Rat submandibular glands secrete nanovesicles with NTPDase and 5'-nucleotidase activities

Débora A. González · Patricia Egido · Noelia B. Balcarcel ·
Claude Hattab · Martín M. Barbieri van Haaster ·
Julie Pelletier · Jean Sévigny · Mariano A. Ostuni

Received: 19 September 2014 / Accepted: 4 December 2014 / Published online: 19 December 2014
© Springer Science+Business Media Dordrecht 2014

Abstract Extracellular nucleotides modulate a wide number of biological processes such as neurotransmission, platelet aggregation, muscle contraction, and epithelial secretion acting by the purinergic pathway. Nucleotidases as NTPDases and ecto-5'-nucleotidase are membrane-anchored proteins that regulate extracellular nucleotide concentrations. In a previous work, we have partially characterized an NTPDase-like activity expressed by rat submandibular gland microsomes, giving rise to the hypothesis that membrane NTPDases could be released into salivary ducts to regulate luminal nucleotide

concentrations as was previously proposed for ovarian, prostatic, and pancreatic secretions. Present results show that rat submandibular glands incubated *in vitro* release membrane-associated NTPDase and ecto-5'-nucleotidase activities. Electron microscopy images show that released membranes presenting nucleotidase activity correspond to exosome-like vesicles which are also present at microsomal fraction. Both exosome release and nucleotidase activities are raised by adrenergic stimulation. Nucleotidase activities present the same kinetic characteristics than microsomal nucleotidase activity, corresponding mainly to the action of NTPDase2 and NTPDase3 isoforms as well as 5'-nucleotidase. This is consistent with Western blot analysis revealing the presence of these enzymes in the microsomal fraction.

Electronic supplementary material The online version of this article (doi:10.1007/s11302-014-9437-0) contains supplementary material, which is available to authorized users.

D. A. González (✉) · P. Egido · N. B. Balcarcel ·
M. M. Barbieri van Haaster
Cátedra de Biofísica, Facultad de Odontología, Universidad de
Buenos Aires, M. T. de Alvear 2142 (1122), Ciudad Autónoma de
Buenos Aires, Argentina
e-mail: debora@biofis.odon.uba.ar

J. Pelletier · J. Sévigny
Centre de recherche du CHU de Québec, Québec, QC, Canada

J. Sévigny
Département de microbiologie-infectiologie et d'immunologie,
Faculté de Médecine, Université Laval, Québec, QC, Canada

C. Hattab · M. A. Ostuni
InsERM UMR-S1134, 6 rue Alexandre Cabanel, 75015 Paris, France

C. Hattab · M. A. Ostuni
Université Paris Diderot, Sorbonne Paris Cité, UMR-S1134 Paris,
France

C. Hattab · M. A. Ostuni
Institut National de la Transfusion Sanguine, Paris, France

C. Hattab · M. A. Ostuni
Laboratoire d'excellence GR-Ex, Paris, France

Keywords Submandibular gland · Extracellular nucleotides ·
NTPDases · Nucleotidases · Nanovesicles · Extracellular
vesicles

Abbreviations

Con-A	Concanavalin-A
E-NTPDase	Ecto-nucleoside triphosphate diphosphohydrolase
NTP	Nucleoside triphosphates
NDP	Nucleoside diphosphates
P27	Microsomal fraction
P100	Post-microsomal fraction
P ₂₇₀₀₀	27,000×g pellets from incubated gland
PM	Plasma membrane
PBS	Phosphate-buffered solution
PS	Physiological solution
RER	Rough endoplasmic reticulum
SMG	Submandibular gland

Introduction

Extracellular nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine-5'-triphosphate (UTP), and uridine diphosphate (UDP), signalling via purinergic(P2) receptors, participate in a wide number of biological processes such as neurotransmission, platelet aggregation, muscle contraction, and epithelial secretion. The regulation of the concentrations of these nucleotides at the cell surface is therefore crucial for these functions. The most important family of ectonucleotidases that hydrolyses these molecules belongs to the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family [1, 2]. Eight members of this family have been identified, which are either located at the plasma membrane (PM) and/or associated with intracellular membranes [3, 4]. NTPDase1, NTPDase2, NTPDase3, and NTPDase8 have their catalytic site facing the extracellular environment where they hydrolyse nucleoside triphosphates (NTP) and nucleoside diphosphates (NDP), including ATP and ADP, respectively. The hydrolysis of AMP to adenosine and Pi is catalysed mainly by the ecto-5'-nucleotidase [5].

Digestive-associated glands as salivary glands and pancreas express different actors of the purinergic system. In submandibular gland (SMG), metabotropic (P2Y₁, P2Y₂) and ionotropic (P2X₄, P2X₇) receptor subtypes were found, both in basolateral and luminal membranes in acinar and ductal cells [6–8]. It was demonstrated that P2X₇ activation regulates ionic currents and saliva volume and also induces reactive oxygen species production and inflammatory responses [9–11].

Salivary glands also express different NTPDase isoforms that contribute to regulate nucleotide concentration in these systems [12]. NTPDase1 was identified by immunohistochemistry in mucous acinar and ductal cells and also zymogen granules of mouse submandibular salivary gland [13]. NTPDase2 and ecto-5'-nucleotidase are co-expressed with NTPDase3 in mouse parotid and submandibular serous acinar cells but not in ductal cells [14]. Although all these enzymes are considered as PM-anchored proteins, the expression of NTPDase1, NTPDase2, and NTPDase3 has been also described in Golgi and endoplasmic reticulum (ER) membranes [15–18]. Particularly, NTPDase1 and 5'-nucleotidase expressed by pancreatic acini are released within the pancreatic juice to contribute with the control of ATP, ADP, and adenosine levels in the duct system [19, 20].

