Characterization of a Functional NTPDase in the Endoplasmic Reticulum of Rat Submandibular Salivary Gland

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Summary
Nucleotidase activity and Ca-uptake were characterized in endoplasmic reticulum (ER) enriched rat submandibular gland (SMG) microsomal preparations. (i) Ca-uptake had characteristics of an ER Ca-ATPase. (ii) Nucleotidase activity was equally stimulated by calcium, magnesium and manganese, but with different Km values. (iii) Specific inhibitors of P-type Ca-ATPases were ineffective on nucleotidase activity, demonstrating that this activity was not related to calcium uptake and did not correspond to classical Ca2+ pumps. (iv) ATP and UTP were more efficient substrates, whereas ADP and UDP were hydrolyzed at significantly slower rate. (v) Nucleotidase activity was sensitive to mild detergent solubilization and insensitive to ionophore addition. (vi) Nucleotidase activity was strongly inhibited by suramin, a nucleoside triphosphate diphosphohydrolase (NTPDase) inhibitor. (vii) Nucleotidase activity exponentially diminished as function of time. All these observations are consistent with a NTPDase identity. The presence of a NTPDase was demonstrated by immunohistochemistry in rat SMG. Immunoreactivity was stronger in ductal cells than in mucous and serous acini. Although this enzyme was observed in the plasma membrane, colocalization with the ER marker calnexin revealed a specific subcellular localization in this organelle of all three types of cell. The putative function of this NTPDase activity in salivary glands is discussed.

Key words
Apyrase • CD39 • Ca-uptake • ATPase activity • Submandibular gland

Introduction
Submandibular glands (SMG) belong to the major salivary gland group, with important functions involved in the oral health and the digestion process. They are widely used as a model to study the secretory mechanisms in the epithelial cells. Regulation of salivary secretion is complex and secretory agents usually act by cAMP and Ca2+ pathways, which could regulate the same process with opposite or synergic results.

The Ca2+ pathway implies the fine-tuning regulation of cytosolic calcium levels. Ca2+ influx from the extracellular liquid and Ca2+ release from intracellular calcium stores are strongly related with the cation extrusion and the stores refilling. Several pumps and calcium channels have been identified in submandibular glands. Muallem and coworkers have identified in the rat submandibular gland different calcium ATPases as the plasma membrane Ca-ATPase (PMCA) and the sarco-endoplasmic reticulum Ca-ATPase (SERCA) (Lee et al. 1997a), and calcium channels like the IP3-sensitive and the ryanodyne-sensitive (Lee et al. 1997b).
The functional characterization of the SERCA was usually addressed by measuring the enzymatic activity and the ionic transport in submandibular endoplasmic reticulum microsomes (Alonso et al. 1971, Hurley and Martinez 1985, 1986, Hurley and Ryan 1988), but a clear correlation between Ca^{2+} uptake and ATPase activity was never well established. At present the question remains what is the actual identity of the enzymes that hydrolyze ATP in those microsomes.

Another class of ATP hydrolyzing enzymes is the family of ecto-nucleoside triphosphate diphosphohydrolases (NTDPases) that hydrolyze ATP and other nucleotides with largely higher hydrolysis rate than the Ca-ATPase pumps, but without coupled cation transport (for detailed review see Plesner 1995, Robson et al. 2006). At present, eight members of this family were identified, which are located to the plasma membrane and/or to intracellular membranes (Robson et al. 2006).

NTDPases are ubiquitous in eukaryotic cells but their functions are not fully understood. Cumulate evidence supports the involvement of these enzymes in the regulation of nucleotide concentration and adenosine recycling process. Furthermore, their spatial and temporal expression in several cell types could be at the origin of different pathophysiological processes (reviewed in Robson et al. 2006).

Several articles characterizing an NTPDase like activity in the rat parotid gland were published (Dowd et al. 1989, 1993, 1996, 1999, Teo et al. 1993, Murphy et al. 1994), but there are only a few papers concerning this kind of activity in rat submandibular gland (Valenzuela et al. 1989, Murphy et al. 1994).

