Ectonucleotidases in the digestive system: focus on NTPDase3 localization

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Lavoie EG, Gulbransen BD, Martín-Satúé M, Aliagas E, Sharkey KA, Sévigny J. Ectonucleotidases in the digestive system: focus on NTPDase3 localization. Am J Physiol Gastrointest Liver Physiol 300: G608–G620, 2011. First published January 13, 2011; doi:10.1152/ajpgi.00207.2010.— Extracellular nucleotides and adenosine are biologically active molecules that bind members of the P2 and P1 receptor families, respectively. In the digestive system, these receptors modulate various functions, including salivary, gastric, and intestinal epithelial secretion and enteric neurotransmission. The availability of P1 and P2 ligands is modulated by ectonucleotidases, enzymes that hydrolyze extracellular nucleotides into nucleosides. Nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5′-nucleotidase are the dominant ectonucleotidases at physiological pH. While there is some information about the localization of ecto-5′-nucleotidase and NTPDase1 and -2, the distribution of NTPDase3 in the digestive system is unknown. We examined the localization of these ectonucleotidases, with a focus on NTPDase3, in the gastrointestinal tract and salivary glands. NTPDase1, -2, and -3 are responsible for ecto-ATPase activity in these tissues. Semiquantitative RT-PCR, immunohistochemistry, and in situ enzyme activity revealed the presence of NTPDase3 in some epithelial cells in serous acini of salivary glands and mucous acini and duct cells of sublingual salivary glands, in cells from the stratified esophageal and forestomach epithelia, and in some enteroendocrine cells of the gastric antrum. Interestingly, NTPDase2 and ecto-5′-nucleotidase are coexpressed with NTPDase3 in salivary gland cells and stratified epithelia. In the colon, neurons express NTPDase3 and glial cells express NTPDase2. Ca2+ imaging experiments demonstrate that NTPDases regulate P2 receptor ligand availability in the enteric nervous system. In summary, the specific localization of NTPDase3 in the digestive system suggests functional roles of the enzyme, in association with NTPDase2 and ecto-5′-nucleotidase, in epithelial functions such as secretion and in enteric neurotransmission.

nucleoside triphosphate diphosphohydrolases; ecto-5′-nucleotidase; CD73; P2 receptors; enteric nervous system

EXTRACELLULAR NUCLEOTIDES and nucleosides are involved in a number of biological functions, such as neurotransmission, platelet aggregation, muscular contraction, and epithelial secretion (9). These functions are mediated by extracellular nucleotides that are activating members of the P2 receptor family (ionotropic P2X receptors and metabotropic P2Y receptors) and extracellular adenosine acting on P1 receptors (8). Membrane-bound enzymes known as ectonucleotidases hydrolyze extracellular nucleotides into nucleosides, thereby controlling the concentration of P2 and P1 agonists (54). Nucleoside triphosphate diphosphohydrolase (NTPDase)-1, -2, -3, and -8 are the major ectonucleotidases implicated in the hydrolysis of tri- and diphosphonucleotides at physiological pH (41). These plasma membrane-located NTPDases differ slightly in their catalytic properties. For instance, NTPDase1 hydrolyzes ATP as well as ADP, NTPDase2 acts mostly as an ATPase, and NTPDase3 and -8 display intermediate hydrolysis profiles (29). AMP, the final product of NTPDase action, is further hydrolyzed to adenosine by ecto-5′-nucleotidase.

In the digestive system, extracellular nucleotides and nucleosides regulate several functions, including salivary secretion (18, 25, 38), intestinal epithelial secretion (13, 40, 53), gastrointestinal motility (5), and enteric neurotransmission (7, 22). By controlling P2 and P1 receptor agonist levels, ectonucleotidases such as NTPDases and ecto-5′-nucleotidase act as regulators of these functions. Indeed, there is evidence that several cell types express functional ectonucleotidases in the digestive system. Macrophages of the intestinal submucosa (39), pancreatic acinar cells, and mucous acini and myoepithelial cells of the submandibular salivary glands (SMG) express NTPDase1 (26). Myoepithelial cells (26) and enteric glia (6) express NTPDase2, while blood vessels express NTPDase1 and -2 (44) and ecto-5′-nucleotidase (28). Functionally, vascular expression of NTPDase1 and ecto-5′-nucleotidase protects against intestinal ischemia-reperfusion injuries (20, 23). Chadwick and Frischauf (10) reported the presence of NTPDase3 mRNA in the digestive tract and pancreas, but the cellular expression of NTPDase3 in the digestive system remains to be determined. Since some NTPDase-like activity, for example, in the serous acini of salivary glands (14) and the gastric epithelium (42), cannot be accounted for by the localization of NTPDase1 and -2, we hypothesize that NTPDase3 is responsible for this activity. The aim of the present work was to localize NTPDase3 in the digestive system. The localization of NTPDase1 and -2 and ecto-5′-nucleotidase was also examined in the tissues expressing NTPDase3 to obtain a more complete and detailed localization for the major ectonucleotidases in the gastrointestinal tract.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and protocols were approved by the Animal Care Committees of Université Laval and the University of Calgary and the Institutional Animal Ethics Committee.
of the University of Barcelona. Adult C57BL/6 mice (Charles River, Pointe-Claire, QC, Canada) were used for all histological studies. Male albino guinea pigs (225–300 g; Charles River) were used for Ca2+ imaging and immunohistochemistry.

