considered. Enpp4 and Enpp5 can be discarded by the absence of significant activity towards ATP and 2',3'-cGAMP of the human and murine orthologs, and in the case of Enpp5 also by the absence of activity on 4-nitrophenyl-dTMP. Smpdl3a can be discarded by the acid pH optima of the activities on ATP and 4-nitrophenyl-dTMP. However, with the only data shown in Table 5, it cannot be discarded that a very minor part of the activity of RLNPP/PDE on 4-nitrophenyl-dTMP and/or on 2',3'-cGAMP at pH 9 (as in the assays of the amino acid effect on the inactivation by EDTA, and of 2',3'-cGAMP hydrolase) could be due to Enpp4 or Smpdl3a, respectively.

Table 2 Effects of amino acid analogs on the inactivation of RLNPP/PDE by EDTA

Amino acid analog (5 mM)	Acceleration factor (AF)	$k_{i(\text{ap})}$ ¹ increase (100[AF-1]) (%)
D-Aspartate	5.3	430
D-Alanine	2.3	130
Pyruvate	1.6	60
Glycylglycine	1.5	50
α -Ketoglutarate	1.4	40
Ammonium acetate	1.3	30
Aminoethane	1.2	20
Aminomethane	1.2	20
2-Aminopropane	1.2	20
Butyrate	1.2	20
Ammonium chloride	1.2	20
Glycylglycylglycine	1.2	20
L-Lactate	1.2	20
Malonate	1.2	20
Oxaloacetate	1.2	20
2-Aminobutane	1.1	10
ϵ -Aminohexanoate	1.1	10
Glutarate	1.1	10
L-Malate	1.1	10
Propionate	1.1	10
Succinate	1.1	10
Sodium acetate	1.0	0
γ -Aminobutyrate	1.0	0
1-Aminopropane	1.0	0
Ethylene glycol	1.0	0
Formamide	1.0	0
Urea	1.0	0
β -Alanine	0.9	-10
p-Aminobenzoate	0.8	-20

¹The values of $k_{i(\text{ap})}$ were obtained from inactivation experiments similar to those shown in Fig. 3

Nevertheless, the activity of Enpp4-like enzymes on 4-nitrophenyl-dTMP is not documented other than by an incidental mention (Albright et al. 2012) and that on ATP is considered negligible (Albright et al. 2014). On the other hand, the presence of significant Smpdl3a activity in RLNPP/PDE is not supported by the very low activity towards 4-nitrophenyl-phosphorylcholine as compared to that on 4-nitrophenyl-dTMP (Table 3), as both substrates are hydrolyzed at the same rate by human SMPDL3A (Traini et al. 2014). Therefore, most part, if not all of the phosphodiesterase activity of RLNPP/PDE on 4-nitrophenyl-dTMP (whose inactivation by EDTA was accelerated by amino acids) as well as the 2',3'-cGAMP hydrolase activity, are due to rat Enpp3.

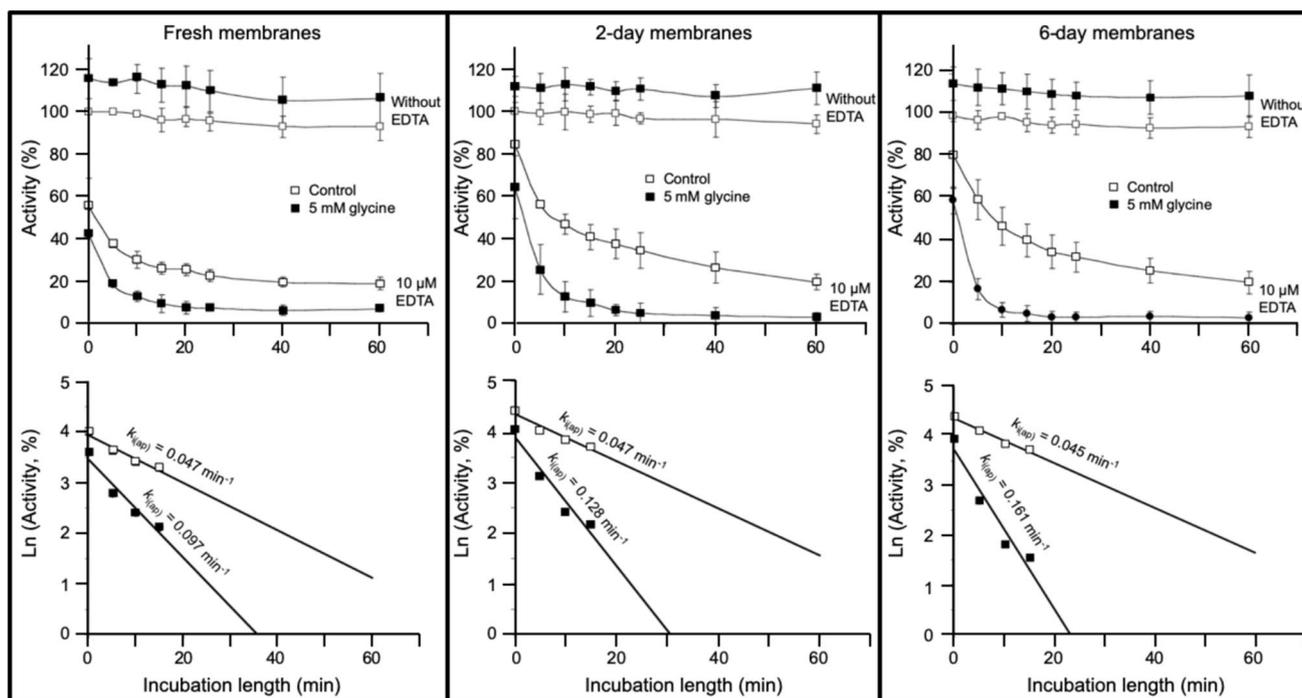


Fig. 6 Acceleration by glycine of the time-dependent inactivation of RLNPP/PDE by EDTA in a crude preparation of liver membranes. With fresh membranes, the experiment was started immediately after preparation and finished in less than two hours. In the other cases, the

membranes were kept at 4°C for two or six days before the experiments. The values of $k_{i(ap)}$ were estimated after a large rapid decrease of activity that was stronger in the case of fresh membranes. These values can be underestimations

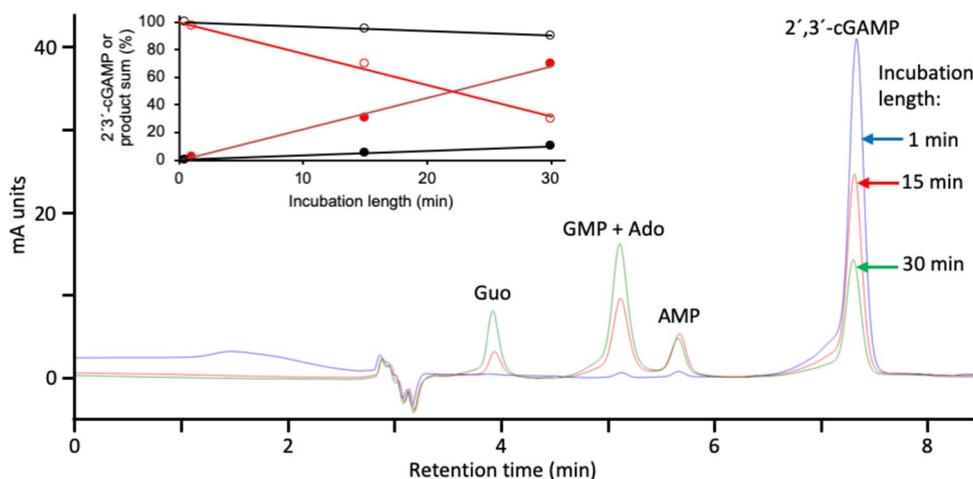


Fig. 7 RLNPP/PDE hydrolysis of 2',3'-cGAMP. A reaction mixture of 100 μL containing 50 mM Tris-HCl, pH 9, 10 μM 2',3'-cGAMP and 10 μL of RLNPP/PDE was incubated at 37°C. At the indicated times, 20 μL were analyzed by HPLC. The insert shows the quantitative results of reaction mixtures with either 1 μL (black line and symbols) or 10 μL (red line and symbols) of enzyme. The data show the remaining substrate (open circles) and the sum of AMP, GMP, Ado and Guo formed (closed circles). 5'-Nucleotides were direct products of RLNPP/PDE and nucleosides were secondary products due to a contaminating nucleotidase activity

structural features of the R group that diminish the acceleration factor or AF value (Table 1 and Fig. 3), including the following cases (AF values from Table 1 are given in parenthesis). The addition of a methylene group produced in most cases a ≈ 1.5 -fold decrease of AF value, as indicated