In this work we characterize an ATPase activity of endoplasmic reticulum microsomes from rat submandibular gland, which has the characteristics of an ecto-nucleotidase activity and that it is not related to calcium uptake. We also identify the cellular and subcellular localization of a NTPDase immunoreactivity in this gland.

**Methods**

**Animals**

Male Wistar rats weighing 250-280 g were fed with standard chow diet and water ad libitum, and kept with 12 h light / 12 h dark period. They were treated in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS 123).

**Microsomes preparation**

Microsomes preparation protocol was modified from that previously described by Hurley and Martinez (1986). Briefly, rats were allowed free access to standard chow pellets and water except for the night before sacrifice when only food was withheld. For each tissue preparation 2-4 rats were deeply anaesthetized with ether and exsanguinated via the abdominal aorta. The submandibular glands were removed, separated from the adjoining sublingual glands, weighed, minced on ice and homogenized in cold buffer (10 mM HEPES, pH 7.0, 0.3 M sucrose and 1 mM of the protease inhibitor PMSF) using a teflon and glass homogenizer. The homogenate was centrifuged for 2 min at 1000 x g to remove nuclei and cell debris. Supernatant was centrifuged for 20 min at 10,000 x g, the pellet containing mitochondrial fraction was discarded and resulting supernatant was centrifuged for 30 min at 27,000 x g. The final pellet was resuspended in the same buffer and proteins were quantified using the dye-binding BioRad assay.

**Electron microscopy**

Microsome preparation was fixed in 2.5 % glutaraldehyde during 60 min at room temperature and postfixed 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.

**Electrophoresis and Western blotting**

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Proteins (15 µg per lane) were separated on 10 % polyacrylamide gel under non-reducing conditions. They were transferred to 0.45 µm pore nitrocellulose membranes (Sartorius AG, Göttingen, Germany) and probed with monoclonal anti-hNTPDase1 antibody (BU61 clone, Ancell, Bayport, MN, USA) (1:750 dilution); or with a rabbit anti-calnexin monoclonal antibody (Becton Dickinson, Le Pont de Claix, France). (1:4000 dilution). Bands were visualized using peroxidase-conjugated anti-mouse or anti-rabbit IgG respectively (Sigma, Saint-Quentin Fallavier, France) and the Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA).

**Deglycosylation**

Microsome preparations were subjected to deglycosylation using PNGase F (New England Biolab,
Ipswich, MA, USA) following manufacturer instructions.

**ATPase activity**

The microsomal ATPase activity was measured at pH 7.2 and 37 °C following Pi liberation. The media contained 50 mM MOPS-Tris, pH 7.2, 100 mM KCl, 3 mM ATP and different amounts of CaCl₂ or MgCl₂, and EGTA to obtain the desired cation concentration. Reactions were started by addition of 0.1 mg vesicular protein per ml. After 2 min incubations the reactions were stopped by addition of cold trichloroacetic acid at 5% final concentration. Pi production was determined by a colorimetric method (Baginski et al. 1967). Free cation concentrations were calculated as previously described (Fabiato and Fabiato 1979).

\[ ^{45}\text{Ca}] \text{Ca-uptake} \]

Ca-uptake was measured in 1 ml of medium of the following composition: 100 mM KCl, 50 mM MOPS/Tris, pH 7.2, 3 mM ATP, 3 mM MgCl₂, 0.1 mM CaCl₂, also containing 1500 ± 500 cpm/nmol of \[^{45}\text{Ca} \] (New England Nuclear, Boston, MA, USA), and 0.1 mg of membrane protein. The reaction mixture lacking membrane proteins was preincubated for 5 min at 37 °C. Ca-uptake was started by addition of membrane proteins and aliquots of the reaction mixture were passed through Millipore filters (0.45 µm pore diameter) at the given time periods. The filters were immediately washed with 5 ml of 3 mM LaCl₃ in order to diminish the unspecific binding. Radioactivity was determined by liquid scintillation counting in a toluene-based scintillation cocktail.