Materials

Levamisole, nucleotides, (NH₄)₂S, and paraformaldehyde were purchased from Sigma-Aldrich (Oakville, ON, Canada); aqueous hematoxylin from Accurate Chemical and Scientific (Westbury, NY); dextran (200,000–300,000 mol wt) from MP Biomedicals (Solon, OH); and Mowiol 4-88 from Calbiochem (VWR CANLAB, Mississauga, ON, Canada). Sodium metatungstate (POM1) was purchased from Tocris Bioscience (Ellisville, MO).

Semiquantitative RT-PCR

Total RNA was isolated from various digestive tract tissues in the mouse [parotid, SMG, sublingual salivary glands (SLG), esophagus, stomach, small intestine (duodenum, jejunum, and ileum), and colon] using TRIzol reagent (Invitrogen, Burlington, ON, Canada). Total RNA was quantified using the Qubit quantification platform (Invitrogen). For removal of any contaminating genomic DNA, a DNase1 (New England Biolabs, Pickering, ON, Canada) digestion step was performed before the RT reaction on 1 μg of total RNA with the Superscript III (Invitrogen) using oligo(dT)₂₀ as primer, according to the manufacturer’s instructions. One microliter of the 20-μl RT reactions was used for the amplification reaction using a Taq DNA polymerase (New England Biolabs). Primer sequences used for semi-quantitative RT-PCR experiments are indicated in Table 1.

Antibodies

Unless indicated otherwise, all the primary antibodies used in this study have been previously characterized and validated: rabbit C9F (15, 24) and guinea pig mN1-2α (36) to mouse NTPDase1; rabbit mN2-3β (2) to mouse NTPDase2; guinea pig mN3-3α (36) to mouse NTPDase3; rabbit rN3-3α, (48) to rat NTPDase3, which cross-reacts with guinea pig NTPDase3; guinea pig rN3-5α, (48) to rat ecto-5’-nucleotidase; as well as commercially available rabbit anti-aquaporin-5; mouse anti-S100 (Millipore, Temecula, CA), rabbit anti-protein gene product 9.5 (PGP9.5; Neuromics, Edina, MN), and rabbit anti-aquaporin-5, mouse anti-S100 (Millipore, Temecula, CA), rabbit anti-protein gene product 9.5 (PGP9.5; Neuromics, Edina, MN), and rabbit anti-aquaporin-5 (catalog no. ab53085, Abcam, Cambridge, UK). Secondary antibodies were biotinylated goat anti-guinea pig and goat anti-rabbit, Cy3 donkey anti-guinea pig, and Cy5 donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488 goat anti-rabbit and goat anti-mouse and Alexa Fluor 594 goat anti-guinea pig (Invitrogen). Immunochemistry, Immunofluorescence, and Enzyme Histochemistry

Tissue processing. For immunohistochemistry and enzyme histochemistry, freshly dissected esophagus, stomach, and colon were embedded in optimal cutting temperature (OCT) freezing medium (Tissue-Tek, Sakura Finetek, Torrance, CA), snap-frozen in isopentane in dry ice, and stored at -80°C. Sections (6 μm) were prepared and routinely fixed in 10% phosphate-buffered formalin mixed with cold acetone (Fisher Scientific, Ottawa, ON, Canada) before further processing. For staining of salivary glands and stomach, mice were perfused with 4% paraformaldehyde following standard protocol, then tissue samples were excised, immersed in sucrose, and included in OCT freezing medium, sectioned, and processed for staining. Sections were counterstained with aqueous hematoxylin, mounted on Mowiol mounting medium, and photographed under a microscope (model BX51, Olympus) or with the spinning-disk confocal system (WaveFX, Quorum Technologie). Immunofluorescence of whole mounts was imaged on a confocal microscope (FluoView FV1000, Olympus America, Melville, NY) using a ×60 Plan Neofluar N.1.42 numerical aperture oil-immersion lens. Optical sections (1 μm) were acquired through each field of view.