In a previous work, we have partially characterized an NTPDase-like activity expressed by rat SMG microsomes highly enriched in the rough ER. Immunohistochemical experiments confirmed the localization of NTPDases in PM and intracellular membranes both in ductal and acinar cells [21]. Those results let us to hypothesize that SMG cells could liberate NTPDases to the ducts to regulate ATP and ADP concentrations and modulate purinergic signaling.

Materials and methods

Reagents and solutions

Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Antibodies

All the primary antibodies used in this study have been previously characterized and validated: rabbit anti-rat NTPDase1 (rN1-6_LI5); rabbit anti-rat NTPDase2 (rN2-6_L); rabbit anti-rat NTPDase3 (rN3-1_LI5); guinea pig anti-rat NTPDase8 (rN8-8_CI5); and rabbit anti-rat ecto-5'-nucleotidase (rNu-9_SI5) [22–25]. More details concerning these antibodies are available at <http://ectonucleotidases-ab.com/>.

Submandibular glands

Animals were treated in accordance with European Committee guidelines concerning the care and use of laboratory animals. Wistar rats were sacrificed by exposure to ether and subsequent cervical dislocation. The submandibular glands were removed and separated from the surrounding connective tissue and sublingual glands.

Membrane fractions

Microsomes were prepared as previously reported [21]. Clean glands were weighed, minced on ice, and homogenized in cold buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0, 0.3 M sucrose, and 1 mM of the protease inhibitor PMSF) using a Teflon/glass homogenizer. The homogenate was centrifuged for 20 min at 10,000×g and the supernatant for 30 min at 27,000×g. This pellet was suspended in the same buffer to obtain the microsomal fraction (P-27), and supernatant was centrifuged again for 1 h at 100,000×g to obtain a PM-enriched fraction (P-100). The protein concentration was determined using the Pierce BCA protein assay (Thermo Scientific) with bovine serum albumin as standard.

Hydrolytic activity in isolated membranes

Nucleotidase activity was measured at pH 7.2 and 37 °C following Pi release. The media contained 50 mM 3-(N-Morpholino)propanesulfonic acid -tris(hydroxymethyl)aminometano (MOPS-Tris), pH 7.2, 100 mM KCl, 1 mM ethylene glycol tetraacetic acid (EGTA), and 3 mM MgCl₂. EGTA was added to chelate contaminant calcium and avoid the activation of sarco-endoplasmic reticulum calcium ATPase. Reactions were started by addition of ATP to media with 0.04–0.08 mg vesicular protein per

milliliter, preincubated for 5 min at 37 °C. At the selected times, the reactions were stopped by equal volume of 10 % cold trichloroacetic acid. Sodium azide (10 mM), suramin (different concentrations), or concanavalin-A (50 µg/ml) was included together with the microsomes when their effects on ATPase activity were evaluated. Pi production was determined with the colorimetric method of Baginski, based on the formation of a complex between Pi and ammonium molybdate [26].

Hydrolytic activity from submandibular gland

Each gland cut into pieces of 30–40 mg was first washed by immersion for 15 min in cold physiological solution (PS) (115 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM HEPES, 25 mM NaHCO₃, pH 7.4), bubbled with carbogen (95 % O₂:5 % CO₂), and then incubated for 30 min in PS at 37 °C with gentle shaking, with different additions according the experiment. When glands were removed after incubations, media were centrifuged for 15 min at 2,000×g to separate cells or debris. Then, supernatants were centrifuged for 60 min at 27,000×g and pellets washed in PS, centrifuged again, and suspended in PS. Finally, 3 mM MgATP (or MgADP or MgAMP) was added to gland containing media or to suspended pellets, and several samples were taken at different time up to 15 min to measure Pi concentration. Same results were obtained when pellets were suspended in the MOPS-Tris buffer at pH 7.2 used in experiments with isolated membranes.

Western blot

Protein samples (35 µg) from SMG whole homogenate, microsomal fraction (P27), and post-microsomal fraction (P100) were loaded on a NuPAGE® Novex® Bis-Tris 4–12 % gel or on a 10 % acrylamide gel, under non-reducing conditions, transferred to a nitrocellulose membrane, and incubated overnight at 4 °C under agitation with primary antibodies. Pre-immunized serum was used as negative control. Immune complexes were visualized with secondary peroxidase-conjugated antibodies, using a chemiluminescent kit (GE Healthcare Europe, Saclay, France). In some experiments, protein samples were deglycosylated with PNGase F following the manufacturer's protocol but strongly diminishing the amounts of reducing agent to avoid antibodies inactivation (New England BioLabs, Ipswich, USA).

Electron microscopy

Microsome preparation was fixed in 2.5 % glutaraldehyde for 60 min at room temperature and fixed in 1 % osmium tetroxide for 60 min. It was dehydrated and embedded in LX-112 resin, and sections were contrasted with 2 % uranyl acetate and observed in a JEOL 1200 EX electron microscope operated at 80 kV accelerated voltage. Extracellular vesicles suspended in phosphate-buffered solution (PBS; pH 7.2) were

seeded (5 µl) on a grid covered by acrylic membrane and allowed to settle for 20 min at room temperature. Excess PBS was removed by wicking with filter paper before fixation with 2 % paraformaldehyde, 2 % glutaraldehyde, and 0.05 M phosphate solution for 2 min. Grids were washed three times with distilled water prior to application of 1 % phosphotungstic acid counterstain for 1 min. Excess liquid was allowed to dry overnight at room temperature. Grids were analyzed with a Zeiss EM 109T transmission electron microscope equipped with digital camera Gatn ES1000W.