Essentiality of the α -amino-carboxyl structure for the effects of amino acids on the acceleration of RLNPP/PDE inactivation by EDTA

Comparisons of selected amino acids highlight simple

structural features of the R group that diminish the acceleration factor or AF value (Table 1 and Fig. 3), including the following cases (AF values from Table 1 are given in parenthesis). The addition of a methylene group produced in most cases a ≈ 1.5 -fold decrease of AF value, as indicated

Fig. 8 RLNPP/PDE hydrolysis of ATP. A reaction mixture of 200 μ L containing 50 mM Tris-HCl, pH 9, 100 μ M ATP and 1 μ L of RLNPP/PDE was incubated at 37°C. At the indicated times, 20 μ L were analyzed by HPLC. The insert shows the quantitative results of the reaction mixture in terms of AMP+Ado formed. AMP was a direct product of RLNPP/PDE and Ado was a secondary product due to a contaminating nucleotidase activity

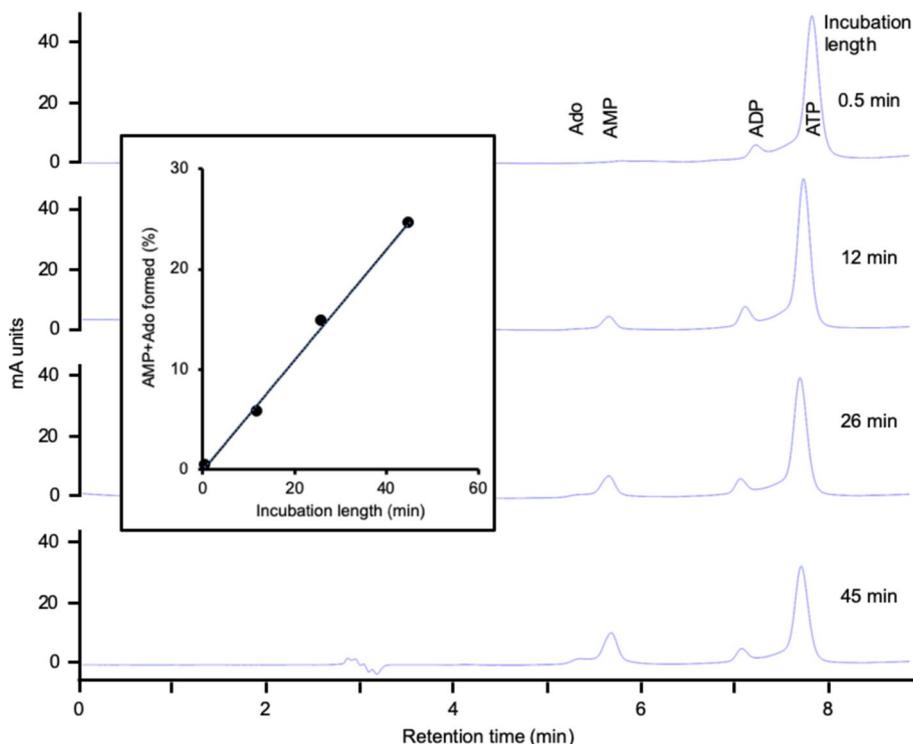


Table 3 RLNPP/PDE hydrolytic activities

Substrate	Activity ¹ (nmol min ⁻¹ mL ⁻¹)
4-Nitrophenyl-dTMP (20 mM)	1836 ± 238
4-Nitrophenyl-phosphorylcholine (20 mM)	15 ± 7
ATP (100 μ M)	157 ± 17
2',3'-cGAMP (10 μ M)	2.7 ± 0.6

¹Activities were assayed at pH 9 and 37°C. The hydrolysis of the 4-nitrophenyl derivatives was assayed by measuring the formation of nitrophenol at 405 nm. The hydrolysis of ATP and 2',3'-cGAMP were assayed by HPLC (Fig. 8 and Fig. 7, respectively)

by comparison of glycine (6.6) with alanine (3.7), aspartate (5.3) with glutamate (3.4), and asparagine (3.7) with glutamine (2.7). An exception to this rule comes from the

comparison of valine (2.0) with leucine (2.1) or isoleucine (1.8), perhaps due to the ramified carbon chain of the R group. The amidation of a carboxyl group produced a \approx 1.3-fold decrease of AF value, as indicated by comparison of aspartate (5.3) with asparagine (3.7), and glutamate (3.4) with glutamine (2.7). The addition of an aromatic ring produced also a \approx 1.3-fold decrease of AF value, as indicated by comparison of alanine (3.7) with phenylalanine (3.1), and serine (4.4) with tyrosine (3.2). The removal of a hydroxyl group produced a 1.2–1.7-fold decrease, as indicated by comparison of serine (4.4) with alanine (3.7), and hydroxyproline (2.7) with proline (1.6).

Comparisons of amino acids with analogs in which the α -amino-carboxyl functional group is modified, highlight the essential character of this structure, including the following cases (AF values from Table 1, for L-amino acids,

Table 4 Selected proteins identified in RLNPP/PDE by LC-MS/MS. Data taken from Table S1, except gene names that were taken from the Uniprot database

Uniprot name	Description	Coverage %	Total peptides	Total spectral count	Spectral abundance factor	Gene name
ENPP3_RAT	Ectonucleotide pyrophosphatase /phosphodiesterase family member 3	15.31	12	23	0.026	Enpp3
A6JJ18_RAT	bis(5'-adenosyl)-triphosphatase	30.84	6	13	0.029	Enpp4
ENPP5_RAT	Ectonucleotide pyrophosphatase /phosphodiesterase family member 5	19.08	5	6	0.013	Enpp5
ASM3A_RAT	Acid sphingomyelinase-like phosphodiesterase 3a	8.31	2	5	0.011	Smpd13a

Table 5 Comparison of RLNPP/PDE activities with those of relevant enzymes detected in RLNPP/PDE by LC-MS/MS

Enzyme	Nucleotide pyrophosphatase on ATP	Phosphodiesterase on 4-nitrophenyl-dTMP	Phosphodiesterase on 2',3'-cGAMP
RLNPP/PDE	Active at pH 7.5–8.0 (Ribeiro et al. 2000) and at pH 9 (Fig. 7)	Active with alkaline pH optimum (Romero 2003)	Active at pH 9 (Fig. 7)
Enpp3	Rat enzyme is active at pH 9.5 (Dohler et al. 2018)	Rat enzyme is active with alkaline pH optimum (Deissler et al. 1995)	Human and murine orthologs are active at pH 4–9 (Mardjuki et al. 2024)
Enpp4	Human ortholog shows very minor activity (Albright et al. 2014)	Human ortholog is active at unknown pH (Albright et al. 2012)	Murine ortholog shows negligible activity (Mardjuki et al. 2024) ¹
Enpp5	Human and murine orthologs show negligible activity (Gorelik et al. 2017)	Rat enzyme shows negligible activity (Ohe et al. 2003)	Murine ortholog shows negligible activity (Mardjuki et al. 2024) ¹
Smpd13a	Human and murine orthologs are active with acid pH optimum (Gorelik et al. 2016; Traini et al. 2014)	Human ortholog is active with acid pH optimum (Traini et al. 2014)	Murine ortholog is active at pH 4–10 (Hou et al. 2023)

¹Enpp1 and Enpp3 account for all the 2',3'-cGAMP hydrolase activity in mouse cells

or Table 2, for analogs, are in parenthesis). The removal of the α -amino group produced a strong, 3–5-fold decrease of AF as indicated by comparison of glycine (6.6), aspartate (5.3), alanine (3.7) and glutamate (3.4) with their respective deaminated compounds acetate (1.3), succinate (1.1), propionate (1.1) and glutarate (1.1). The substitution of the α -amine by an α -oxo group produced a strong, 2–4-fold decrease of AF as indicated by comparison of aspartate (5.3), alanine (3.7) and glutamate (3.4) with their respective α -oxo derivatives oxaloacetate (1.2), pyruvate (1.6) and α -ketoglutarate (1.4). The substitution of the α -amine by an α -hydroxyl group, produced a strong, 3–5-fold decrease of AF as indicated by comparison of aspartate (5.3) and alanine (3.7) with their α -hydroxy derivatives L-malate (1.1) and L-lactate (1.2). The removal of the α -carboxyl group produced a strong, 2.5–5.5-fold decrease of AF as indicated by comparison of glycine (6.6) and alanine (3.7) with their decarboxylated derivatives aminomethane (1.2) and aminoethane (1.2). Increasing the covalent distance between the carboxyl and amine groups produced a strong, 4–7-fold decrease of AF as indicated by comparison of glycine (6.6) to glycylglycine (1.5), glycylglycylglycine (1.2), β -alanine (0.9), γ -aminobutyrate (1.0) and ϵ -aminohexanoate (1.1).