Enzyme histochemistry. Ectonucleotidase activities in digestive tissue sections were localized using the Wachstein-Meisel lead phosphate precipitation method, as described elsewhere (36). Briefly, fixed tissue sections were preincubated for 30 min at 25°C in Tris-maleate buffer (2 mM CaCl₂, 250 mM sucrose, 50 mM Tris-maleate, pH 7.4) supplemented with 2.5 mM levamisole as an alkaline phosphatase inhibitor. Enzymatic reaction for the hydrolysis of 200 μM or 1 mM nucleotides was performed for 1 or 2 h at 37°C in the same buffer supplemented with 5 mM MnCl₂, 2 mM Pb(NO₃)₂, and 3% dextran T250 (wt/vol). For control experiments, substrate was omitted. Reaction products were revealed by incubation of tissue sections with 1% (NH₄)₂S (vol/vol) for 1 min.

Immunohistochemistry. Immunohistochemistry (peroxidase-based activity) experiments were performed as previously described (36). Briefly, tissue sections were incubated at 4°C for 18 h with the indicated primary antibodies and then at 25°C for 1 h with the appropriate biotinylated secondary antibodies. Preimmune sera were routinely included as controls for the antibodies produced by us.

Double-staining immunofluorescence. Double-staining experiments were performed as previously described (36). Briefly, tissue sections were processed for staining of the first antigen as follows: the first primary antibody was incubated at 4°C for 18 h, then the secondary antibody was added for 1 h at 25°C. Staining of the second antigen was performed under similar conditions, except the second primary antibody was incubated at 25°C for 1.5 h.

Whole-Mount Preparation and Immunohistochemical Labeling

Mice were killed by cervical dislocation [whereas guinea pigs were anesthetized with halothane (5% in O₂; Benson Medical Industries, Minneapolis, MN)], and the distal colon was removed and placed in a Sylgard-coated petri dish containing PBS with 3 μM nicardipine. The colon was opened along the mesenteric border, pinned flat (mucosa-side-up), and treated with Zamboni’s fixative overnight at 4°C. After extensive rinsing with PBS, the mucosa, submucosa, and circular muscle were removed by microdissection, leaving only the myenteric plexus with adherent longitudinal muscle. These longitudinal muscle-myenteric plexus (LMPM) whole-mount preparations were processed for immunofluorescence as described by Gulbransen and Sharkey (22).

Table 1. Primers used for semiquantitative RT-PCR

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<td>actβ</td>
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<table>
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Ca\(^{2+}\) Imaging

Ca\(^{2+}\) imaging was carried out as described by Gulbransen et al. (21). Briefly, colons were excised from male albino guinea pigs, and LMMP whole-mount preparations were dissected in ice-cold oxygenated physiological saline solution consisting of (in mM) 135 NaCl, 5 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 10 glucose, 10 HEPES, 5 sodium bicarbonate, and 1 sodium pyruvate. LMMPs were enzymatically treated [mixture of collagenase type II (Invitrogen) and dispase II (Roche, Laval, QC, Canada)] for 15 min at room temperature, rinsed, and loaded with 4 \(\mu\)M fluo 4-AM (Invitrogen) in a dark, oxygenated environment at 37°C for 45 min. Then LMMPs were rinsed and left at room temperature for 20 min to allow for fluo 4-AM destercification before imaging.

Images were acquired at 2 Hz through the \(\times20\) water-immersion objective (UMPlanFL, 0.5 numerical aperture) of an upright motorized fixed-stage microscope (model BX61WI, Olympus) using Imaging Workbench 6 software (INDEC Biosystems, Santa Clara, CA) and a charge-coupled device digital camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu City, Japan). LMMPs were maintained at \(-34°C\), and stimuli were applied using a gravity-flow perfusion system (Automate Scientific, Berkeley, CA) at a rate of \(-2–3\) ml/min.

The change in fluorescence over time was calculated as described by Gulbransen et al. (21), and traces [generated using Prism 4 (GraphPad Software)] represent means \(\pm\) SE of all glial regions of interest within a myenteric ganglion. Statistical differences were determined by ANOVA; \(P < 0.05\) was considered significant.