Results

Ecto-ATPase activity from SMG

SMG was incubated in a physiological solution, and Pi production was measured after 3 mM MgATP addition. Nucleotidase activity had similar kinetics to that previously observed in microsomes and could be attributed to NTPDases [21] (Fig. 1). These results are consistent with the presence of ecto-ATPases in the rat SMG, either embedded in the basolateral or luminal membranes or released into the incubation medium.

When SMG fragments incubated without nucleotides were removed, the addition of MgATP, MgADP, or MgAMP to 1,000×g centrifugated media showed the apparent presence of NTPDases and 5'-nucleotidase (not shown). Since nucleotidase activities in supernatants were high enough to support the hypothesis of gland secretion, we aimed to determine the following:

1. Whether enzymes were released in “soluble” forms or inserted in membranes
2. Whether enzymes release could be increased by salivary secretion agonists
3. The identity of the enzymes contributing to nucleotidase activity

Hydrolytic activity released by SMG

NTPDase1, NTPDase2, NTPDase3, and NTPDase8 and ecto-5'-nucleotidase are membrane enzymes, so if they are released to extracellular media, they should be part of some type of vesicles. According to this, nucleotidase activity was found on 27,000×g pellet (P₂₇₀₀₀) obtained from gland incubation media after a first centrifugation at 2,000×g to separate detached cells and cellular debris.

We also found that ATPase, ADPase, and AMPase activities in P₂₇₀₀₀, as well as protein concentration increased when gland fragments were incubated with the adrenergic

Fig. 1 Submandibular gland nucleotidase activity. After 30 min of SMG incubation in PS, Pi production was measured at different times after addition of 3 mM MgATP. Data are mean $\pm$ SEM, $n=3$. Pi production rates at different times were fitted to exponential decay function indicating that 85 % of initial ATP hydrolysis rate decayed with a $T_{1/2}$ around 3 min. *White circle*: total Pi production ($\mu\text{mol}/\text{mg}$ wet gland). *Black circle*: Pi production rate (μmol Pi/mg wet gland/min)

secretagogues isoproterenol (Fig. 2 and Table 1) or epinephrine (not shown). Proteins in the pellet were 9.2 ± 0.5 and 15.9 ± 1.5 ng/mg (mean SEM, $n=9$) of wet gland after incubation without or with 0.1 mM isoproterenol, respectively, that is, an increase of 1.8 times. In the supernatant remained a low ATPase activity constant over time which was not increased by isoproterenol.

ATPase activity (Figs. 1 and 2) can be interpreted as the sum of two components, which could originate from two different enzymes. The major fraction (≥ 80 %) was unstable decaying exponentially with a $T_{1/2}$ of 2–3 min and could be attributed to NTPDase2. This assumption is based on ATP-dependent inactivation reported first for some rat Mg-ATPases or ecto-ATPases [27–30] and more recently, since NTPDases have been identified and cloned, for human NTPDase2 [31]. The stable (final) ATPase activity was similar to ADPase activity and could be due to NTPDase1, NTPDase3, or NTPDase8.

Unstable ATPase activity attributed to NTPDase2 was the largest in the pellet and the most increased by isoproterenol. Although the mechanism of NTPDase2 inactivation during ATP hydrolysis is unknown, the enzyme was stabilized by addition of the tetravalent lectin concanavalin-A (Con-A) to the incubation medium before substrate [31]. We measured pelleted nucleotidase activity with 3 mM ATP in the absence or presence of 50 $\mu\text{g}/\text{ml}$ Con-A (Fig. 3). As expected, Con-A prevented time-dependent inhibition of basal and isoproterenol-dependent ATPase activity, reinforcing the proposal of NTPDase2 as the main secreted ATPase.

Microsomal ATPase activity

Microsomal NTPDases could be a reserve of enzymes for the plasma membrane and, in view of our results, to be released into the duct. We measured Pi production during incubation

Fig. 2 Time dependence of baseline and isoproterenol-stimulated nucleotidase activity released to incubation medium. After 30 min of gland incubation in the absence (*black circle*) or in the presence of 100 μM isoproterenol (*black square*), incubation media were centrifuged 15 min at $2,000\times g$ and 60 min at $27,000\times g$; the last pellets were suspended into fresh PS containing 3 mM ATP. **a** Pi production was measured at several incubation time points. **b** Pi production rates at different times were fitted to an exponential decay function. The difference between total (*black square*) and baseline (*black circle*) activities represents the nucleotidase activity liberated by the action of isoproterenol (*white triangle*). Data are mean $\pm$ SEM, $n=10$

Table 1 ATPase, ADPase, and AMPase activities in 27,000×g pellet from incubation media obtained after gland incubation without (B) or with (S) 0.1 mM isoproterenol. Initial (I), final (F), and time sensitive (I-F)