In summary, the α -amino-carboxyl structure typical of the common amino acids is necessary to accelerate RLNPP/PDE inactivation by EDTA. On the other hand, the presence of an imino instead of the amino group produced little effect on AF as indicated by the comparison of proline (1.6) or hydroxyproline (2.7) with for instance the aliphatic amino acids (1.8–2.1). It seems likely that the D configuration of amino acids is similarly efficient as the L one, since the AF values obtained with D-alanine (2.3) and D-aspartate (5.3) were like those observed with L-alanine (3.7) and L-aspartate (5.3).

With a few exceptions, amino acid size correlates negatively, and stability constant of amino acid-Zn²⁺ complex correlates positively with the acceleration of RLNPP/PDE inactivation by EDTA

Comparisons among amino acids indicated that some features that represent augmentation of the R group (addition of methylene or aromatic ring, carboxyl amidation) diminished the AF value. Therefore, correlation analyses were performed between the increase of $k_{i(\text{ap})}$ and two size-related parameters (crude data listed in Table S2). To analyze the correlation with respect to molecular weight, all the amino acids studied in Fig. 3 were considered, which yielded a very weak, non-significant negative correlation (Fig. 9a). To analyze the correlation with respect to partial molar volume of the whole amino acid, glutamine and tyrosine could not be considered due to lack of data. In this case the negative correlation was somewhat better but still weak and non-significant (Fig. 9b). Nevertheless, in both correlation analyses, the point corresponding to tryptophan was clearly separated from the rest of amino acids. Therefore, the correlations were reanalyzed omitting tryptophan data. The resulting negative correlations were stronger and significant, particularly with partial molar volume (Fig. 9a and b; see data shown in red color).

The correlation between the increase of $k_{i(\text{ap})}$ and relative amino acid hydrophobicity was also analyzed. Since different hydrophobicity scales have been proposed, an average hydrophobicity calculated from twelve different normalized scales was used (Peters and Elofsson 2014). In this normalization, hydrophobic amino acids get negative values. Only a very weak, negligible and non-significant correlation was observed (Fig. 9c). Correlation was tested also with the

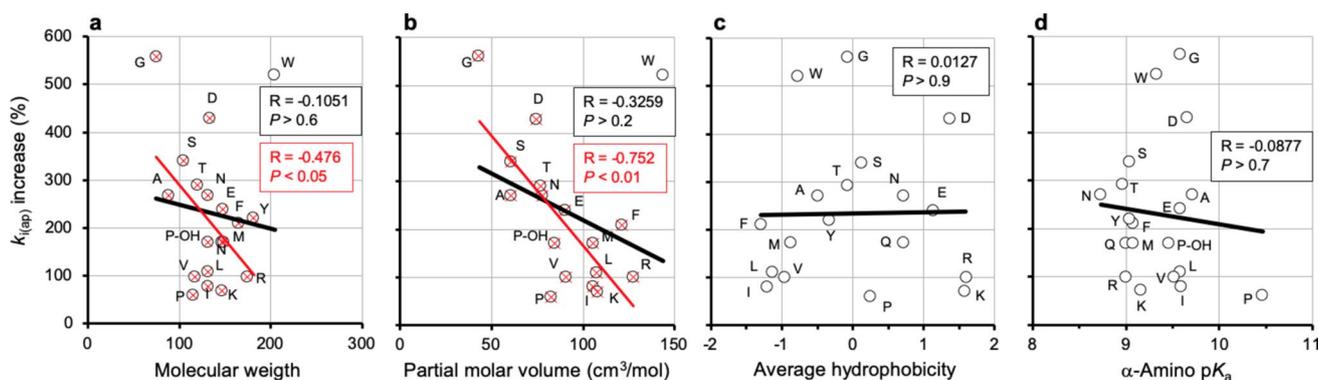


Fig. 9 Correlation analyses of $k_{i(\text{ap})}$ increase with amino acid parameters: molecular weight, partial molar volume, hydrophobicity and α -amino pK_a . Molecular weights were taken from PubChem database (Kim et al. 2023); partial molar volumes and pK_a values from (Haynes et al. 2017), hydrophobicity averages from twelve different normalized scales from (Peters and Elofsson 2014). Panels **a** and **b** display two datasets: (i) circles and black regression line (all the amino acids for which data were available) and (ii) red crosses (over-

lapped with circles) and regression line (same amino acids except one clear outlier which corresponds to tryptophan). Panels **c** and **d** display a single dataset (all the amino acids for which data were available). Crude data are shown in Table S2. The resulting Pearson correlation coefficients and P values are indicated in the panels. Amino acids are identified by the one-letter code. P-OH, hydroxyproline. Some values of $k_{i(\text{ap})}$ increase can be underestimations (see the legend to Fig. 3)

twelve scales from where the average hydrophobicity was calculated (Peters and Elofsson 2014). In the best cases, weak non-significant, either positive or negative correlations were observed, which justify the choice of average hydrophobicity in Fig. 9c.

The study of the amino acid effects on RLNPP/PDE inactivation by EDTA was carried out at pH 9.0, which is near the typical value of the α -amino pK_a . It was reasoned that this could lead to different ionization degrees of this group, which is essential for the effect of amino acids (see above). Therefore, the possible correlation between the increase of $k_{i(\text{ap})}$ and the α -amino pK_a value was also analyzed (Fig. 9d; crude data listed in Table S2), but the results indicated the absence of any significant relationship even when some outliers were omitted (not shown).

It seemed likely that the pro-inactivating effect of amino acids would involve somehow an interaction with Zn^{2+} ions. Therefore, correlation analyses were also run between the increase of $k_{i(\text{ap})}$ and the stability constants of free amino acid- Zn^{2+} complexes. In the NIST database (Smith et al. 2004), stability constants are recorded at different temperatures and ionic strength, but data for all amino acids under the same conditions are not available. Two different datasets were composed (see Table S2) by choosing constants either at 37°C and ionic strength (μ) 0.15 (no data for hydroxyproline and tyrosine) or at 25°C and μ 0.10 (no data for glutamine, glutamate and isoleucine). The correlations obtained with both datasets are shown in Fig. 10. Both cases gave similar results. A weak, non-significant positive correlation was observed when all the datapoints were considered (Fig. 10, both panels; see data colored black). However, after removing two or three very clear outliers in each case

(corresponding to aspartate, proline and hydroxyproline), strong and significant positive correlations were apparent (Fig. 10, both panels; see data shown in red color).

In summary, the correlations shown in Figs. 9a and b and 10 establish a combined relationship, whether causal or not, of the acceleration effect evoked by amino acids with their size and with their affinity for Zn^{2+} . In general, the smaller the amino acid and the higher its affinity for Zn^{2+} , the stronger is the acceleration produced of the time-dependent inactivation of RLNPP/PDE by EDTA. However, a few meaningful exceptions are worth of note. In Fig. 9a and b, tryptophan, which is the largest amino acid displays one of the stronger effects, only slightly below glycine which is the smallest amino acid. In Fig. 10 (both panels), aspartate, which is by far the amino acid with the highest affinity for Zn^{2+} , accelerates enzyme inactivation by EDTA somewhat less than glycine and tryptophan. Also exceptional were proline and hydroxyproline, which produced lower $k_{i(\text{ap})}$ increases than their relatively high affinities for Zn^{2+} would predict. All these exceptions indicate that the effect of the amino acids cannot be rationalized on a single parameter.