**Immunohistochemistry**

Submandibular glands from four rats were removed and cleaned from connective tissue. They were immediately fixed in 4% paraformaldehyde during 4 h at room temperature and embedded in paraffin. Paraffin sections were dehydrated and endogenous peroxidase activity was inactivated by the peroxidase-antiperoxidase (PAP) technique. Sections were incubated with a mouse anti-human NTPDase1 (hNTPDase1) monoclonal antibody (BU61 clone, 1:100 dilution, Ancell, Bayport, MN, USA) overnight at 4 °C. Peroxidase complexed secondary antibody was purchased from Dakopatts Ltd. (Trappes, France). Sections were counterstained with Mayer’s hematoxylin (E.M.S.) (Euromedex, Souffelweyersheim, France).

**Colocalization studies**

Paraffin sections for confocal microscopy were dehydrated and incubated with a rabbit anti-calnexin monoclonal antibody (1:100) for 1 h (Becton Dickinson, Le Pont de Claix, France). After washing, sections were incubated with a secondary anti-rabbit immunoglobulin antibody coupled with ALEXA Fluor 488 (1:200) (Molecular Probes, OR, USA) and then incubated overnight at 4 °C with the anti-hNTPDase1 antibody (1:100). Following the second washing, secondary anti-mouse immunoglobulins antibody coupled with ALEXA Fluor 546 (Molecular Probes) was applied for 60 min (1:150 dilution). After several washes, slides were covered with a hydrophilic and antifading mounting media (Fluoromount G, E.M.S.). Final observations were made with a Zeiss Axiovert 200M confocal microscope with a 63x oil immersion objective. LSM 510 image software (Heidelberg, Germany) was used to select observation fields (identical intensities profile of each fluorochrome), to collect the results (histograms, fluorograms, colocalizations of both dyes), to derive data recording and to perform the analysis. Step size for the sectioning was 0.4 µm for the deconvolved images with a pinhole value fixed at 1 µm. Similar incubations without the primary antibody were also performed as negative controls.

**Reagents and solutions**

C₁₂E₈ was purchased from Calbiochem (La Jolla, CA, USA). Unless otherwise noted, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Statistics**

Data are presented as the mean ± S.E.M. Experimental data were evaluated using one-way ANOVA. Scheffe’s homogeneity test was performed in order to determine differences between means of each experimental group and control counterpart. Differences were considered significant for p<0.05.

**Results**

**Electron microscopy and Western blot**

Figure 1a shows typical image of section from microsomal preparation embedded in resin. Vesicles surrounded by large dots correspond to ribosomes with typical characteristics of rough ER microsomes. Dotted surfaces were also observed and corresponded to surfaces
of vesicles that have not been sectioned. It has to be mentioned that neither mitochondria nor mitochondrial fragments were observed in these microsomal preparations.

The ER origin of these microsomes was confirmed by Western blot with the presence of a characteristic band of 88 kDa that corresponds to the ER specific protein calnexin (Fig. 1b).

[$^{45}$Ca]Ca uptake

Figures 1c and 1d show time-dependent Ca uptake by SMG microsomes. Figure 1c demonstrates ATP dependence of this uptake, resulting in an increase of calcium inside the vesicles, observed only in the presence of ATP. A fast release of Ca$^{2+}$ was triggered by addition of Ca$^{2+}$ ionophore A23187.

The effect of two SERCA inhibitors on Ca-uptake are shown in the Figure 1d. Thapsigargin and sodium orthovanadate diminished the Ca$^{2+}$ uptake to SMG microsomes by about 90%. The mitochondrial Ca-uniporter inhibitor ruthenium red and the PMCA inhibitor compound 48/80 had no effect on SMG microsomes Ca$^{2+}$ uptake (data not shown). These are the first results about the action of ATPase inhibitors on the rat submandibular Ca$^{2+}$ uptake. They confirm the microsomal origin of the ER membranes while minimizing the possibility of mitochondrial or plasma membrane contamination.