	Basal (B)	+ Isoproterenol (S)	Isoproterenol dependent (S–B)	$\frac{S}{B}$
	$\mu\text{mol Pi/min/mg wet gland (\% related to I)}$			
Initial ATPase activity (I)	$1.6\text{e-}4 \pm 4.5\text{e-}6$ (100 %)	$5.8\text{e-}4 \pm 2.1\text{e-}5$ (100 %)	$4.2\text{e-}4$ (100 %)	3.6
ATPase activity after 8-min incubation (F)	$3.2\text{e-}5 \pm 2.0\text{e-}6$ (20 %)	$8.6\text{e-}5 \pm 9.1\text{e-}6$ (15 %)	$5.3\text{e-}5$ (13 %)	2.7
Time-sensitive ATPase activity (I-F)	$1.3\text{e-}4 \pm 2.5\text{e-}6$ (80 %)	$4.9\text{e-}4 \pm 1.4\text{e-}5$ (85 %)	$3.6\text{e-}4$ (87 %)	3.8
ADPase activity*	$2.8\text{e-}5 \pm 2.1\text{e-}6$ (18 %)	$7.3\text{e-}5 \pm 1.9\text{e-}6$ (13 %)	$4.5\text{e-}5$ (11 %)	2.6
AMPase activity*	$2.0\text{e-}5 \pm 1.6\text{e-}6$ (13 %)	$4.6\text{e-}5 \pm 2.2\text{e-}6$ (8 %)	$2.6\text{e-}5$ (6 %)	2.3

ADPase and AMPase activities were measured by triplicate with 3 mM MgADP or MgAMP and were constant over 15 min. For each column, percentages are referred to initial ATPase activity

* Mean $\pm$ SEM, $s=3$

time with different ATP concentrations (Fig. 4). As observed when glands were incubated with 3 mM MgATP (Figs. 1 and 2), accumulated Pi did not follow linear functions with time, revealing decreasing hydrolysis rates. After 2-min incubation, the relation “[Pi]/initial [ATP]” was around 36 and 4 % for 0.1 and 3.0 mM ATP, respectively. Data were fitted with exponential functions which were then derived to obtain initial velocities. The inset in Fig. 4 shows Hanes plot used to calculate apparent values for maximum initial velocity ($V_m = 1.99 \pm 0.05 \mu\text{mol Pi mg}^{-1} \text{min}^{-1}$) and the Michaelis-Menten constant ($K_m = 0.30 \pm 0.05 \text{ mM}$). K_m values for rat NTPDase1, NTPDase2, and NTPDase3 were reported as 0.076, 0.203, and 0.311 mM ATP, respectively [32]. Microsomal apparent K_m is compatible with simultaneous activation

F) ATPase activity values resulted from exponential adjustments of the curves in Fig. 2b ($\pm$ SE)

of several NTPDases, especially NTPDase2 and NTPDase3 which have same order of K_m values.

NTPDase1, NTPDase2, and NTPDase3 have different sensitivity to the inhibitor suramin, an antagonist of P2 receptors, NTPDase1 being markedly more resistant [32]. Using the Cheng-Prusoff equation for a competitive inhibition, Iqbal et al. calculated the K_i values for NTPDase1, NTPDase2, and NTPDase3 being 0.300, 0.065, and 0.013 mM, respectively [32]. Here, we measured ATPase activity with 3 mM ATP in the presence of suramin concentrations spanning more than three orders of magnitude and observed that the rate of Pi production diminished along all the tested inhibitor range (Fig. 5). Data were fitted with a biphasic sigmoidal curve, and two IC_{50} (0.019 and 0.507 mM) were calculated. Using the apparent $K_m = 0.3 \text{ mM}$ deduced from Fig. 4, the K_i values estimated in our microsomes using Cheng-Prusoff equation were 0.018 and 0.046 mM, similar to those reported for

Fig. 3 Concanavalin-A reduced the time-dependent inhibition of nucleotidase activity. After 30 min of gland incubation in the absence (black circle) or in the presence of 100 μM isoproterenol (black square), incubation media were centrifuged for 15 min at 2,000×g and 60 min at 27,000×g; the pellets were suspended into fresh PS and preincubated in the presence of concanavalin-A. Pi production from 3 mM MgATP was measured at different incubation times. Data are mean $\pm$ SEM, $n=3$

Fig. 4 Effect of initial ATP concentration on microsomal nucleotidase activity. Pi versus time in microsomes with different ATP concentrations: 0.1 (white square), 0.3 (white circle), 0.6 (white triangle up), 1.5 (white triangle down), and 3.0 (white rectangle) mM. Curves were obtained by free fitting with exponential functions to obtain initial velocities. Inset: Hanes representation. Data are mean $\pm$ SEM, $n \geq 3$

Fig. 5 Effect of suramin on microsomal nucleotidase activity. Microsomes were preincubated for 5 min at 37 °C in standard medium with different suramin concentrations before addition of 3 mM ATP for 1 min reaction (mean±SEM, three independent experiments by duplicate). The curve is a “two-site competition function” fitted with Sigmaplot 11 software $y = \min + (\max - \min) \left[\frac{F_1}{1 + 10^{(x - \text{LOGEC}_{50})}} + \frac{1 - F_1}{1 + 10^{(x - \text{LOGEC}_{50})_2}} \right]$ min=8.0, max=102.7, EC₅₀₁=1.9e-5 M, EC₅₀₂=5.1e-4 M, F_1 (fraction)=0.5; $R^2=0.98$

NTPDase2 and NTPDase3 and markedly lower than the one reported for NTPDase1.