Mechanistic interpretation of the effect of free amino acids as accelerators of the time-dependent inactivation of Enpp3 by EDTA: evidence is compatible with a model proposing the occurrence of a conformational change evoked by amino acids

The acceleration elicited by free amino acids that themselves do not inhibit or inactivate Enpp3 (Figs. 3 and 5) can occur in principle through two different mechanisms.

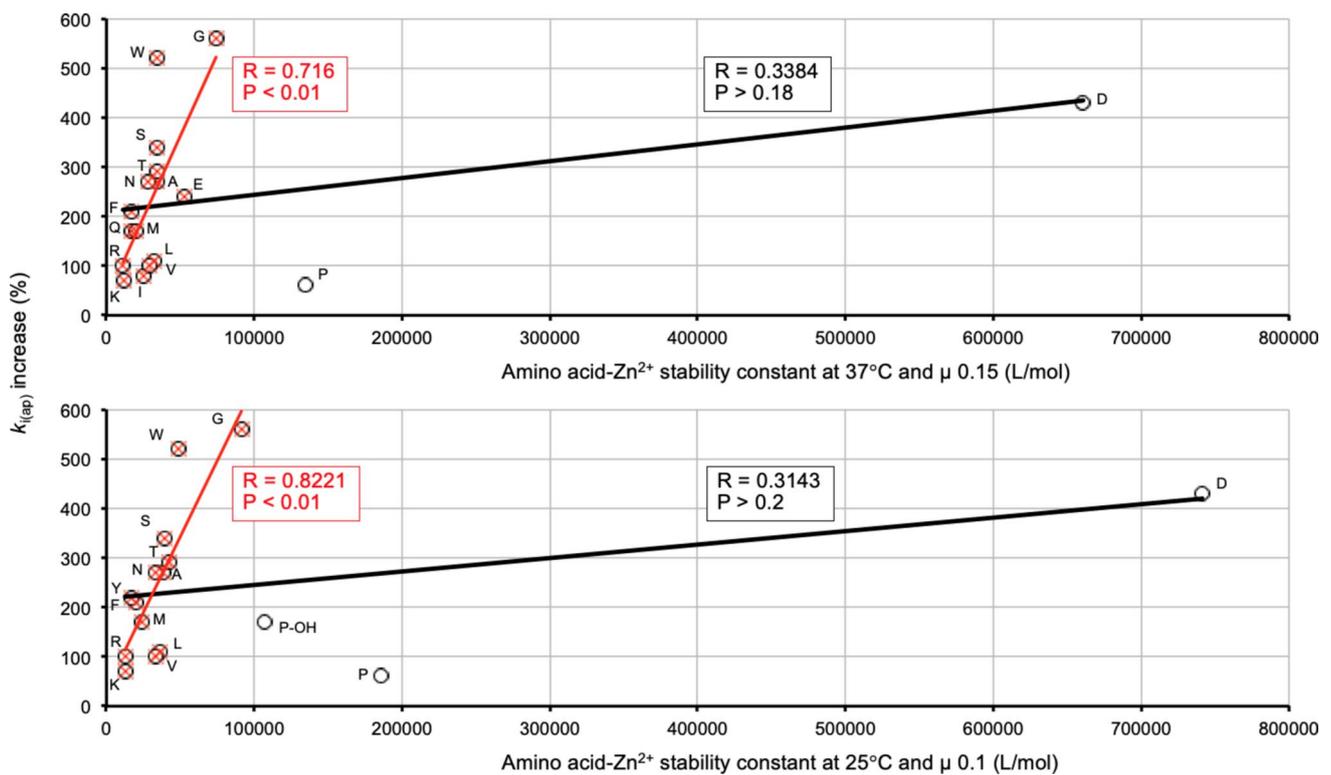


Fig. 10 Correlation analyses of $k_{i(ap)}$ increase with amino acid- Zn^{2+} stability constants. The stability constants of amino acid- Zn^{2+} complexes, at either 37°C and μ 0.15 (upper panel) or at 25°C and μ 0.10 (lower panel), were taken from (Smith et al. 2004). Each panel shows two datasets: (i) circles and black regression line (all the amino acids for which stability constants were available) and (ii) red crosses

(overlapped with circles) and regression line (same amino acids except two or three clear outliers). Crude data are shown in Table S2. The resulting Pearson correlation coefficients and P values are indicated in the panels. Amino acids are identified by the one-letter code. P-OH, hydroxyproline. Some values of $k_{i(ap)}$ increase can be underestimations (see the legend to Fig. 3)

In one case, the amino acids would bind the enzyme in one (or more) sterically-delimited sites (possibly including contact with enzyme-bound Zn^{2+} through the α -amino-carboxyl structure) and evoke a conformational change that loosens enzyme-bound Zn^{2+} and/or makes it more accessible to EDTA. Another mechanism that could take place would be that, rather than binding to Enpp3, amino acids could bind in solution to EDTA- Zn^{2+} complexes formed from Zn^{2+} slowly released from the enzyme. The formation of the ternary EDTA- Zn^{2+} -amino acid complexes would stabilize and possibly accelerate Zn^{2+} chelation.

Both mechanisms are not mutually exclusive and can perhaps coexist. However, the experiments shown in Fig. 6 argue strongly against the formation of EDTA- Zn^{2+} -amino acid complexes in solution as the only explanation for the pro-inactivating effect of amino acids. In that experiment, the effect of EDTA alone was not dependent on the time elapsed from membrane preparation to assay, whereas the acceleration evoked by glycine increased with time. There is no reason why the formation of EDTA- Zn^{2+} -amino acid complexes in solution should be affected by the time elapsed after membrane preparation.

Therefore, as a model to interpret the results, it is proposed that there is a direct interaction of free amino acids with rat Enpp3, which, through a conformational change, diminishes the stability of the protein- Zn^{2+} complex, and facilitates the removal of the ions by EDTA with the consequent loss of catalytic activity.

About the possible physiological relevance of an effect of amino acids on the conformation of Enpp3

Mammalian Enpp3 enzymes are multidomain integral membrane proteins. Most of their structure, after small cytosolic (CD) and transmembrane (TM) domains, faces the extracellular medium containing two N-terminal tandem somatomedin B-like domains (SMB), a phosphodiesterase domain (PDE) and a C-terminal nuclease-like domain (NUC) (Borza et al. 2021). The PDE domain contains the active site with the two essential Zn^{2+} ions coordinated by several residues. Most of our experiments were performed using the Enpp3 solubilized by limited trypsinization under native conditions, which likely corresponds to the Enpp3 protein devoid

of the CD and TM domains. Extrapolation of the results to enzyme in its physiological membrane location is supported by experiments demonstrating that the amino acid-dependent acceleration of the time-dependent inactivation of Enpp3 by EDTA takes place also in crude membranes. The effect was observed with freshly obtained membranes, but its intensity increased when tests were delayed a few days after membrane preparation (Fig. 6).

In its natural location facing the extracellular medium, Enpp3 is exposed not to a single amino acid, but to mixtures typical of serum which fluctuate depending on nutritional status. Tests run with amino acid mixtures at different concentrations (0.25–4-fold those prevailing in portal blood of fasted rats; Fig. 5) showed that the amino acid effect on Enpp3 *in vivo* may depend on the nutritional status of the animal.

Roles of Enpp1 and Enpp3 orthologs dependent or not on enzyme activity and on protein contacts: potential relevance of a conformational change evoked by amino acids

Although the results of this work are primarily related to Enpp3, the functions of both Enpp1 and Enpp3 ortholog proteins are discussed here, as both enzyme kinds display a marked similitude in their structures and catalytic activities (Borza et al. 2021; Gorelik et al. 2018; Stefan et al. 2005). For this reason, after attributing to Enpp3 the amino acid-dependent acceleration of inactivation by EDTA, one wonders whether the same response could be observed with Enpp1.

Despite their similitude, it must be remarked that Enpp1 and Enpp3 differ in their tissular expression profiles, remarkably with a different subcellular distribution in rat hepatocytes, where Enpp1 is located in the basolateral and Enpp3 in the apical surface (Scott et al. 1997).