ATPase activity

To functionally characterize the ATPase activity in our micromal preparation, we tested: i) the dependence on Ca$^{2+}$ and/or Mg$^{2+}$, (ii) the sensitivity to specific inhibitors of P-type, F-type, and V-type ATPases, (iii) the substrate preference pattern, (iv) the sensitivity to the action of detergents and (v) the dependence on incubation time.

Figure 2a shows ATPase activity of SMG microsomes as function of either free Mg$^{2+}$ (with no added calcium) or free Ca$^{2+}$ (without added magnesium). Maximal activities were similar for both conditions (around 1 µmol Pi produced/mg protein/min), but the Km values calculated from plots were different with values of 28±5 µM and 320±51 µM for Ca$^{2+}$ and Mg$^{2+}$, respectively. ATPase activity was also stimulated by Mn$^{2+}$ (open squares). Maximal activity was reached in the presence of millimolar concentrations of Ca$^{2+}$ or Mg$^{2+}$ irrespective of other cation concentrations (data not shown).

Fig. 1. ER origin and calcium uptake by rat submandibular microsomes (a) Electron micrograph of a thin section of the microsome preparation showed typical rough microsomes (arrows indicated the ribosomes bound to the vesicles, bar=200 nm). (b) Western blot for identification of Calnexin. Immunoreactivity of microsomes from rat submandibular endoplasmic reticulum with a rabbit anti-calnexin monoclonal antibody. (c) Calcium uptake was measured at 37 °C, pH 7.2, 50 µM $^{45}$Ca (CaCl$_2$), 100 mM KCl, 3 mM MgCl$_2$, with (triangles) or without (circles) 3 mM ATP. As indicated by arrow, 5 µM calcium ionophore A23187 was added. (d) Experiments were made with identical control condition (triangles) but in the presence of 1 mM sodium orthovanadate (squares) or 2 µM thapsigargin (circles). Each point is a result of at least 5 independent experiments. Results are expressed as mean ± S.E.M.
Table 1. Effects of inhibitors on ATP hydrolysis by rat submandibular microsomes.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ATPase activity (% of control)</th>
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<tbody>
<tr>
<td>Thapsigargin (2 µM)</td>
<td>89.7 ± 12.6</td>
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<tr>
<td>Sodium orthovanadate (1 mM)</td>
<td>89.8 ± 7.8</td>
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<tr>
<td>Ouabain (0.1 mM)</td>
<td>95.5 ± 10.5</td>
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<tr>
<td>Compound 48/80 (1 mg/ml)</td>
<td>89.3 ± 9.3</td>
</tr>
<tr>
<td>Sodium azide (10 mM)</td>
<td>67.4 ± 11.5*</td>
</tr>
<tr>
<td>Suramin (3 mM)</td>
<td>19.7 ± 2.0**</td>
</tr>
<tr>
<td>Suramin (3 mM) + sodium orthovanadate (1mM)</td>
<td>13.4 ± 2.9**</td>
</tr>
<tr>
<td>C12E8 (detergent/protein = 3 w/w)</td>
<td>28.0 ± 2.0**</td>
</tr>
<tr>
<td>Ionophore (A23187 5 µM)</td>
<td>98.0 ± 4.5</td>
</tr>
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</table>

Control ATPase activity (100 %) was 1.24±0.09 µmol Pi/mg protein/min. Results are expressed as mean ± S.E.M. (n=5). Data were analyzed by one-way ANOVA. * p<0.05, ** p<0.01.