Sodium azide (NaN₃) is known to inhibit NTPDase1, NTPDase3, and NTPDase8 to variable extents but not NTPDase2 [3]. Figure 6a shows Pi production by microsomes incubated with 3 mM ATP in the absence or presence of 10 mM NaN₃. From the relation “[Pi]/initial [ATP]” at the end of the experiment, we estimated ATP hydrolysis that was always <10 %. Calculation from data in Fig. 6a, b shows exponential fitting of dPi/dt versus time, to obtain the initial activities (A_i), final activities (A_f), and time to reach 50 % of the fall ($T_{1/2}$). As proposed above, two functional components can be identified in the absence of Con-A, (a) an unstable initial ATPase activity ($[A_i - A_f]$), progressively reduced during the hydrolysis reaction and not affected by NaN₃, representing approximately 60 % of the total activity and consistent with NTPDase2 and (2) a stable ATPase activity (A_f) inhibited ~50 % by N₃Na. $T_{1/2}$ was around 1 min in both conditions, indicating that the inhibitor had no significant effect on inactivation mechanism. A similar analysis was made in plasma membrane vesicles from rat myometrium, where the authors reported the simultaneous activation by ATP of a labile enzyme and of a stable enzyme inhibited by NaN₃ [33]. Figure 6 also shows that ATPase activity remained constant when Con-A was added to incubation medium before ATP, indicating that unstable activity can be attributed to a glycosylated protein, presumably NTPDase2 as discussed above.

Electron microscopy

NTPDase activity released to the incubation media presented the same kinetic characteristics to that of microsomal NTPDase activity. These results strongly support the

Fig. 6 Effect of incubation time, crosslinking, and sodium azide addition on microsomal nucleotidase activity. **a** Accumulated Pi as a function of time when microsomes ([protein]=0.03–0.05 mg/ml) were incubated with 3 mM ATP, in the absence (black square) ($6 \leq n \leq 14$) or presence ($n=3$) of 10 mM N₃Na (triangles) or 50 μg/ml Con-A (white circle). Data are mean±SEM. **b** Pi production rate calculated by differentiation of data in **a**. Data fitted with exponential functions gave $k=0.65 \pm 0.15$ (black square) and $0.43 \pm 0.06 \text{ min}^{-1}$ (white triangle). Curves were compared using GraphPad Prism software. Both k constants did not present significant differences ($p < 0.1$)

hypothesis that, as was described for the pancreas [19], NTPDases could be released into microvesicles during salivary secretion. Using electron microscopy approach, we analyzed pellets obtained from incubation media without or with isoproterenol after centrifugation at 27,000×g (Fig. 7a). We found spherical vesicles <150 nm in diameter in both pellets, although we dare not differentiate them in number or quality. Interestingly, similar vesicles were found in the P27 of the homogenized gland (microsomes) obtained with the same centrifugation speed (Fig. 7b).

Western blot in isolated membranes from homogenized glands

We performed specific Western blot analysis to identify the enzymes responsible of released nucleotidase activity. We analyzed the microsomal fraction obtained from the 27,000×g pellet (P27) where rough endoplasmic reticulum

Fig. 7 Electron microscopy images. **a** Pellet obtained from gland incubation medium after isoproterenol treatment (P₂₇₀₀₀). **b** Microsomal fraction (P27) where arrows indicate nanovesicles. Bars represent 200 nm

membranes (RER) predominate and from the PM-enriched fraction resulting from centrifugation at 100,000×*g* of the 27,000×*g* supernatant (P100).

In our previous work [21], we used an antibody directed to a conserved region of plasma membrane NTPDases to reveal their presence in microsomes. Additionally, such NTPDases were found in cytosol and PM of acinar and ductal cells with stronger reactivity in the luminal pole. Here, we tested specific antibodies against NTPDase1, NTPDase2, NTPDase3, and NTPDase8 and also ecto-5'-nucleotidase, in P27 and P100. Figure 8 shows the presence of NTPDase1, NTPDase2, and NTPDase3 and also ecto-5'-nucleotidase in both fractions, as well as the absence of NTPDase8. The semiquantitative method used herein did not allow detecting significant differences between the amount of NTPDase2 and NTPDase3 present in the microsomal-enriched fraction (P27) and the plasma membrane-enriched fraction

(P100). Three NTPDase isoforms as well as ecto-5'-nucleotidase were present in the P27 fraction in a glycosylated form, as demonstrated by the effect of PNGase deglycosylase (P27dg, Fig. 8). However, only NTPDase 1 isoform was expressed in a complete deglycosylated form in the microsomal fraction (P27) as shown by high amounts of 55-kDa deglycosylated protein in this fraction. Both NTPDase2 and NTPDase3 showed similar pattern for microsomal (P27) and membrane-enriched (P100) fractions. They showed a majority band corresponding to whole glycosylated form higher than 70 kDa, accordingly with expected size. However, a lower and thin band could also be observed, corresponding maybe to a partially glycosylated or truncated form. Supplementary Fig. 1 shows control experiments performed replacing polyclonal antibodies by the corresponding pre-immunize serums, at the same work dilution. There were no unspecific signals using pre-immunized serums.