Enpp1 orthologs are the most studied members of the ENPP family, with many roles in health and disease mostly dependent on the catalytic activities of the enzyme. (i) A well-known example of this is the regulation of calcification in bone and other tissues susceptible to mineralization. The hydrolysis of extracellular ATP by Enpp1 orthologs generates pyrophosphate (PPi), which is an inhibitor of hydroxyapatite deposition and prevents overmineralization. Deficiency or inactivating mutations of ENPP1 are clearly related to the occurrence of several calcification disorders (Borza et al. 2021; Ferreira et al. 2023; Orriss et al. 2016; Roberts et al. 2019; Terkeltaub 2006; Terkeltaub 2001). (ii) Also dependent on the activity of Enpp1 enzymes is their role in purinergic signaling due to the hydrolysis and generation of agonists (Ruiz-Fernández de Córdoba et al. 2023; Stefan et al. 2006). (iii) Another activity-dependent function of ENPP1 is its immunoregulatory role through the cGAS

(2',3'-cGAMP synthase)-STING pathway (Decout et al. 2021), where it intervenes by hydrolyzing 2',3'-cGAMP (Carozza et al. 2022; Li et al. 2014). This has stimulated the search of ENPP1 inhibitors with potential application in cancer immunotherapy (Carozza et al. 2020; Cogan and Bakhoun 2020; Onyedibe et al. 2019; Rauf et al. 2023; Ruiz-Fernández de Córdoba et al. 2023). (iv) Finally, an important function attributed to ENPP1 is the inhibition of the insulin receptor tyrosine kinase, which has a role in the insulin resistance of non-insulin-dependent diabetes mellitus (Arianti et al. 2021; Goldfine et al. 1998; Goldfine et al. 2008; Goldfine et al. 1999; Maddux et al. 1995; Roberts et al. 2019; Teno et al. 1999). In contrast to the clear dependence of functions i-iii on the catalytic activities of ENPP1, the inhibition of the insulin receptor is caused by protein-to-protein contact that hinders the insulin-induced conformational changes that evoke receptor autophosphorylation (Maddux and Goldfine 2000). In this case there is contradictory evidence, still unresolved, for the dependence of the inhibition of the insulin receptor on the enzyme activity of ENPP1 (Chin et al. 2009; Grupe et al. 1995; Stefan et al. 1996), but anyhow such inhibition has been repeatedly related to a direct contact between both membrane proteins (Di Paola et al. 2011; Dimatteo et al. 2013; Kulesza et al. 2023; Maddux and Goldfine 2000; Maddux et al. 1995; Tassone et al. 2018).

Enpp3 homologues have also been attributed with multiple roles. This covers functions that are clearly dependent on enzyme activity, as well as some that have not yet been characterized in this regard. (i) Enpp3 is highly expressed in basophils and mast cells and, through its ATP hydrolytic activity, it has been shown to regulate negatively the ATP-dependent chronic allergic responses (Bühning et al. 2001; Tsai et al. 2015). (ii) High Enpp3 expression has also been observed in epithelial cells of the small intestine, and its ATP hydrolytic activity contributes to the maintenance of the interferon-producing plasmacytoid dendritic cells, which are very sensitive to ATP-induced cell death (Furuta et al. 2017). (iii) Another activity-dependent function of ENPP3 is immunoregulation through the cGAS-STING pathway, mediated by the hydrolysis of 2',3'-cGAMP (Mardjuki et al. 2024), an activity that has been confirmed in this work (Fig. 7). (iv) Evidence links Enpp3 orthologs to the female reproductive system, as it is highly expressed in the uterine epithelium. ENPP3 undergoes cyclic changes in the human endometrium, where it affects embryo adhesion and invasion by modifying implantation-factors expression (Boggavarapu et al. 2016; Chen et al. 2018). In rats, Enpp3 expression has also been related to the morphological changes and inflammatory response during ovulation and luteinization (Li et al. 2017). (v) Enpp3 expression has also been shown to be involved in the invasive properties of tumor cells of glioma (Deissler et al. 1999) and colon carcinoma (Yano

et al. 2003), but the mechanism for these effects is unknown. (vi) Finally, there is circumstantial evidence that Enpp3 may be also related to diabetes, though a direct link to the insulin receptor, as that known to occur in the case of ENPP1, has not been reported (Ghanaat-Pour et al. 2007; Goldsworthy et al. 2013; Liu et al. 2019; Ye et al. 2024).

The effects of free amino acids on RLNPP/PDE affect enzyme activity only in the presence of EDTA, a metal chelator. This is most unlikely to have a reflection under physiological conditions, where EDTA would be absent. However, the results obtained in vitro with the combined action of amino acids and EDTA are diagnostic of a change of conformation caused by amino acid binding to the enzyme, which may indeed occur in the absence of EDTA. Although this conformational change would not modify Enpp3 catalytic activities under physiological conditions, one can consider speculatively whether such conformational change may be relevant to any Enpp3 role dependent on protein contacts with(out) intervention of its enzyme activity. On the other hand, if the amino acid effect, here reported with Enpp3, were extended to the structurally and enzymatically similar Enpp1, it would be conceivable that the effect of ENPP1 on the insulin receptor, which depends on protein-to-protein contacts, were affected by the change of conformation evoked by free amino acids. The same could apply if the above-mentioned relationship of Enpp3 to diabetes were mediated by contact with the insulin receptor. The potential relevance of these hypothesis is highlighted by the known role of free amino acids on the pathogenesis of insulin resistance (Chen et al. 2022; Gar et al. 2018; Kolic et al. 2023; Krebs et al. 2002; Lee et al. 2018; Patti et al. 1998; Saleem et al. 2019; Seibert et al. 2015; Tremblay et al. 2007).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00726-024-03431-4>.

Acknowledgement We are grateful to Felipe Clemente, from the Proteomic Unit (CAI Biological Techniques) of Complutense University of Madrid (Madrid, Spain), for his expert advice.

Author contributions J.M.R. and J.C.C. conceived, designed and drafted the manuscript, and carried out formal analyses of data. A.F., M.J.C. and J.C.C. supervised experiments performed by other coauthors. A.R., G.C.L., A.F., J.M., R.F., J.L.G. and M.J.C. performed experiments and obtained data. All authors evaluated and reviewed the manuscript, and agreed to the submission.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This research was funded by the Dirección General de Investigación, Ministerio de Ciencia y Tecnología, Spain, Grant No. BMC2001-0719.

Data availability The data presented in this study are available in the article and supplementary material. Further inquiries can be directed to the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Albright RA, Chang WC, Robert D, Ornstein DL, Cao W, Liu L, Redick ME, Young JI, De La Cruz EM, Braddock DT (2012) NPP4 is a procoagulant enzyme on the surface of vascular endothelium. *Blood* 120:4432–4440. <https://doi.org/10.1182/blood-2012-04-425215>
- Albright RA, Ornstein DL, Cao W, Chang WC, Robert D, Tehan M, Hoyer D, Liu L, Stabach P, Yang G, De La Cruz EM, Braddock DT (2014) Molecular basis of purinergic signal metabolism by ectonucleotide pyrophosphatase/phosphodiesterases 4 and 1 and implications in stroke. *J Biol Chem* 289:3294–3306. <https://doi.org/10.1074/jbc.M113.505867>
- Arianti R, Ariani NL, Muhammad AA, Sadewa AH, Farmawati A, Sunarti Hastuti P, Kristóf E (2021) Influence of Single Nucleotide Polymorphism of ENPP1 and ADIPOQ on Insulin Resistance and Obesity: A Case-Control Study in a Javanese Population. *Life* 11:552. <https://doi.org/10.3390/life11060552>
- Bischoff E, Tran-Thi TA, Decker KF (1975) Nucleotide pyrophosphatase of rat liver. A comparative study on the enzymes solubilized and purified from plasma membrane and endoplasmic reticulum. *Eur J Biochem* 51:353–361. <https://doi.org/10.1111/j.1432-1033.1975.tb03935.x>
- Boggavarapu NR, Lalitkumar S, Joshua V, Kasvandik S, Salumets A, Lalitkumar PG, Gemzell-Danielsson K (2016) Compartmentalized gene expression profiling of receptive endometrium reveals progesterone regulated ENPP3 is differentially expressed and secreted in glycosylated form. *Sci Rep* 6:33811. <https://doi.org/10.1038/srep33811>
- Borza R, Salgado-Polo F, Moolenaar WH, Perrakis A (2021) Structure and function of the Ecto-Nucleotide Pyrophosphatase-Phosphodiesterase (ENPP) family: tidying up diversity. *J Biol Chem* 298:101526. <https://doi.org/10.1016/j.jbc.2021.101526>
- Bühring HJ, Seiffert M, Giesert C, Marxer A, Kanz L, Valent P, Sano K (2001) The basophil activation marker defined by antibody 97A6 is identical to the ectonucleotide pyrophosphatase/phosphodiesterase 3. *Blood* 97:3303–3305. <https://doi.org/10.1182/blood.v97.10.3303>
- Cameselle JC, Costas MJ, Günther Sillero MA, Sillero A (1984) Two low K_m hydrolytic activities on dinucleoside 5',5''- P^1 , P^4 -tetraphosphates in rat liver. Characterization as the specific dinucleoside tetraphosphatase and a phosphodiesterase I-like enzyme. *J Biol Chem* 259:2879–2885. [https://doi.org/10.1016/S0021-9258\(17\)43230-3](https://doi.org/10.1016/S0021-9258(17)43230-3)