Table 1 shows the effect of different ATPase inhibitors. No significant inhibition of ATPase activity was obtained by the SERCA inhibitor thapsigargin, the P-type ATPases inhibitor sodium orthovanadate, the PMCA inhibitor compound 48/80, or the Na/K ATPase inhibitor ouabain. However, sodium azide, an unspecific NTPDase inhibitor (Knowles and Nagy 1999, Schetinger et al. 2001), slightly diminished ATPase activity (67.4 % of control), while suramin, a NTPDase inhibitor (Iqbal et al. 2005), produced a strong inhibition of this activity. Figure 2b shows the concentration-dependent inhibition profile from which IC50 could be calculated giving values of 55±10 µM and 585±160 µM for suramin and sodium azide, respectively. The simultaneous addition of suramin and sodium orthovanadate at saturating concentrations produced an additional 5 % inhibition, whereas addition of calcium ionophore A23187 did not change ATPase activity.

Hydrolysis of different tri- and dinucleotides are presented in Figure 2c. The SMG microsomes nucleotidase activity is principally a tri-nucleotidase activity. ATP and UTP are largely hydrolyzed compared to ADP or UDP. ATP/ADP and UTP/UDP hydrolysis ratio are 4.39 and 4.93, respectively. Figure 2d shows that ATPase activity decayed exponentially. Differential activity values were obtained from the derivative of the Pi production as function of time (not shown)

These results strongly suggest that this ATPase activity resulted from an NTPDase. Moreover, addition of detergent C12E8 significantly inhibited the ATPase activity of SMG microsomes (28 % of control, Table 1). If this ATPase activity was the product of a SERCA type enzyme, mild detergent solubilization would augment activity by abolishing inhibition by intravesicular Ca2+ concentration. Furthermore, it has been previously described that detergent had a strong inhibitory effect on NTPDase activity (Wang and Guidotti 1998).

Cation and substrate specificity, inhibitors effect, and detergent sensitivity indicate the presence of one or several NTPDases, probably belonging to the NTPDase1 related enzymes (NTPDases 1, 2, 3 and 8), which are usually located in the plasma membrane. However, some papers also reported NTPDase1 activity in subcellular compartments in eukaryotic cells (Zhong et al. 2001, D’Alessio et al. 2003), thus opening the question about the possible intracellular function of these enzymes.

Immunohistochemistry

Based on the highly conserved amino acid sequence of different NTPDases among species, we tested the specificity of a commercially available anti-human NTPDase1 (hNTPDase1) monoclonal antibody.

Western blot performed under non-reducing conditions PAGE revealed that this antibody was able to recognize a band of 78 kDa in human platelet lysate as well as in rat whole homogenate and microsomal fraction (Fig. 3a, lanes 1-3). It should be noted that 54 kDa bands are proportionally most important in SMG microsomal fraction compared to SMG whole homogenate, indicating that microsomal fraction presented a significant amount of non-glycosylated enzyme (Lemmens et al. 2000). After PNGase F digestion, microsomal fraction showed a reduced 78 kDa band and the presence of a new band of about 28 kDa (Fig. 3, lane 4).

Figures 3b-e show rat SMG immunoreactivity against anti-hNTPDase1 monoclonal antibody. As expected, immunoreactivity was strongly revealed in blood vessels, which are well known to express high levels of NTPDases. Cells of epithelial ducts (Figs 3b,c) and mucous acini (Figs 3b,d) are homogeneously labeled, whereas only 20 % of serous acini were anti hNTPDase1 positive (Figs 3b,e). Figures 3c-e reveal that immunoreactivity was located both in the plasma membrane and in the cytosol of rat SMG cells with stronger reactivity in the plasma membrane of luminal pole (Figs 1c-e, arrow heads). NTPDase immunoreactivity was principally located in the luminal pool of duct cells, whereas labeling in serous and mucous acini is distributed through the
cells. This difference might be accounted by the nuclear localization of duct cells near the luminal membrane, while acinar cells nuclei are distributed throughout the cytoplasm.

Distribution of NTPDase immunoreactivity presented herein was different from that described in the mouse SMG where mucous acini were weakly stained while duct cells were not reactive to anti CD39 polyclonal antibody at all (Kittel et al. 2004).