Fig. 8 Western blots of NTPDase isoforms in rat SMG microsomal and plasma membrane-enriched fractions. Samples (35 µg protein) from rat SMG microsomal fraction (P27) or plasma membrane-enriched fraction (P100) were immunoblotted using different nucleotidase polyclonal antibodies (dilution 1/4,000; 1/1,000 or 1/500) as indicated. Deglycosylated SMG microsomal fractions (P27dg) were obtained using PNGase F enzyme as indicated by the manufacturer

Discussion

As ATPases were identified in ovarian, prostate, and pancreas secretion fluids, the authors have proposed that these enzymes could be secreted to regulate luminal nucleotide concentrations [19, 20, 34–36]. Enzyme release would be associated to microvesicles, although the mechanisms are unclear of how the vesicles are leaving the cell and how the correct orientation of the enzymes is achieved.

Here, we show that incubation of SMG in a physiological solution at 37 °C produced the release of membrane-associated NTPDase and 5'-nucleotidase-like activities. ATP hydrolysis has the same kinetic characteristics as observed in microsomes. Electron microscope images show that released membranes are exosome-like vesicles with a diameter <150 nm. Similar vesicles were found among microsomes, where rough ER predominates. The release of membrane-associated functional nucleotidases was stimulated by the adrenergic secretagogues isoprenaline and epinephrine. To our knowledge, this is the first report of NTPDase and 5'-nucleotidase release by salivary cells and autonomic regulation of such liberation.

We performed a kinetic analysis of NTPDase activity in the microsomal fraction. First, we compared ATP and suramin dependence of ATPase activity with data obtained for recombinant rat NTPDases [32]. Our results gave an apparent K_m value (0.30 mM) that falls between those reported for NTPDases 2 and 3, which are markedly higher than the value from NTPDase1. This result is compatible with simultaneous activation of NTPDase2 and NTPDase3, with similar affinity for ATP. The sensitivity to suramin is also consistent with the inhibition of NTPDase2 and NTPDase3, being NTPDase1 much more resistant and probably representing $\leq 10\%$ of total microsomal ATPase activity. We also analyzed the effects of time and NaN_3 on ATPase activity in SMG microsomes and observed again that at least two different NTPDases contributed to ATP and ADP hydrolysis. An unstable and azide-resistant activity can be assigned to NTPDase2, like the human ecto-ATPase [31], and the stable and azide-sensitive activity could be attributed mainly to NTPDase3, both co-expressed with ecto-5'-nucleotidase in mouse submandibular acini [14]. Western blots with specific antibodies indicate that NTPDase1, NTPDase2, and NTPDase3 and ecto-5'-nucleotidase are all present in the microsomal fraction (P27) and in the PM-enriched post-microsomal fraction (P100). Kinetic analysis confirmed the presence of active forms of NTPDase2 and NTPDase3 as well as ecto-5'-nucleotidase. NTPDase1 could be responsible of a minor fraction of microsomal ATPase activity, what is consistent with previous results showing that this enzyme is not functional when localized in intracellular membranes [18].

The inactivation mechanism seen for some NTPDases is still unknown. The lectin Con-A, which is known to crosslink

glycoproteins, reduces initial activity but prevents substrate inactivation in human NTPDase2 [31] and enhances human NTPDase3 activity [37], although the latter displays a linear time course of ATP hydrolysis in the absence of Con-A. We observed that preincubation with Con-A prevented inactivation when both submandibular fragments and microsomes were incubated with 3 mM ATP, consistent with previous and present results showing the expression of glycosylated NTPDase2 in rat SMG microsomes (Fig. 8) [21].

Microsomes consist mainly of RER vesicles. In the living cell, ecto-enzymes inserted into RER membranes should have their active sites into the reticulum to have the correct orientation when they arrive at the PM. The rupture of the RER membranes during tissue homogenization generates the formation of spherical vesicles surrounded by ribosomes, although some of them could be reversed [38] allowing measurement of the activities studied in this work. However, Fig. 7b shows that among microsomes pelleted at $27,000\times g$, there are small vesicles without ribosomes which may correspond to the vesicles originally contained within multivesicular bodies (MVBs) and called exosomes when released into the extracellular medium. Endosomes formed by PM invagination would generate small vesicles by new invaginations, giving rise to the MVBs. So, internalized ecto-enzymes would be oriented with their active site to the extracellular medium in the released vesicles when the MVBs merge with the PM [39].

In submandibular glands, there are P2Y_1 , P2Y_2 , P2X_4 , and P2X_7 , both on acinar and ductal cells. Intraductal injection of ATP or UTP decreased the salivary Cl^- concentration significantly in a dose-dependent manner, a process associated with regulation of CFTR channels. However, an ATP concentration high enough to have the physiological effect could not be detected in saliva, suggesting that its effect could be prevented by a high ecto-ATPase activity in the ducts [40]. P2X_7 receptors would be the main responsible for the ATP response in this tissue. Purinergic receptor agonist-stimulated salivation was suppressed by more than 70 % in submandibular glands from P2X_7 null mice [10]. At least one EC_{50} is $>100\text{ }\mu\text{M}$ ATP for this receptor [8, 41], which is consistent with the high ATP K_m we measured for microsomal NTPDases.

Intracellular NTPDases could be a stock of PM enzymes, and there are some previous reports about intracellular localization of cell surface NTPDase isoforms. In this way, it was suggested that P2X receptor activation stimulates insertion of intracellular latent NTPDase2 into the plasma membrane, in a way to sustain hydrolysis capacity given enzyme inactivation [16]. Ivanenkov et al. reported that human NTPDase3 becomes catalytically active in the ER or in the ER–Golgi intermediate compartment [15]. Additionally, it was shown that alternative splicing of the rat NTPDase2 gene produces an NTPDase2b isoform, which has combined cell surface and intracellular localization [17]. NTPDase1 could be present but

inactive in our intracellular membranes as was proposed by Zhong et al. [18] who reported that the enzyme is not active until it is fully glycosylated in PM.