RESULTS

We first evaluated NTPDase distribution in the digestive system using semiquantitative RT-PCR on mouse digestive tissues to assess the expression of NTPDase1, -2, and -3 and ecto-5'-nucleotidase. All mouse tissues tested were found to express mRNA by semiquantitative RT-PCR in the gastrointestinal tract and salivary glands. PCR products of NTPDase1, -2, and -3 and ecto-5'-nucleotidase (entpd3) mRNA by semiquantitative RT-PCR in the gastrointestinal tract and salivary glands. PCR products of NTPDase1 (entpd1), -2 (entpd2), and -3 (entpd3) and ecto-5'-nucleotidase (nt5e), obtained after amplification of total RNA with specific primers (see Table 1), were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light. \(\beta\)-Actin (\(ACT\beta\)) is shown as control. Arrows indicate 200-bp band of the ladder.

Next, we used immunolabeling to analyze NTPDase3 protein expression in salivary glands and the digestive tract. NTPDase1 and -2 and ecto-5'-nucleotidase expression were also analyzed on NTPDase3-expressing structures and cells. NTPDase8 expression was not analyzed because of the unavailability of specific antibodies against the mouse isoform.

Salivary Glands

ATPase activity, as determined by enzyme histochemistry, was localized at the periphery of the acini, coinciding with the localization of connective tissue. Serous acinar cells of parotid glands and SMG, as well as mucous acinar cells of SLG, also displayed ATPase activity (Fig. 2, ATP). The combined immunohistochemical localization of NTPDase1, -2, and -3 largely correlated with ATPase activity. NTPDase3 protein was found to localize in serous acini of the parotid and SMG, as well as in mucous acini of the SLG (Fig. 2, NTPDase3). Interestingly, NTPDase3-expressing acinar cells in all salivary glands were immunoreactive for NTPDase2 and ecto-5'-nucleotidase. NTPDase3 and ecto-5'-nucleotidase were also expressed in sublingual duct epithelial cells (Fig. 2, NTPDase3 and ecto-5'-NT). Nevertheless, ATPase activity in these cells was barely detectable. NTPDase2 immunoreactivity was also located in the myoepithelial cells surrounding the acini and at the blood vessel periphery, as reported by Kittel et al. (26). NTPDase1 localization corresponded to the ATPase activity unaccounted for by the expression of NTPDase2 and -3, whereas ecto-5'-nucleotidase localization corresponded to the AMPase activity (data not shown).

To confirm the NTPDase3 localization observed by immunohistochemistry and verify the plasma membrane subcellular localization of this enzyme in salivary gland acini, double-immunofluorescence staining was performed with an antibody to aquaporin-5, a plasma membrane marker of serous acinar cells of the parotid gland and SMG, as well as mucous acinar cells of the SLG (27). As shown in Fig. 3, the basolateral and apical regions of the plasma membrane expressing aquaporin-5 were immunoreactive for NTPDase3.

Esophageal and Gastric Epithelia

Immunoreactivity for NTPDase3 was located in the mucosal squamous stratified epithelium of the esophagus and forestomach (Fig. 4, NTPDase3). While all cell layers of the esophagus express NTPDase3, NTPDase3 localization in the forestomach was limited to the basal cell layer. NTPDase2 was expressed along with NTPDase3 in esophageal and forestomach epithelia, in keeping with the ATPase activity data (Fig. 4, ATP). Interestingly, ecto-5'-nucleotidase was also expressed in these epithelia, but with some distinctions. In the esophagus, ecto-5'-nucleotidase was seen in the epithelial cells expressing NTPDase3 and -2, with slightly more staining in differentiated cells than in basal cells (Fig. 4A, ecto-5'-NT). In the forestomach, there was a clear distinction between the expression of ecto-5'-nucleotidase and the expression of either of the two NTPDases, as the ecto-5'-nucleotidase was clearly located in the upper differentiated epithelial cells (Fig. 4B, ecto-5'-NT). AMPase activity correlated with the immunolocalization of ecto-5'-nucleotidase (Fig. 4, AMP). In addition, immunoreactivity for NTPDase2 and -3 and ecto-5'-nucleotidase was also detected in the apical epithelial layer in contact with the lumen.
Fig. 2. NTPDase3 is expressed with NTPDase2 and ecto-5′-nucleotidase (ecto-5′-NT) in mouse salivary glands. Enzyme histochemistry and immunohistochemical assays performed on serial tissue sections of parotid and submandibular and sublingual salivary glands (SMG and SLG) show ATPase activity and ectonucleotidase immunoreactivity. ATPase activity [ATP (200 μM)] is detected in serous acini of parotid glands and SMG, as well as in mucous acini of SLG; connective tissues also display ATPase activity. Serous acini of parotid, SMG, and mucous acini of SLG display NTPDase3 immunoreactivity. NTPDase2 and ecto-5′-nucleotidase are expressed with NTPDase3 on acini. NTPDase3 and ecto-5′-NT are detected in duct cells of the SLG, where no ATPase activity is detected. Arrows indicate positively labeled plasma membranes of acini; arrowheads indicate positive duct cells. Nuclei were counterstained with aqueous hematoxylin. Insets correspond to control without substrate or preimmune serum staining, respectively. Scale bar, 20 μm.