Immunofluorescence and colocalization

Present observations of NTPDase1-like immunoreactivity in the cytosol (Figs 3c-e) suggest localization in the endoplasmic reticulum. This hypothesis was tested by immunofluorescence colocalization experiments using two antibodies reacting to NTPDase1 and to calnexin (specific endoplasmic reticulum protein) (Bergeron et al. 1994). Figure 4 shows anti-calnexin immunoreactivity (a,d,g), anti NTPDase labeling (b,e,h) and colocalization images (c,f,i) from duct cells (a,b,c), mucous acini (d,e,f) and serous acini (g,h,i). The three cell types show positive anti-NTPDase immunofluorescence (red) and a strong reaction to anti-calnexin antibodies (green). Anti-calnexin immunoreactivity presents the typical perinuclear pattern of ER localization.

Colocalization analysis was made using the same fluorescence intensity for both signals and pixel-to-pixel superposition was only considered as a valid colocalization point. Figure 4 (c,f,i) shows that NTPDase1 immunoreactivity strongly colocalized with calnexin in duct cells (c), mucous acinar cells (f) and serous acinar cells (i).

Discussion

We studied active Ca$^{2+}$ transport and ATPase activity in rat submandibular gland using an endoplasmic reticulum enriched microsomes preparation (Hurley and Martinez 1986).

Ca$^{2+}$-uptake by the microsomes was completely abolished by P-type ATPases or SERCA inhibitors. These results give the first information about specific inhibitor actions on submandibular Ca$^{2+}$ uptake, and agree with the presence of a SERCA pump in submandibular gland (Lee et al. 1997a). However, both
rate and maximal amount of accumulated Ca$^{2+}$ were lower than those measured in the well-known sarcoplasmic reticulum (SR) from rabbit skeletal muscle (Gonzalez et al. 2003). Since SR contains only the SERCA1 isoform, this difference might result from the expression of SERCA isoforms SERCA3 and SERCA2 in the SMG (Lee et al. 1997a). It is also possible that this difference might be the result of a minor SERCA concentration in microsomes from SMG.

High sensitivity to thapsigargin discards the possibility of a secretory pathway calcium ATPase (SPCA) since the pumps involved in this process are thapsigargin insensitive (Van Baelen et al. 2004, Vanoeveren et al. 2005).

The mean of the work was to determine whether the ATPase activity measured in the microsomes is only a result of a SERCA activity. In skeletal muscle, addition of calcium ionophore A23187 to impermeable vesicles enriched in SERCA enhances the ATPase activity since it prevents calcium accumulation and consequent ATPase inhibition (Gonzalez et al. 2006). Same effect is usually found by addition of mild detergents, which produces total or partial enzyme solubilization. However, ATPase activity in our vesicles was not modified by ionophore addition, and it was strongly inhibited in the presence of C$_{12}$E$_{8}$. Furthermore, activity was significantly inhibited by sodium azide and by suramin, two drugs that are effective to inhibit NTPDases (Knowles and Nagy 1999, Schetinger et al. 2001, Failer et al. 2003, Iqbal et al. 2005). Simultaneous addition of saturating concentrations of suramin and sodium orthovanadate revealed additive effects, supporting the hypothesis of at least two different ATP-hydrolyzing enzymes in the microsomal preparation.

When characterizing cation dependence of ATPase activity we found that, although having more affinity for calcium, it could be also activated by magnesium or manganese when calcium was absent as described for NTPDases (Mihaylova-Todorova et al. 2002) and SPCA (Van Baelen et al. 2004), respectively. Km values for these cations were significantly slower than described Km values in SERCA (Gonzalez et al. 2006) or SPCA (Van Baelen et al. 2004, Vanoeveren et al. 2005), but similar to those reported for NTPDases (Mihaylova-Todorova et al. 2002). The manganese activation was previously described for different plasma membrane Mg-ATPase (Beeler et al. 1985, Zhao and Dhalla 1988) but in those reports activation rates were even lower for Mn compared to Mg. Conversely, results presented herein showed similar activation rates for both cations in accordance with recent data obtained with a Trypanosoma Brucei NTPDase (De Souza Leite et al. 2007).

