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Kinetic and biochemical characterization of an ecto-nucleotide pyrophosphatase/phosphodiesterase (EC 3.1.4.1) in cells cultured from submandibular salivary glands of rats

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ABSTRACT

The participation of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity in the nucleotide hydrolysis by salivary gland cells of rats was evaluated using *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) as a substrate for this enzyme. We investigated the biochemical characteristics of this ectoenzyme in cells cultured from submandibular salivary glands of rats. Primary cell cultures demonstrated ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activities, which could be observed by extracellular hydrolysis of *p*-Nph-5′-TMP and other biochemical characteristics such as dependence of metal ions, dependence of pH alkaline and inactivation by a metal ion chelator. The K_m value for the hydrolysis of *p*-Nph-5′-TMP was 280.7 ± 34.2 μM (mean ± S.D., $n = 4$) and V_{max} was 721.31 ± 225 nmol *p*-nitrophenol/min/mg (mean ± S.D., $n = 4$). We suggest that E-NPP is co-localized with an ecto-ATP diphosphohydrolase/ecto-NTPDase and an ecto-5′-nucleotidase, since these enzymes probably act under different conditions. It may be postulated that the physiological role for these ecto-enzymes is to terminate the action of the co-transmitter ATP, generating adenosine.

1. Introduction

Many publications have discussed the role of adenosine triphosphate (ATP) as an extracellular mediator and neurotransmitter in various systems, including salivary glands.[1,2] Extracellular ATP exerts a broad range of physiological responses, including neurotransmission,[1] modulation of vascular tone, platelet aggregation, neutrophil aggregation, superoxide release, production of pro-inflamatory cytokines[3,4] and transepithelial chloride secretion by activation of plasma membrane receptors.[5] ATP and other extracellular nucleotides influence epithelial cell functions via a variety of P2 receptors.[6] These receptors can be ligand-gated ion channels (P2X-type) or coupled to heterotrimeric G proteins (P2Y-type). In salivary glands, four distinct P2 subtypes of ATP receptors have been identified in the ductal and acinar cells: $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$.[7] At the end of its action, the ATP signaling molecule must be recaptured by the cell or hydrolyzed by enzymatic system(s).

The extracellular hydrolysis of ATP to adenosine by ectonucleotidases has been reported in several cell types.[6,8–10] These enzymatic activities can regulate the extracellular

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concentration of adenine nucleotides and nucleosides, thus modulating their local effects. Degradation of ATP and other nucleotides can occur through a cascade of cell-surface-bound enzymes such as ecto-ATPase (EC 3.6.1.3), ecto-apyrase/ATP diphosphohydrolase/NTPDase (EC 3.6.1.5), ecto-pyrophosphatase/phosphodiesterase/NPP (EC 3.1.4.1) and ecto-5′-nucleotidase (EC 3.1.3.5).[1] These enzymes seem to have multiple roles in extracellular nucleotide metabolism and in the regulation of intercellular signalling.[11] E-NTPDase describes a family of mammalian enzymes that catalyze the hydrolysis of γ- and β-phosphate residues of nucleosides 5′-tri and 5′-diphosphates, and these enzymes require the presence of divalent cations for their catalytic activity.[1] We, recently, described the presence of an NTPDase as an ecto-enzyme in cell cultures obtained from submandibular glands of adult rats.[12]

The family of E-NPPs is known to hydrolyze 5′-phosphodiester bonds in nucleotides and their derivatives, resulting in the release of 5′-monophosphates.[11,13] E-NPPs hydrolyze a broad range of substrates such as UDP-galactose, NAD^+, cAMP, ATP and ADP. On the other hand, AMP is not a substrate for NPPs, as expected from the absence of phosphodiester or pyrophosphate bonds.[13] The *p*-nitrophenyl 5′-thymidine monophosphate (*p*-Nph-5′-TMP) has been used as an artificial substrate marker for E-NPPs characterization, generating *p*-nitrophenol as a final product. Current evidence suggests that E-NPPs have multiple and largely related physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility, activity of ecto-kinases and, probably, regulation of the insulin receptor.[11] These families of enzymes demonstrate not only overlapping tissue distributions, but also overlapping substrate specificities and may also present overlapping function.[1] Nucleotides can exert different responses in diverse tissues, for this reason cells can co-exhibit two or more different families of enzymes, probably with slightly different nucleotide hydrolysis properties. NPP4-5 have a predicted type I membrane orientation, but have not yet been functionally characterized.[13]