NTPDase3 localization in the digestive system

of the esophagus (Fig. 4A); however, neither ATPase nor AMPase activity could be detected in that structure (Fig. 4A, ATP and AMP).

NTPDase3 immunoreactivity was also present in the glandular portion of the mouse gastric epithelia (Fig. 5A). This staining was limited to a specific cell type in the antral region of the stomach that was similar in abundance and distribution to enteroendocrine cells. As gastrin-secreting G cells are the major enteroendocrine cell type in that region of the stomach, a double-immunofluorescence assay for NTPDase3 and gastrin expression was performed. The data presented in Fig. 5B show that some gastrin-expressing cells were NTPDase3-positive. However, not all NTPDase3-expressing cells were positive for gastrin (data not shown), suggesting that other types of enteroendocrine cells express NTPDase3. Lack of somatostatin immunoreactivity would thereby appear to exclude D cells (data not shown). In the lamina propria, in addition to nerve terminals expressing NTPDase3, other cell types, such as blood vessels and immune cells, exhibit ATPase activity, which corresponds to the immunolocalization of NTPDase1 and -2 (data not shown). Some enteroendocrine gastric cells, as well as the luminal side of the gastric pits, expressed ecto-5′-nucleotidase, as determined by immunohistochemistry and AMPase activity (Fig. 5A).

Enteric Nervous System

The enteric nervous system innervates the digestive tract from the esophagus to the anal sphincter. Enzyme histochemistry and immunohistochemistry experiments performed on serial sections of the mouse gastrointestinal tract showed ATPase activity throughout the entire length of the enteric nervous system. Representative histochemical images of ATPase activity in mouse stomach and colon are shown in Fig. 6, A and B, respectively. In addition to the nervous structures, smooth muscle cells and blood vessels also exhibited ATPase activity. NTPDase3 and -2 were coexpressed within the myenteric and submucosal plexuses, as well as in the nerve terminals of the smooth muscle layer and mucosa (Fig. 6). AMPase activity and ecto-5′-nucleotidase immunoreactivity...
activity were not detected in enteric nervous structures (data not shown). ATPase activity unaccounted for by the detection of NTPDase2 and -3 coincided with the immunolocalization of NTPDase1 (data not shown). Immunohistochemistry on tissue sections demonstrated that NTPDase3 is expressed within the enteric nervous system. However, tissue sections did not allow identification of the cellular constituents of the enteric nervous system expressing NTPDase3. Therefore, we stained whole-mount preparations of mouse colonic myenteric plexus to determine the cellular localization of NTPDase3. Myenteric ganglia were highly immunoreactive for NTPDase3, which outlined the cellular constituents of the myenteric plexus, namely, the enteric neurons and glia (Fig. 7A). NTPDase3 immunoreactivity was primarily localized to the membranes of PGP9.5-immunoreactive enteric neurons, with punctate NTPDase3 staining throughout the cytoplasm in the vast majority (989 of 1,000) of enteric neurons (Fig. 7A). Given the close proximity of enteric neurons and glia, we could not completely rule out the possibility that enteric glia also express NTPDase3, but we found a lack of NTPDase3 staining in the cytoplasm of enteric glia. Intense NTPDase2 immunoreactivity was also present in the myenteric plexus. Dual labeling with NTPDase2 and -3 demonstrated that NTPDase3 is primarily expressed by enteric neurons, while enteric glia mainly express NTPDase2 (Fig. 7B).

**Functional NTPDases Within the Enteric Nervous System**

To determine whether NTPDases expressed within the myenteric plexus are functionally involved in controlling the availability of P2 receptor agonists, we inhibited NTPDase activity with POM1 (50 μM) and used Ca²⁺ imaging to assay the activation of P2 receptors in enteric glial cells. We chose to perform Ca²⁺ imaging experiments in the guinea pig because of the ease of dissection, its well-characterized enteric physiology, and our previous experience with P2 receptor activation imaging in guinea pig enteric glial cells in the colon (21). Before proceeding with such experiments, we used specific markers for enteric glia (S100) and neurons to verify the immunohistochemical localization of NTPDase2 and -3 in the guinea pig myenteric plexus (PGP9.5; Fig. 8). In agreement with our findings in the mouse (cf. Fig. 7), we found that guinea pig myenteric glia express NTPDase2, whereas myenteric neurons express NTPDase3. Furthermore, additional experiments showed that the cellular localization of NTPDase2 and -3 in the guinea pig submucosal plexus is similar to the NTPDase2 and -3 localization in the myenteric plexus (data not shown).