Microsomes nucleotidase activity had a wide range of substrate specificity, presenting significantly higher hydrolysis rate for nucleotide tri phosphates (ATP and UTP) than for nucleotide diphosphates (ADP and UDP). Finally, ATPase activity decayed as a function of time, as was previously described for Ca-independent Mg-ATPases (Beeler et al. 1995), the Ca/Mg ATPase of rat heart plasma membrane (Zhao and Dhalla 1988) or, more specifically, for human NTPDase2 (Knowles and Chiang 2003).
All these results support the hypothesis that the endoplasmic reticulum microsomes from rat submandibular gland express at least two different enzymes responsible for calcium uptake and nucleotidase activity. The biochemical characteristics of nucleotidase activity agree with an ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) activity (Plesner 1995, Heine et al. 1999, Iqbal et al. 2005, Kukulski et al. 2005).

Eight members of E-NTPDase family have been characterized up to date in several tissues. They hydrolyze nucleoside triphosphates and diphosphates with wide affinity range for different substrates (Zimmermann 2001, Kukulski et al. 2005, Wu et al. 2005). NTPDase1 (CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3) and NTPDase8 are typical ecto-enzymes localized in the plasma membrane (Reviewed in Grinthal and Guidotti 2006), and they could be grouped as NTPDase1 related enzymes. NTPDase1 is the prototype member of this group and it has been described as a detergent-sensitive dimeric active form (Wang et al. 1998) that is usually located in the plasma membrane but that can be also expressed in different cytoplasmic compartments in yeast and mammals (Zhong et al. 2001, D’Alesio et al. 2001, Wang et al. 2005). The other four members of this enzyme family (NTPDase4-7) have a specific intracellular localization (Wang and Guidotti 1998, Mulero et al. 1999, Briederbick et al. 2000).

The presence of a NTPDase in three cellular types of the rat submandibular gland was described using an anti-hNTPDase1 antibody (Fig. 3b-e). This antibody was able to recognize two bands from rat submandibular gland homogenates or microsome preparation (Fig. 3a, lanes 2-3) and was demonstrated to cross react with human platelets (Fig. 3a, lane 1). Moreover, after deglycosylation, microsome preparation presented a third band corresponding to deglycosylated form (Fig. 3a, lane 4). Immunohistochemistry revealed a strong immunoreactivity at the plasma membrane of blood vessels as well as acinar and duct cells. However, there was also important cytosolic anti-NTPDase1 immunoreactivity in salivary cells. Since cytosolic NTPDase localization well correlated with the specific ER marker calnexin (Fig. 4), it could be speculated that NTPDase described herein is one of the four intracellular members of the NTPDase family (NTPDase4-7). The two splicing variants of NTPDase4 (LALP70) hydrolyze a variety of nucleoside tri- and diphosphates and show insignificant catalytic activity towards ATP or ADP as substrates (Wang and Guidotti 1998, Briederbick et al. 2000). ER- and Golgi-allocated NTPDase5 (CD39L4) and NTPDase6 (CD39L2) preferentially hydrolyze nucleoside diphosphate (Trombetta & Helenius 1999, Mulero et al. 1999, Braun et al. 2000). Finally, NTPDase7 (LALP1) has an intracellular membranes distribution and hydrolyzes a variety of nucleoside tri- and diphosphates with a higher preference for UTP than for ATP (Shi et al. 2001). The substrate specificity of nucleotidase activity measured in submandibular gland microsomes (Fig. 2c) excludes the hypothesis that main activity was the product of any of these intracellular isoforms. The other members of NTPDase family are usually found in the plasma membrane. However, Zhong et al. (2001) demonstrated that, when transfected with a CD39-like apyrase, Cos7 cells presented this enzyme in the endoplasmic reticulum. It should be taking into account that maturation process of several plasma membrane proteins occurs in the ER. These authors showed an incomplete glycosylation pattern and a reduced activity (Zhong et al. 2001). Present results support the hypothesis that this may be the case in ER