- Carozza JA, Brown JA, Böhnert V, Fernandez D, AlSaif Y, Mardjuki RE, Smith M, Li L (2020) Structure-Aided Development of Small-Molecule Inhibitors of ENPP1, the Extracellular Phosphodiesterase of the Immunotransmitter cGAMP. *Cell Chem Biol* 27:1347–1358. <https://doi.org/10.1016/j.chembiol.2020.07.007>
- Carozza JA, Cordova AF, Brown JA, AlSaif Y, Böhnert V, Cao X, Mardjuki RE, Skariah G, Fernandez D, Li L (2022) ENPP1's regulation of extracellular cGAMP is a ubiquitous mechanism of attenuating STING signaling. *Proc Natl Acad Sci U S A* 119:e2119189119. <https://doi.org/10.1073/pnas.2119189119>
- Chance B, Maehly AC (1955) Assay of catalases and peroxidases. *Methods Enzymol* 2:764–775. [https://doi.org/10.1016/S0076-6879\(55\)02300-8](https://doi.org/10.1016/S0076-6879(55)02300-8)
- Chen Q, Xin A, Qu R, Zhang W, Li L, Chen J, Lu X, Gu Y, Li J, Sun X (2018) Expression of ENPP3 in human cyclic endometrium: a novel molecule involved in embryo implantation. *Reprod Fertil Dev* 30:1277–1285. <https://doi.org/10.1071/rd17257>
- Chen S, Miki T, Fukunaga A, Eguchi M, Kochi T, Nanri A, Kabe I, Mizoue T (2022) Associations of serum amino acids with insulin resistance among people with and without overweight or obesity: A prospective study in Japan. *Clin Nutr* 41:1827–1833. <https://doi.org/10.1016/j.clnu.2022.06.039>
- Chin CN, Dallas-Yang Q, Liu F, Ho T, Ellsworth K, Fischer P, Natasha T, Ireland C, Lu P, Li C, Wang IM, Strohl W, Berger JP, An Z, Zhang BB, Jiang G (2009) Evidence that inhibition of insulin receptor signaling activity by PC-1/ENPP1 is dependent on its enzyme activity. *Eur J Pharmacol* 606:17–24. <https://doi.org/10.1016/j.ejphar.2009.01.016>
- Cogan D, Bakhoun SF (2020) Re-awakening Innate Immune Signaling in Cancer: The Development of Highly Potent ENPP1 Inhibitors. *Cell Chem Biol* 27:1327–1328. <https://doi.org/10.1016/j.chembiol.2020.11.001>
- Corder CN, Lowry OH (1969) The zinc requirement of dinucleotide pyrophosphatase in mammalian organs. *Biochim Biophys Acta* 191:579–587. [https://doi.org/10.1016/0005-2744\(69\)90351-9](https://doi.org/10.1016/0005-2744(69)90351-9)
- Decout A, Katz JD, Venkatraman S, Ablasser A (2021) The cGAS-STING pathway as a therapeutic target in inflammatory diseases. *Nat Rev Immunol* 21:548–569. <https://doi.org/10.1038/s41577-021-00524-z>
- Deissler H, Lottspeich F, Rajewsky MF (1995) Affinity purification and cDNA cloning of rat neural differentiation and tumor cell surface antigen gp130RB13-6 reveals relationship to human and murine PC-1. *J Biol Chem* 270:9849–9855. <https://doi.org/10.1074/jbc.270.17.9849>
- Deissler H, Blass-Kampmann S, Bruyneel E, Mareel M, Rajewsky MF (1999) Neural cell surface differentiation antigen gp130(RB13-6) induces fibroblasts and glioma cells to express astroglial proteins and invasive properties. *FASEB J* 13:657–666. <https://doi.org/10.1096/fasebj.13.6.657>
- Di Paola R, Caporarello N, Marucci A, Dimatteo C, Iadicicco C, Del Guerra S, Prudente S, Sudano D, Miele C, Parrino C, Piro S, Beguinot F, Marchetti P, Trischitta V, Frittitta L (2011) ENPP1 Affects Insulin Action and Secretion: Evidences from In Vitro Studies. *PLoS One* 6:e19462. <https://doi.org/10.1371/journal.pone.0019462>
- Dimatteo C, Marucci A, Palazzo A, Cisternino C, Marsano RM, Trischitta V, Di Paola R (2013) Role of somatomedin-B-like domains on ENPP1 inhibition of insulin signaling. *Biochim Biophys Acta* 1833:552–558. <https://doi.org/10.1016/j.bbamcr.2012.10.017>
- Dohler C, Zebisch M, Strater N (2018) Crystal structure and substrate binding mode of ectonucleotide phosphodiesterase/pyrophosphatase-3 (NPP3). *Sci Rep* 8:10874. <https://doi.org/10.1038/s41598-018-28814-y>
- Ferreira CR, Carpenter TO, Braddock DT (2023) ENPP1 in Blood and Bone: Skeletal and Soft Tissue Diseases Induced by ENPP1 Deficiency. *Annu Rev Pathol* 19:507–540. <https://doi.org/10.1146/annurev-pathmechdis-051222-121126>
- Furuta Y, Tsai SH, Kinoshita M, Fujimoto K, Okumura R, Umemoto E, Kurashima Y, Kiyono H, Kayama H, Takeda K (2017) E-NPP3 controls plasmacytoid dendritic cell numbers in the small intestine. *PLoS One* 12:e0172509. <https://doi.org/10.1371/journal.pone.0172509>
- Gar C, Rottenkolber M, Prehn C, Adamski J, Seissler J, Lechner A (2018) Serum and plasma amino acids as markers of prediabetes, insulin resistance, and incident diabetes. *Crit Rev Clin Lab Sci* 55:21–32. <https://doi.org/10.1080/10408363.2017.1414143>
- Ghanaat-Pour H, Huang Z, Lehtihet M, Sjöholm A (2007) Global expression profiling of glucose-regulated genes in pancreatic islets of spontaneously diabetic Goto-Kakizaki rats. *J Mol Endocrinol* 39:135–150. <https://doi.org/10.1677/jme-07-0002>
- Goldfine ID, Maddux BA, Youngren JF, Frittitta L, Trischitta V, Dohm GL (1998) Membrane glycoprotein PC-1 and insulin resistance. *Mol Cell Biochem* 182:177–184. <https://doi.org/10.1023/A:1006878001180>
- Goldfine ID, Maddux BA, Youngren JF, Trischitta V, Frittitta L (1999) Role of PC-1 in the etiology of insulin resistance. *Ann N Y Acad Sci* 892:204–222. <https://doi.org/10.1111/j.1749-6632.1999.tb07797.x>
- Goldfine ID, Maddux BA, Youngren JF, Reaven G, Accili D, Trischitta V, Vigneri R, Frittitta L (2008) The role of membrane glycoprotein plasma cell antigen 1/ectonucleotide pyrophosphatase phosphodiesterase 1 in the pathogenesis of insulin resistance and related abnormalities. *Endocr Rev* 29:62–75. <https://doi.org/10.1210/er.2007-0004>
- Goldsworthy M, Absalom NL, Schröter D, Matthews HC, Bogani D, Moir L, Long A, Church C, Hugill A, Anstee QM, Goldin R, Thursz M, Hollfelder F, Cox RD (2013) Mutations in MII2, an H3K4 methyltransferase, result in insulin resistance and impaired glucose tolerance in mice. *PLoS One* 8:e61870. <https://doi.org/10.1371/journal.pone.0061870>
- Gorelik A, Illes K, Superti-Furga G, Nagar B (2016) Structural Basis for Nucleotide Hydrolysis by the Acid Sphingomyelinase-like Phosphodiesterase SMPDL3A. *J Biol Chem* 291:6376–6385. <https://doi.org/10.1074/jbc.M115.711085>
- Gorelik A, Randriamihaja A, Illes K, Nagar B (2017) A Key Tyrosine Substitution Restricts Nucleotide Hydrolysis by the Ecto-enzyme NPP5. *FEBS J* 284:3718–3726. <https://doi.org/10.1111/febs.14266>
- Gorelik A, Randriamihaja A, Illes K, Nagar B (2018) Structural Basis for Nucleotide Recognition by the Ecto-enzyme CD203c. *FEBS J* 285:2481–2494. <https://doi.org/10.1111/febs.14489>
- Grarup N, Urhammer SA, Ek J, Albrechtsen A, Glümer C, Borch-Johnsen K, Jørgensen T, Hansen T, Pedersen O (2006) Studies of the relationship between the ENPP1 K121Q polymorphism and type 2 diabetes, insulin resistance and obesity in 7,333 Danish white subjects. *Diabetologia* 49:2097–2104. <https://doi.org/10.1007/s00125-006-0353-x>
- Grupe A, Alleman J, Goldfine ID, Sadick M, Stewart TA (1995) Inhibition of insulin receptor phosphorylation by PC-1 is not mediated by the hydrolysis of adenosine triphosphate or the generation of adenosine. *J Biol Chem* 270:22085–22088. <https://doi.org/10.1074/jbc.270.38.22085>
- Haynes WM, Lide DR, Bruno TJ (2017) CRC Handbook of Chemistry and Physics, 97th edn. CRC Press, Boca Raton, FL
- Hou Y, Wang Z, Liu P, Wei X, Zhang Z, Fan S, Zhang L, Han F, Song Y, Chu L, Zhang C (2023) SMPDL3A is a cGAMP-degrading enzyme induced by LXR-mediated lipid metabolism to restrict