Having confirmed that NTPDase2 and -3 are the primary ecto-ATPases expressed within the myenteric plexus, we tested the ability of ATP to activate P2 receptors in enteric glia under control conditions or upon the attenuation of NTPDase activity.
using POM1 (50 μM; Fig. 9) as an inhibitor. Under both conditions, a 30-s application of 100 μM ATP elicited a robust increase in free cytosolic Ca\(^{2+}\) in enteric glial cells (Fig. 9A). Under control conditions, glial Ca\(^{2+}\) levels typically returned to baseline within 60 s. However, upon NTPDase inhibition, glial Ca\(^{2+}\) responses were significantly extended and persisted beyond 60 s (Fig. 9, A and B). To quantitate the lengthening of response, we analyzed the area under the curve (AUC) of sequential responses to 100 μM ATP and found that the mean glial Ca\(^{2+}\) response to ATP was significantly larger in the presence of an NTPDase inhibitor [AUC(ΔF/F)s, where F is fluorescence and s is time in seconds]: for the first ATP response, AUC = 39 ± 5 for control and 108 ± 18 for POM1-treated cells (P < 0.001); for the second ATP response, AUC = 30 ± 6 for control and 84 ± 13 for POM1-treated cells (P < 0.01); for the third ATP response, AUC = 21 ± 3 for control and 71 ± 10 for POM1-treated cells (P < 0.01); for the fourth ATP response, AUC = 25 ± 8 for control and 56 ± 11 for POM1-treated cells [P > 0.05 (n = 6 ganglia for each group), as determined by ANOVA], suggesting that NTPDases limit the bioavailability of P2 ligands in the enteric nervous system (Fig. 9C).

**DISCUSSION**

In this work, we used immunolabeling and in situ enzymatic assays to present a detailed analysis of the expression and specific localization of NTPDase3 along the digestive system.
We demonstrate that enteric neurons and certain epithelial cells indeed express NTPDase3. More specifically, NTPDase3 immunoreactivity was detected in the epithelia of salivary glands, i.e., in serous acini of the parotid glands and SMG and in mucous acini and ducts of the SLG. In addition, epithelial cells of the esophagus and forestomach and some enteroendocrine cells of the gastric antrum express NTPDase3. We also report the localization of NTPDase2 and ecto-5′-nucleotidase in NTPDase3-expressing structures. Our NTPDase1 staining confirms its localization as reported in blood vessels (44), in immune cells of the gastrointestinal lamina propria (39), and in SMG mucous acini and myoepithelial cells (26). As no NTPDase1 immunoreactivity was detected in NTPDase3-expressing cells, no NTPDase1 staining was shown in the present work. Nevertheless, it is noteworthy that the residual ATPase activity that cannot be attributed to NTPDase2 or -3 coincides with the immunolocalization of NTPDase1 (data not shown), suggesting that NTPDase1, -2, and -3 are together responsible for the major part of membrane-located ATPase activity detected in the tissues analyzed here. Interestingly, on the basis of previous observations (30; unpublished data), the ADPase, UTPase, and UDPase activities of NTPDase3 were consistently detected in the same cells in which ATPase activity was detected, in agreement with the known biochemical properties of NTPDase3 (29, 31). The ability to hydrolyze all these nucleotides (i.e., ATP, ADP, UTP, and UDP) is obviously an important action of NTPDase3, as there are various P2Y receptors that respond to these ligands, which play important functions in the digestive tract.

The NTPDase3-immunoreactive cells in the digestive system were of the same types as those previously reported in other systems. NTPDase3 was located in a subclass of neurons in the rat brain (3) and in epithelial cells of the human pulmonary (17) and murine reproductive systems (36). In the reproductive system, NTPDase3 expression is limited to epithelial cells of the seminal vesicle, epididymis (principal cells), oviduct, and secretory epithelia of the prostate and Cowper’s gland (36). NTPDase3 is also expressed in the islets of Langerhans and appears to modulate endocrine pancreatic secretion (30). The coexpression of NTPDase3 with NTPDase2 in the epithelia lining the salivary gland, esophagus, and forestomach is a rather singular observation, since examples of cells expressing multiple NTPDases are rare. Two such cell types are Leydig cells (36) and myoepithelial cells of the SMG (26), where NTPDase1 and -2 expression was reported. Coexpression of NTPDases in the same cells raises the possibility of physical-functional interactions between different NTPDases at the cell surface. Indeed, since NTPDases form oligomers (16, 19, 49), NTPDase2 and -3 might conceivably heterodimerize in the epithelial cells mentioned above. Further studies are necessary to address this point.