**Fig. 4.** CD39 and calnexin colocalization by confocal microscopy immunofluorescence. Calnexin immunostaining was revealed by an anti-calnexin Ab coupled to Alexa Fluor 488 (green a, d, g) and that of CD39 by an anti-CD39 monoclonal Ab coupled to Alexa 546 (red b, e, h). Cytoplasmic anti-calnexin immunoreactivity was found in duct cells (a), mucous acinar cells (d) and serous acinar cells (g). Duct (b), mucous (e) and serous (h) cells were anti CD39 immunostained. White points in figures c, f, and i indicate that both proteins colocalize at a narrow perinuclear area. L – lumen, N – nucleus, SG – secretory granules. Bar=5 µm.
enriched fractions from rat SMG. Indeed, (i) the NTPDase activity of SMG microsomal ER-enriched fraction was tenfold lower than recently reported NTPDase activity from rat SMG acinar cells (Henz et al. 2006) and (ii) we observed the presence of a 54 kDa band corresponding to a deglycosylated form of the enzyme in the ER, compared with the 78 kDa band (glycosylated protein) which is the most abundant form in the whole homogenate of SMG (Fig. 3a, lanes 2-4). Suramin inhibition pattern and substrate specificity could indicate the presence of an NTPDase2 or NTPDase3 (Iqbal et al. 2005, Kukulski et al. 2005), but biochemical characterization of NTPDase activity is not enough to distinguish among NTPDases 1, 2, 3 or 8.

The question about the putative function of this NTPDase in the ER of SMG remains open since, on one hand, it was proposed that high nucleotidase activity in the intracellular compartments could deplete ATP and potentially inhibit other ATP-dependent components and processes (Braakman et al. 1992), but on the other hand, the presence of an active intracellular NTPDase activity was associated with several secretory processes. It was found in secreted vesicles observed in the lumen of pancreatic acini (Beaudoin et al. 1986) as well as in the pancreatic juice (Yegutkin et al. 2006), the oviduct (Rosenberg et al. 1977, Knowles and Nagy 1999) and the prostatic fluid (Ronquist and Brody 1985). NTPDases could play an important role in vesicle trafficking and protein glycosylation (Trombetta and Helenius 1999, Beaudoin et al. 1986) by modifying substrate concentrations which regulate activities of enzymes involved in these processes. This hypothesis is supported by NTPDase distribution in SMG, where ductal and mucous acinar cells are directly involved in the secretory process of highly glycosylated mucins. Another non-exclusive hypothesis is that functional NTPDases from submandibular ER might play an active role in regulation of ATP concentration in duct saliva. It was recently demonstrated that ATP is secreted by pancreatic acinar cells and that ATP concentration is modified during its passage through the ductal system by the action of NTPDase-like enzymes secreted to ductal lumen (Yegutkin et al. 2006). Luminal ATP concentration is crucial to control calcium signal triggered by purinergic receptor (P2R). Indeed, it was demonstrated in both pancreatic and salivary acinar and duct cells that expression and sensitivity of P2R were heterogeneous (Lee et al. 1997c, Li et al. 2003, Yegutkin et al. 2006), with predominant expression of ATP/UTP-sensitive receptors compared to ADP/UDP-sensitive receptors in duct cells.

Our results demonstrated that both SERCA and NTPDase like enzymes are present in submandibular gland ER. The former is responsible to calcium uptake restoring cytosolic calcium to baseline levels before stimulation. The later could be involved in the control of ductal ATP concentration which, in turns, would modulate purinergic signaling cascade.

Conflict of Interest
There is no conflict of interest.

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