- cGAS-STING DNA sensing. *Immunity* 56:2492–2507. e2410. <https://doi.org/10.1016/j.immuni.2023.10.001>
- Kato K, Nishimasu H, Oikawa D, Hirano S, Hirano H, Kasuya G, Ishitani R, Tokunaga F, Nureki O (2018) Structural insights into cGAMP degradation by Ecto-nucleotide pyrophosphatase phosphodiesterase 1. *Nat Commun* 9:4424. <https://doi.org/10.1038/s41467-018-06922-7>
- Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, Li Q, Shoemaker BA, Thiessen PA, Yu B, Zaslavsky L, Zhang J, Bolton EE (2023) PubChem 2023 update. *Nucleic Acids Res* 51:D1373–d1380. <https://doi.org/10.1093/nar/gkac956>
- Kolic J, Sun WG, Johnson JD, Guess N (2023) Amino acid-stimulated insulin secretion: a path forward in type 2 diabetes. *Amino Acids* 55:1857–1866. <https://doi.org/10.1007/s00726-023-03352-8>
- Kong AT, Leprevost FV, Avtonomov DM, Mellacheruvu D, Nesvizhskii AI (2017) MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry-based proteomics. *Nat Methods* 14:513–520. <https://doi.org/10.1038/nmeth.4256>
- Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, Nowotny P, Roth E, Waldhäusl W, Roden M (2002) Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes* 51:599–605. <https://doi.org/10.2337/diabetes.51.3.599>
- Kulesza T, Typiak M, Rachubik P, Rogacka D, Audzeyenka I, Saleem MA, Piwkowska A (2023) Pit 1 transporter (SLC20A1) as a key factor in the NPP1-mediated inhibition of insulin signaling in human podocytes. *J Cell Physiol* 238:1921–1936. <https://doi.org/10.1002/jcp.31051>
- Lee SG, Yim YS, Lee YH, Lee BW, Kim HS, Kim KS, Lee YW, Kim JH (2018) Fasting serum amino acids concentration is associated with insulin resistance and pro-inflammatory cytokines. *Diabetes Res Clin Pract* 140:107–117. <https://doi.org/10.1016/j.diabres.2018.03.028>
- Li L, Yin Q, Kuss P, Maliga Z, Millan JL, Wu H, Mitchison TJ (2014) Hydrolysis of 2'3'-cGAMP by ENPP1 and design of nonhydrolyzable analogs. *Nat Chem Biol* 10:1043–1048. <https://doi.org/10.1038/nchembio.1661>
- Li FX, Yu JJ, Liu Y, Miao XP, Curry TE Jr (2017) Induction of Ecto-nucleotide Pyrophosphatase/Phosphodiesterase 3 During the Periovarian Period in the Rat Ovary. *Reprod Sci* 24:1033–1040. <https://doi.org/10.1177/1933719116676394>
- Liu GM, Zeng HD, Zhang CY, Xu JW (2019) Key genes associated with diabetes mellitus and hepatocellular carcinoma. *Pathol Res Pract* 215:152510. <https://doi.org/10.1016/j.prp.2019.152510>
- López-Gómez J, Costas MJ, Meireles Ribeiro J, Fernández A, Romero A, Ávalos M, Cameselle JC (1998) Glycine-enhanced inhibition of rat liver nucleotide pyrophosphatase/phosphodiesterase-I by EDTA: a full account of the reported inhibition by commercial preparations of acidic fibroblast growth factor (FGF-1). *FEBS Lett* 421:77–79. [https://doi.org/10.1016/s0014-5793\(97\)01536-6](https://doi.org/10.1016/s0014-5793(97)01536-6)
- Maddux BA, Goldfine ID (2000) Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor alpha-subunit. *Diabetes* 49:13–19. <https://doi.org/10.2337/diabetes.49.1.13>
- Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, Spencer S, Grupe A, Henzel W, Stewart TA et al (1995) Membrane glycoprotein PC-1 and insulin resistance in non-insulin-dependent diabetes mellitus. *Nature* 373:448–451. <https://doi.org/10.1038/373448a0>
- Maddux BA, Chang YN, Accili D, McGuinness OP, Youngren JF, Goldfine ID (2006) Overexpression of the Insulin Receptor Inhibitor, PC-1/NPP1, Induces Insulin Resistance and Hyperglycemia. *Am J Physiol Endocrinol Metab* 290:E746–749. <https://doi.org/10.1152/ajpendo.00298.2005>
- Mardjuki R, Wang S, Carozza J, Zirak B, Subramanyam V, Abhiraman G, Lyu X, Goodarzi H, Li L (2024) Identification of the extracellular membrane protein ENPP3 as a major cGAMP hydrolase and innate immune checkpoint. *Cell Rep* 43:114209. <https://doi.org/10.1016/j.celrep.2024.114209>
- Martin RG, Ames BN (1961) A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J Biol Chem* 236:1372–1379. [https://doi.org/10.1016/S0021-9258\(18\)64180-8](https://doi.org/10.1016/S0021-9258(18)64180-8)
- Ohe Y, Ohnishi H, Okazawa H, Tomizawa K, Kobayashi H, Okawa K, Matozaki T (2003) Characterization of nucleotide pyrophosphatase-5 as an oligomannosidic glycoprotein in rat brain. *Biochem Biophys Res Commun* 308:719–725. [https://doi.org/10.1016/s0006-291x\(03\)01454-2](https://doi.org/10.1016/s0006-291x(03)01454-2)
- Onyedibe KI, Wang M, Sintim HO (2019) ENPP1, an Old Enzyme with New Functions, and Small Molecule Inhibitors-A STING in the Tale of ENPP1. *Molecules* 24:4192. <https://doi.org/10.3390/molecules24224192>
- Orriss IR, Arnett TR, Russell RG (2016) Pyrophosphate: a key inhibitor of mineralisation. *Curr Opin Pharmacol* 28:57–68. <https://doi.org/10.1016/j.coph.2016.03.003>
- Patti ME, Brambilla E, Luzzi L, Landaker EJ, Kahn CR (1998) Bidirectional modulation of insulin action by amino acids. *J Clin Invest* 101:1519–1529. <https://doi.org/10.1172/JCI1326>
- Peters C, Elofsson A (2014) Why is the biological hydrophobicity scale more accurate than earlier experimental hydrophobicity scales? *Proteins* 82:2190–2198. <https://doi.org/10.1002/prot.24582>
- Rauf A, Akram Z, Naveed M, AlMasoud N, Alomar TS, Saleem M, Waheed A, Ribaud G (2023) Studies on the Inhibition of Ecto-nucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) by 2-(3,4-Dihydroxyphenyl)-7,8-dihydroxy-3-methoxychromen-4-one, a Flavonoid from *Pistacia chinensis*. *Chemistry* 5:2094–2103. <https://doi.org/10.3390/chemistry5040142>
- Ribeiro JM, López-Gómez J, Vergeles JM, Costas MJ, García-Díaz M, Fernández A, Flores A, Cameselle JC (2000) Rat liver nucleotide pyrophosphatase/phosphodiesterase is an efficient adenylyl transferase. *Biochem J* 346:25–31. <https://doi.org/10.1042/bj3460025>
- Ribeiro JM, Canales J, Costas MJ, Cabezas A, Pinto RM, García-Díaz M, Martín-Cordero P, Cameselle JC (2023) Genomic Distribution of Pro-Virulent cpdB-like Genes in Eubacteria and Comparison of the Enzyme Specificity of CpdB-like Proteins from *Salmonella enterica*, *Escherichia coli* and *Streptococcus suis*. *Int J Mol Sci* 24:4150. <https://doi.org/10.3390/ijms24044150>
- Ritchie C, Carozza JA, Li L (2022) Biochemistry, Cell Biology, and Pathophysiology of the Innate Immune cGAS-cGAMP-STING Pathway. *Annu Rev Biochem* 91:599–628. <https://doi.org/10.1146/annurev-biochem-040320-101629>
- Roberts F, Zhu D, Farquharson C, Macrae VE (2019) ENPP1 in the Regulation of Mineralization and Beyond. *Trends Biochem Sci* 15:3010–3037. <https://doi.org/10.1016/j.tibs.2019.01.010>
- Romero A (2003) Efecto de los aminoácidos comunes sobre la inactivación de la nucleótido pirofosfatasa por el quelante de metales EDTA: Evidencia de un cambio conformacional debido a los aminoácidos libres. PhD Thesis, Universidad de Extremadura, Spain,
- Ruiz-Fernández de Córdoba B, Martínez-Monge R, Lecanda F (2023) ENPP1 Immunobiology as a Therapeutic Target. *Clin Cancer Res* 29:2184–2193. <https://doi.org/10.1158/1078-0432.Ccr-22-1681>
- Saleem T, Dahpy M, Ezzat G, Abdelrahman G, Abdel-Aziz E, Farghaly R (2019) The Profile of Plasma Free Amino Acids in Type 2 Diabetes Mellitus with Insulin Resistance: Association with Microalbuminuria and Macroalbuminuria. *Appl Biochem Biotechnol* 188:854–867. <https://doi.org/10.1007/s12010-019-02956-9>
- Scott LJ, Delautier D, Meerson NR, Trugnan G, Goding JW, Maurice M (1997) Biochemical and molecular identification of distinct forms of alkaline phosphodiesterase I expressed on the apical and basolateral plasma membrane surfaces of rat hepatocytes. *Hepatology* 25:995–1002. <https://doi.org/10.1002/hep.510250434>