Salivary Glands

ATPase, ADPase, and AMPase activities were first reported in rat parotid acinar cells by Dowd et al. (14), but the enzymes involved were not determined. The present immunohistochemical data show the expression of two NTPDases, namely, NTPDase3 and -2, as well as of the ecto-5′-nucleotidase, in mouse parotid acini, and these enzymes are likely responsible for the activity described in the rat by Dowd et al.

Fig. 5. Ectonucleotidase localization in enteroendocrine cells of mouse stomach. A: NTPDase3 and ecto-5′-nucleotidase immunoreactivity in enteroendocrine cells of mouse stomach. Serial sections of perfused mouse stomach demonstrate ATPase activity, as well as NTPDase3 and ecto-5′-nucleotidase immunoreactivities, in antral glandular mucosa. ATPase activity [ATP (200 μM)] is localized to lamina propria, blood vessels, nerves, and epithelial cells. Arrows indicate enteroendocrine cells, which present a higher ATPase activity than other epithelial cells. NTPDase3 and ecto-5′-nucleotidase immunoreactivity reveal that few enteroendocrine cells in the antrum region express these enzymes (arrows). Ecto-5′-nucleotidase immunoreactivity is observed in the apical membrane in the gastric pits (arrowheads). AMPase activity [AMP (200 μM)] corroborated ecto-5′-nucleotidase localization. Nuclei were counterstained with aqueous hematoxylin. Insets correspond to controls without substrate and preimmune serum staining, respectively. Scale bar, 20 μm. B: confocal images of a stomach gland showing a cell coexpressing gastrin (green) and NTPDase3 (red) at the apical pole (arrows). DIC, differential interference contrast image. Scale bar, 10 μm.
A major role of NTPDases is the control of P2 receptor activation. Acinar or duct epithelial salivary gland cells express numerous P2 receptors, including P2X4 (46), P2X7 (33), P2Y1 (1), and P2Y2 (47). P2X7 receptors have been the most extensively characterized and play a role in saliva secretion by acinar cells (38). Lévesque et al. (34) demonstrated that NTPDase1 can modulate macrophage P2X7 function. Similarly, NTPDase3 and -2 may be involved in the modulation of P2X7 receptor activation on serous acinar cells. NTPDase1 expressed by pancreatic acini is released within the pancreatic juice (45). This may also occur in salivary glands as saliva, and pancreatic secretions are similar processes. The results of Ishibashi et al. (25) support this hypothesis by demonstrating that the amount of ATP collected from rat SMG ducts following pilocarpine stimulation is underestimated because of its hydrolysis.

In agreement with previous findings, aquaporin-5 was localized to the apical regions of parotid, submandibular, and sublingual acinar epithelia (27). In addition, we detected basolateral aquaporin-5 expression (cf. Fig. 3). C57BL/6 mice were used in the present study, while Konttinen et al. (27) performed localization on Balb/C mice. It is possible that strain variations account for the differential subcellular localization.

The physiological significance of aquaporin-5 coexpression with the ectonucleotidases NTPDase3 and -2 and ecto-5'-nucleotidase is unknown, but since P2 receptor activation regulates aquaporin expression, ectonucleotidases may indirectly participate in this regulation (32, 51). Acinar P2 receptors are presumably regulated by NTPDases, which may, in turn, modulate aquaporin-5 expression in salivary gland acinar cells.

NTPDase3 expression in SLG epithelial duct cells did not correlate with enzymatic activity, as no ATPase activity was detected in SLG duct cells under our experimental conditions. Another ATP diphosphohydrolase has been immunolocalized in pig parotid duct cells (43). Unfortunately, the identity of this protein could not be clearly established, as the antibody used in that study recognized the fourth apyrase-conserved region of porcine NTPDase1. Therefore, any of the seven other members of the E-NTPDase family could have contributed to the observed immunoreactivity. The relevance of duct NTPDase expression needs to be further explored.