Present results demonstrate that adrenergic stimulation of rat submandibular gland increased the release of nucleotidase activities. The kinetic profile indicates that NTPDase2 is the main secreted activity, followed by NTPDase3 and ecto-5'-nucleotidase. Electron microscopy images show nanovesicles compatible with exosomes that would be the vehicle for released enzymes. NTPDases released to the ducts could contribute to regulate luminal ATP, ADP, and adenosine concentrations that activate purinergic receptors.

Acknowledgments This work was supported by the Universidad de Buenos Aires (grant 20020100100364). Miss Noelia Balcarcel is a fellow of the Consejo Interuniversitario Nacional, Argentine. We are grateful to Lic. Lidia Margarita López, from the LANAIS-MIE (UBA-CONICET), for images of secreted nanovesicles.

References

- Kukulski F, Levesque SA, Seigny J (2011) Impact of ectoenzymes on p2 and p1 receptor signaling. *Adv Pharmacol* 61:263–299, PubMed PMID: 21586362
- Zimmermann H, Zebisch M, Strater N (2012) Cellular function and molecular structure of ecto-nucleotidases. *Purinergic Signal* 8(3): 437–502
- Knowles AF (2011) The GDA1_CD39 superfamily: NTPDases with diverse functions. *Purinergic Signal* 7(1):21–45, 126
- Robson SC, Seigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signal* 2(2):409–430, Epub 2008/04/12. eng
- Strater N (2006) Ecto-5'-nucleotidase: structure function relationships. *Purinergic Signal* 2(2):343–350
- Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* 240:31–304, Epub 2004/11/19. eng
- Lee MG, Zeng W, Muallem S (1997) Characterization and localization of P2 receptors in rat submandibular gland acinar and duct cells. *J Biol Chem* 272(52):32951–32955, Epub 1998/01/31. eng
- Turner JT, Landon LA, Gibbons SJ, Talamo BR (1999) Salivary gland P2 nucleotide receptors. *Crit Rev Oral Biol Med* 10(2):210–224, Epub 2000/04/12. eng
- Fontanils U, Seil M, Pochet S, El Ouaili M, Garcia-Marcos M, Dehay JP et al (2010) Stimulation by P2X(7) receptors of calcium-dependent production of reactive oxygen species (ROS) in rat submandibular glands. *Biochim Biophys Acta* 1800(11):1183–1191
- Nakamoto T, Brown DA, Catalan MA, Gonzalez-Begne M, Romanenko VG, Melvin JE (2009) Purinergic P2X7 receptors mediate ATP-induced saliva secretion by the mouse submandibular gland. *J Biol Chem* 284(8):4815–4822, Epub 2008/12/23. eng
- Woods LT, Camden JM, Batek JM, Petris MJ, Erb L, Weisman GA (2012) P2X7 receptor activation induces inflammatory responses in salivary gland epithelium. *American Journal of Physiol Cell Physiol* 303(7):C790–C801
- Henz SL, Ribeiro CG, Rosa A, Chiarelli RA, Casali EA, Sarkis JJ (2006) Kinetic characterization of ATP diphosphohydrolase and 5'-nucleotidase activities in cells cultured from submandibular salivary glands of rats. *Cell Biol Int* 30(3):214–220, Epub 2006/02/17. eng
- Kittel A, Pelletier J, Bigonnesse F, Guckelberger O, Kordas K, Braun N et al (2004) Localization of nucleoside triphosphate diphosphohydrolase-1 (NTPDase1) and NTPDase2 in pancreas and salivary gland. *J Histochem Cytochem* 52(7):861–871, Epub 2004/06/23. eng
- Lavoie EG, Gulbransen BD, Martin-Satue M, Aliagas E, Sharkey KA, Seigny J (2011) Ectonucleotidases in the digestive system: focus on NTPDase3 localization. *Am J Physiol Gastrointest Liver Physiol* 300(4):G608–G620, Epub 2011/01/15. eng
- Ivanenkov VV, Seigny J, Kirley TL (2008) Trafficking and intracellular ATPase activity of human ecto-nucleotidase NTPDase3 and the effect of ER-targeted NTPDase3 on protein folding. *Biochemistry* 47(35):9184–9197, Epub 2008/08/13. eng
- Vlajkovic SM, Wang CJ, Soeller C, Zimmermann H, Thorne PR, Housley GD (2007) Activation-dependent trafficking of NTPDase2 in Chinese hamster ovary cells. *Int J Biochem Cell Biol* 39(4):810–817, Epub 2007/02/20. eng
- Wang CJ, Vlajkovic SM, Housley GD, Braun N, Zimmermann H, Robson SC et al (2005) C-terminal splicing of NTPDase2 provides distinctive catalytic properties, cellular distribution and enzyme regulation. *Biochem J* 385(Pt 3):729–736, Epub 2004/09/15. eng
- Zhong X, Malhotra R, Woodruff R, Guidotti G (2001) Mammalian plasma membrane ecto-nucleoside triphosphate diphosphohydrolase 1, CD39, is not active intracellularly. The N-glycosylation state of CD39 correlates with surface activity and localization. *J Biol Chem* 276(44):41518–41525, Epub 2001/09/08. eng
- Sorensen CE, Amstrup J, Rasmussen HN, Ankorina-Stark I, Novak I (2003) Rat pancreas secretes particulate ecto-nucleotidase CD39. *J Physiol* 551(Pt 3):881–892, Epub 2003/07/02. eng
- Yegutkin GG, Samburski SS, Jalkanen S, Novak I (2006) ATP-consuming and ATP-generating enzymes secreted by pancreas. *J Biol Chem* 281(40):29441–29447, Epub 2006/08/04. eng
- Ostuni MA, Egido P, Peranzi G, Alonso GL, Lacapere JJ, Gonzalez DA (2009) Characterization of a functional NTPDase in the endoplasmic reticulum of rat submandibular salivary gland. *Physiol Res* 58(6):843–854, Epub 2008/12/20. eng
- Fausther M, Lecka J, Kukulski F, Levesque SA, Pelletier J, Zimmermann H et al (2007) Cloning, purification, and identification of the liver canalicular ecto-ATPase as NTPDase8. *Am J Physiol Gastrointest Liver Physiol* 292(3):G785–G795
- Fausther M, Lecka J, Soliman E, Kauffenstein G, Pelletier J, Sheung N et al (2012) Coexpression of ecto-5'-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver. *American J Physiol Gastrointest Liver Physiol* 302(4):G447–G459
- Seigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I et al (2002) Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. *Blood* 99(8):2801–2809
- Vekaria RM, Shirley DG, Seigny J, Unwin RJ (2006) Immunolocalization of ectonucleotidases along the rat nephron. *American J Physiol Renal Physiol* 290(2):F550–F560
- Baginski ES, Foa PP, Zak B (1967) Microdetermination of inorganic phosphate, phospholipids, and total phosphate in biologic materials. *Clin Chem* 13(4):326–332, Epub 1967/04/01. eng
- Beeler TJ, Wang T, Gable K, Lee S (1985) Comparison of the rat microsomal Mg-ATPase of various tissues. *Arch Biochem Biophys* 243(2):644–654, Epub 1985/12/01. eng
- Martin-Romero FJ, Garcia-Martin E, Gutierrez-Merino C (1996) Inactivation of ecto-ATPase activity of rat brain synaptosomes. *Biochim Biophys Acta* 1283(1):51–59, Epub 1996/08/14. eng
- Saborido A, Delgado J, Megias A (1999) Measurement of sarcoplasmic reticulum Ca²⁺-ATPase activity and E-type Mg²⁺-ATPase activity in rat heart homogenates. *Anal Biochem* 268(1):79–88, Epub 1999/02/26. eng