- Seibert R, Abbasi F, Hantash FM, Caulfield MP, Reaven G, Kim SH (2015) Relationship between insulin resistance and amino acids in women and men. *Physiol Rep* 3:e12392. <https://doi.org/10.14814/phy2.12392>
- Smith RM, Martell AE, Motekaitis RJ (2004) NIST Critically Selected Stability Constants by Metal Complexes, NIST Standard Reference Database 46 version 80. National Institute of Standards and Technology, Gaithersburg, MD
- Stefan C, Wera S, Stalmans W, Bollen M (1996) The inhibition of the insulin receptor by the receptor protein PC-1 is not specific and results from the hydrolysis of ATP. *Diabetes* 45:980–983. <https://doi.org/10.2337/diab.45.7.980>
- Stefan C, Stalmans W, Bollen M (1997) Inhibition of the membrane protein PC-1 by commercial acidic fibroblast growth factor is due to a non-protein contaminant. *Biochem J* 325:814. <https://doi.org/10.1042/bj3250811w>
- Stefan C, Jansen S, Bollen M (2005) NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem Sci* 30:542–550. <https://doi.org/10.1016/j.tibs.2005.08.005>
- Stefan C, Jansen S, Bollen M (2006) Modulation of purinergic signaling by NPP-type ectophosphodiesterases. *Purinergic Signal* 2:361–370. <https://doi.org/10.1007/s11302-005-5303-4>
- Tassone EJ, Cimellaro A, Perticone M, Hribal ML, Sciacqua A, Andreozzi F, Sesti G, Perticone F (2018) Uric Acid Impairs Insulin Signaling by Promoting Enpp1 Binding to Insulin Receptor in Human Umbilical Vein Endothelial Cells. *Front Endocrinol* 9:98. <https://doi.org/10.3389/fendo.2018.00098>
- Teno S, Kanno H, Oga S, Kumakura S, Kanamuro R, Iwamoto Y (1999) Increased activity of membrane glycoprotein PC-1 in the fibroblasts from non-insulin-dependent diabetes mellitus patients with insulin resistance. *Diabetes Res Clin Pract* 45:25–30. [https://doi.org/10.1016/s0168-8227\(99\)00056-x](https://doi.org/10.1016/s0168-8227(99)00056-x)
- Terkeltaub RA (2001) Inorganic pyrophosphate generation and disposition in pathophysiology. *Am J Physiol Cell Physiol* 281:C1–C11. <https://doi.org/10.1152/ajpcell.2001.281.1.C1>
- Terkeltaub R (2006) Physiologic and pathologic functions of the NPP nucleotide pyrophosphatase/phosphodiesterase family focusing on NPP1 in calcification. *Purinergic Signal* 2:371–377. <https://doi.org/10.1007/s11302-005-5304-3>
- Traini M, Quinn CM, Sandoval C, Johansson E, Schroder K, Kockx M, Meikle PJ, Jessup W, Kritharides L (2014) Sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A) is a novel nucleotide phosphodiesterase regulated by cholesterol in human macrophages. *J Biol Chem* 289:32895–32913. <https://doi.org/10.1074/jbc.M114.612341>
- Tremblay F, Lavigne C, Jacques H, Marette A (2007) Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu Rev Nutr* 27:293–310. <https://doi.org/10.1146/annurev.nutr.25.050304.092545>
- Tsai SH, Kinoshita M, Kusu T, Kayama H, Okumura R, Ikeda K, Shimada Y, Takeda A, Yoshikawa S, Obata-Ninomiya K, Kurashima Y, Sato S, Umemoto E, Kiyono H, Karasuyama H, Takeda K (2015) The Ectoenzyme E-NPP3 Negatively Regulates ATP-Dependent Chronic Allergic Responses by Basophils and Mast Cells. *Immunity* 42:279–293. <https://doi.org/10.1016/j.immuni.2015.01.015>
- Yano Y, Hayashi Y, Sano K, Shinmaru H, Kuroda Y, Yokozaki H, Yoon S, Kasuga M (2003) Expression and localization of ecto-nucleotide pyrophosphatase/phosphodiesterase I-3 (E-NPP3/CD203c/PD-I beta/B10/gp130RB13-6) in human colon carcinoma. *Int J Mol Med* 12:763–766. <https://doi.org/10.3892/ijmm.12.5.763>
- Ye C, Fu Y, Zhou X, Zhou F, Zhu X, Chen Y (2024) Identification and validation of NAD⁺ metabolism-related biomarkers in patients with diabetic peripheral neuropathy. *Front Endocrinol* 15:1309917. <https://doi.org/10.3389/fendo.2024.1309917>
- Zhou HH, Chin CN, Wu M, Ni W, Quan S, Liu F, Dallas-Yang Q, Ellsworth K, Ho T, Zhang A, Natasha T, Li J, Chapman K, Strohl W, Li C, Wang IM, Berger J, An Z, Zhang BB, Jiang G (2009) Suppression of PC-1/ENPP-1 expression improves insulin sensitivity in vitro and in vivo. *Eur J Pharmacol* 616:346–352. <https://doi.org/10.1016/j.ejphar.2009.06.057>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.