Dowd et al.[14,15] identified and characterized a possible ecto-ATPase activity in rat salivary gland cells. In the submandibular gland cyclic nucleotide phosphodiesterase, PDE1 and PDE5[16,17] were detected in the rat. NPP1 is present on the distal convoluted tubules of the kidney, epithelium of salivary glands ducts, brain capillary endothelium and epididymis.[11]

In a previous study, we described the presence of an E-NTPDase and 5′-nucleotidase in salivary gland cells.[12] In the present study, we demonstrate that salivary gland cells in culture, in addition to these enzymes, were able to promote the extracellular hydrolysis of *p*-Nph-5′-TMP (a substrate marker for the ecto-pyrophosphatase/phosphodiesterase). The kinetic parameters for substrate hydrolysis and the effects of divalent cations, calcium and magnesium on enzymatic activities were determined. We postulated the co-existence of a multiple enzymatic system in the salivary glands for extracellular nucleotide hydrolysis. Our study may provide new information regarding the presence of different families of enzymes with similar functions in the salivary gland cells, which probably act under distinct physiological situations in the regulation of nucleotide signaling pathways.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle medium (DMEM) was purchased from Grand Island Biological Company (Grand Island, NY, USA). Collagenase I-S, nucleotides, Hepes, *p*-Nitrophenyl thymidine 5′-monophosphate, EDTA, Trizma Base, ouabain, lanthanum chloride, levamisole, NEM (*N*-ethylmaleimide) sodium azide and phenylalanine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Cultilab Ltda (São Paulo, SP, Brazil). All the other chemical reagents were of the highest available quality.

2.2. Cell isolation and culture

Salivary gland cell (SGC) clusters were obtained from 35-day-old male Wistar rats. The animals were sacrificed with ether overdose and the submandibular glands were removed quickly, trimmed of fat and fascia tissues and minced in a small volume of Hank's buffer saline solution (HBSS), pH 7.4. The minced salivary glands were dispersed in HBSS supplemented with collagenase I-S (1 mg/ml). The gland cells were dissociated by pipetting 10 times every 20 minutes with a Pasteur pipette for 2 h. The collagenase was removed by centrifugation at $700 \times g$ (5 min) and the cell clusters were then washed with HBSS and centrifuged twice at $40 \times g$ (5 min) to remove the lysed cells and contaminants (red and endothelial cells).

Clusters with 4–5 cells (Fig. 1) (as observed by phase-contrast microscopy) were maintained in a water-saturated atmosphere with 95% air and 5% CO_2 in Dulbecco's modified eagle medium (DMEM) with 5% fetal bovine serum (FBS), pH 7.4 for 24–48 h [modified from[14,18]].

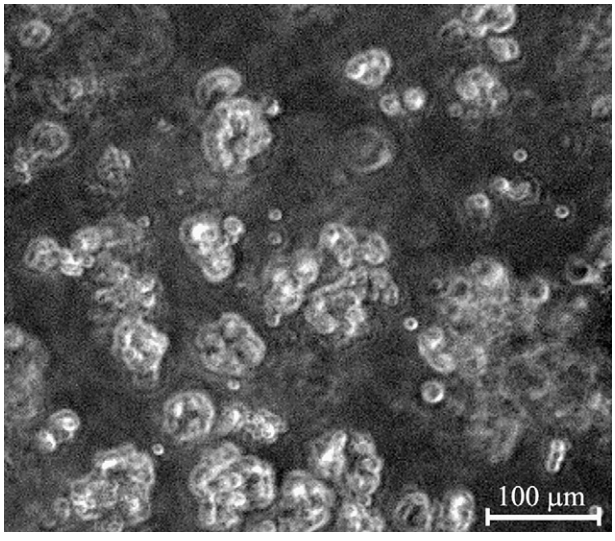

Fig. 1 – Representative picture of submandibular gland cells cultured to 24 h showing cell clusters. The cell culture was performed as described in material and methods of the manuscript. Cells were viewed with a Nikon inverted microscope and images transferred to computer with a digital camera (Sound Vision Inc. Wayland, MA).