30. Zhao D, Dhalla NS (1988) Characterization of rat heart plasma membrane $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase. *Arch Biochem Biophys* 263(2): 281–292, Epub 1988/06/01. eng
31. Knowles AF, Chiang WC (2003) Enzymatic and transcriptional regulation of human ecto-ATPase/E-NTPDase 2. *Arch Biochem Biophys* 418(2):217–227, Epub 2003/10/03. eng
32. Iqbal J, Vollmayer P, Braun N, Zimmermann H, Muller CE (2005) A capillary electrophoresis method for the characterization of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and the analysis of inhibitors by in-capillary enzymatic microreaction. *Purinergic Signal* 1(4):349–358, Epub 2008/04/12. eng
33. Magocsi M, Penniston JT (1991) Ca^{2+} or Mg^{2+} nucleotide phosphohydrolases in myometrium: two ecto-enzymes. *Biochim Biophys Acta* 1070(1):163–172
34. Beaudoin AR, Vachereau A, Grondin G, St-Jean P, Rosenberg MD, Strobel R (1986) Microvesicular secretion, a mode of cell secretion associated with the presence of an ATP-diphosphohydrolase. *FEBS Lett* 203(1):1–2, Epub 1986/07/14. eng
35. Ronquist G, Brody I (1985) The prostasome: its secretion and function in man. *Biochim Biophys Acta* 822(2):203–218, Epub 1985/09/09. eng
36. Rosenberg MD, Gusovsky T, Cutler B, Berliner AF, Anderson B (1977) Purification and characterization of an extracellular ATPase from oviductal secretions. *Biochim Biophys Acta* 482(1):197–212, Epub 1977/05/12. eng
37. Murphy DM, Kirley TL (2003) Asparagine 81, an invariant glycosylation site near apyrase conserved region 1, is essential for full enzymatic activity of ecto-nucleoside triphosphate diphosphohydrolase 3. *Arch Biochem Biophys* 413(1):107–115, Epub 2003/04/23. eng
38. Haaparanta T, Gustafsson JA, Glaumann H (1983) Isolation of mitochondria, lysosomes, and microsomes from the rat ventral prostate with a note on inverted microsomal vesicles. *Arch Biochem Biophys* 223(2):458–467
39. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B et al (2011) Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell and Mol Life Sci : CMLS* 68(16):2667–2688
40. Ishibashi K, Okamura K, Yamazaki J (2008) Involvement of apical P2Y2 receptor-regulated CFTR activity in muscarinic stimulation of Cl^{-} reabsorption in rat submandibular gland. *Am J Physiol Regulat Integr Comp Physiol* 294(5):R1729–R1736
41. Klapperstück M, Büttner C, Schmalzing G, Markwardt F (2001) Functional evidence of distinct ATP activation sites at the human P2X(7) receptor. *J Physiol* 534(Pt 1):25–35