**Esophagus and Forestomach**

The esophageal epithelium expresses NTPDase2 and -3 and ecto-5'-nucleotidase, an expression profile that could lead to an
in vivo accumulation of adenosine in this region. NTPDase3 and -2 expression are more pronounced in the cells in the vicinity of the basal portion of these stratified epithelia, while ecto-5'-nucleotidase is mainly localized in the luminal section of the epithelia. This segmented expression is more pronounced in the forestomach epithelium, where no NTPDase immunoreactivity was detected in the luminal portion, and no ecto-5'-nucleotidase was present in the basal region. Extracellular ATP regulates the ciliary beat frequency of human nasal epithelial cells (37) and frog esophagus epithelial cells (35, 50). As NTPDase2 and -3 and ecto-5'-nucleotidase regulate the availability of extracellular ATP (and adenosine), the expression of these enzymes in the mouse esophagus could regulate ciliary beat frequency.

Enteroendocrine Cells

NTPDase3 is expressed by dispersed enteroendocrine cells of the gastric glandular epithelium. These cells are located in the antral region of the stomach, near the pylorus. Our experiments suggest that a subpopulation of G cells express NTPDase3. However, at least one additional enteroendocrine cell type appears to express NTPDase3, as not all NTPDase3-positive cells were positive for gastrin. Further analysis is necessary to determine the other cell types expressing NTPDase3. In addition, ATP is further hydrolyzed to adenosine by the ecto-5'-nucleotidase expressed by the same, or adjacent, cells, as shown in Fig. 5A. A functional consequence of adenosine formation at the surface of these cells might be the modulation of gastrin secretion by the activation of A1 receptors (55).

Enteric Nervous System

NTPDase3 is expressed by mouse and guinea pig enteric neurons of myenteric and submucosal plexuses, in the muscular layer and mucosal nerve terminals (Figs. 6–8). Since NTPDase3 was detected throughout the plexus, it is possible that NTPDase3 is expressed by all enteric neuron subtypes. Indeed, our data suggest that most, if not all, PGP9.5-immunoreactive myenteric neurons express NTPDase3. The expression of NTPDase2 in mouse and guinea pig enteric glial cells (Figs. 7B and 8C) is in agreement with observations reported by Braun et al. (6) in the rat colon. Purinergic signaling is an important aspect of enteric neurotransmission in sensory and motor neurons, as well as interneurons (4, 5, 11), and its importance is emphasized here by the observation that two members of the NTPDase enzyme family (NTPDase3 in neu-
Fig. 8. NTPDase2 and -3 immunoreactivity in guinea pig myenteric plexus. Images are single optical sections through myenteric ganglia from the distal colon. 
A: NTPDase3 immunoreactivity (red) is primarily confined to enteric neurons and poorly correlates with glia immunoreactive for S100 (green). * are placed within the nuclei of several NTPDase3-immunoreactive neurons. B: neuronal localization is confirmed by colocalization of NTPDase3 (red) with the pan-neuronal marker PGP9.5 (green). *, PGP9.5-immunoreactive neurons colabeled with NTPDase3. C: NTPDase2 immunoreactivity (red) colocalizes with the glial marker S100 (green). Arrows, several areas of colocalization. D: NTPDase2 immunoreactivity (red, arrows) surrounds PGP9.5-immunoreactive neurons (green, *). Scale bars, 20 μm.
rons and NTPDase2 in glia) are expressed in cells intimately contacting one another. ATP released by neurons can therefore be rapidly broken down to ADP by NTPDase2 and -3. ADP is the agonist of P2Y1, P2Y12 and P2Y13, and the expression and functional implication of P2Y1 in the enteric nervous system are well documented (52). P2Y1 activation should be under the control of the NTPDases expressed by the enteric nervous system. In addition, nucleotides released by neurons activate P2Y4 receptors expressed by glial cells (22), and this paracrine communication between neurons and glia would also be under the control of NTPDase2 and -3. In support of this hypothesis, we show that inhibition of NTPDase activity extends nucleotide activation of glial P2 receptors (Fig. 9). Interestingly, neither ecto-5′-nucleotidase expression nor AMPase activity was detected in the enteric nervous system (data not shown).

These results are in accordance with the fact that the main source of extracellular adenosine acting on the presynaptic region of neurons is provided via an equilibrative adenosine transporter (12), and not via ATP hydrolysis.

**Summary**

NTPDase3 is expressed by neurons in the enteric nervous system and by certain epithelial cells of the digestive system. In salivary glands, secretory epithelia of serous and mucous acini (SLG only) express NTPDase3. NTPDase3 is also expressed by the epithelial cells of the upper digestive tract and by some G cells and other yet unidentified subtypes of gastric enteroendocrine cells. In addition, NTPDase3 is often expressed with NTPDase2 and/or ecto-5′-nucleotidase
in the tissues analyzed. Future studies will aim to address the significance of NTPDase3, in association with NTPDase2 and ecto-S'-nucleotidase, in the functions of the digestive system.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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