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Effect of bitter compounds on amylase secretion in murine submandibular glands: Signaling pathway mechanisms

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ABSTRACT

Background: Amylase is synthesized in submandibular glands (SMG) and released into the oral cavity to degrade carbohydrates in the mouth. Bitter taste receptors (T2R) belong to the G-protein coupled receptor (GPCR) family and are expressed in the taste cells and also in the digestive tract.
Methods: The activity of amylase secreted by murine SMG was measured, detecting maltose by Bernfeld's method. Amylase and T2R6 were detected by imunohistochemistry and Western blot. The expression of Ggustducin, Gi, and phospholipase Cβ2 was also studied by Western blot. cAMP levels were measured by radioimmunoassay and inositol monophosphate production was quantified by ELISA.
Results: Theophylline, denatonium and cycloheximide exerted a dose-dependent inhibition on amylase secretion. This effect was reverted by preincubating SMG with an anti-Gαi antibody. cAMP production was increased by the same compounds, an effect that was also abrogated by an anti-Gαi antibody. Bitter compounds reduced inositol monophosphate formation in SMG and H-89, a protein kinase A inhibitor, reverted this action, revealing that this protein kinase down regulates phospholipase C activity.
General significance: We demonstrated that theophylline, denatonium and cycloheximide inhibit salivary amylase secretion, activating an intracellular signaling pathway that involves cAMP and phospholipase C, that cross talks via protein kinase A.

1. Introduction

As accessory exocrine glands of the digestive tract, the salivary glands supply a variety of proteins, fluids and electrolytes that play key roles in maintaining the environment of the oral cavity, and in facilitating the onset of the digestive process. The major salivary glands are comprised by the parotid, the sublingual and the submandibular glands (SMG). In them, the components of saliva are mainly produced by acinar cells and are conveyed to the oral cavity by a cell-lined duct system, where the fluid and electrolyte components are subjected to secondary modifications [1]. Structurally, the SMG of rodents are composed of the acinus and the duct system. In the adult mouse and rat SMG, a portion of the duct system is called the granular convoluted tubule (GCT). The epithelial cells of GCT have abundant secretory granules that contain a variety of biologically active peptides, including nerve growth factor, epidermal growth factor, transforming growth factor, renin and kallikrein [2].

In parotid glands of rodents it has been demonstrated that salivary fluid secretion is regulated by parasympathetic activity mediated through muscarinic receptors in the acinar cells [3]. On the other hand, salivary protein secretion is evoked when neurotransmitters bind to β adrenergic receptors on the basolateral membrane of secretory cells [4]. Less knowledge is available in relation to the regulation of amylase secretion in mouse SMG by the activation of other receptors than those of neurotransmitters and hormones.

T2R belong to the G protein coupled receptors (GPCR) family and they mediate bitter taste perception in mammals. Their activation triggers Ggustducin coupling to the effector enzymes phosphodiesterase (PDE) and phospholipase Cβ2 (PLCβ2) [5,6]. Inositol trisphosphate (IP_3), cAMP and cGMP are among the second messengers generated in the millisecond time range [7,8].

It has been shown that T2R, originally described in taste cells located on the dorsal surface of the tongue, soft palate and pharynx, are also found in rodent stomach and enteroendocrine cells [9]. Since SMG are secretory glands of endodermic origin and belong to the digestive system, it is reasonable to assume that murine SMG could also express

Abbreviations: CV, circumvallate papillae; GCT, granular convoluted tubule; GPCR, G protein coupled receptors; IP_1, inositol monophosphate; IP_3, inositol triphosphate; PDE, phosphodiesterase; PKA, protein kinase A; PLCβ2, phospholipase Cβ2; SMG, submandibular glands; T2R, bitter taste receptors

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T2R. On the basis of functional studies of individual T2R expressed in heterologous cells, it is proposed that the T2R gene receptor family is composed of approximately 30 members that encode bitter receptors [5]. Bitter compounds, which are sensed by these receptors, present a broad structural diversity and pharmacological action and generally can act promiscuously on different receptor subtypes [5]. Among them are quaternary amines (denatonium benzoate or the alkaloids strychnine and quinine), carbamates (phenylthiocarbamide), flavonone glycosides (naringin), acetylated sugars (sucrose octaacetate) and methylxantines (caffeine and theophylline) and many of them are toxic or harmful substances found in plants [10].

The aim of our work is to study the action of different bitter compounds on amylase secretion and activity by murine SMG and the signal transduction pathway activated by these compounds.

2. Materials and methods

2.1. Chemicals

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Solutions were prepared fresh daily. Bitter agonists: theophylline, and denatonium benzoate (Sigma) were kindly provided by Dr. A. I. Spielman (NYU College of Dentistry, NY, USA).

2.2. Animals

Three months old inbred BALB/c mice were purchased from the Instituto Nacional de Tecnología Agropecuaria (INTA, Castelar, Buenos Aires, Argentina). Animals were handled according to the "Guide to the Care and Use of Experimental Animals" (DHEW Publication, NIH 80–23). Mice were fasted 24 h before the experiments were carried out, then anesthetized and euthanized. SMG were surgically obtained, free connective tissue and fat were gently removed, weighted and placed immediately in a Krebs Ringer Bicarbonate (KRB) solution without glucose under carbogen stream (95% O_2, 5% CO_2) for a few minutes before the assay was run. Taste tissue was collected from mice tongues as previously described by punching single circumvallate papillae (CV) with glass capillaries [6]. Underlying muscle and salivary tissue were carefully removed under a dissecting microscope.

2.3. Amylase activity assay

Whole SMG were incubated under carbogen in 500 µl KRB at 37 °C for 30 min. When antagonists or inhibitors where used, they were included from the beginning of the incubation time and agonists were added during 20 min.

Amylase activity secreted into the incubation solution (extracellular) was determined by a modification of Bernfeld's colorimetric technique [11]. Briefly, the supernatant containing secreted amylase was separated and amylase activity was determined by the addition of 1% starch followed by 3, 5-dinitrosalicylic acid in 2 M NaOH solution. Maltose production was detected at 540 nm using a Bio-Rad automatic spectrometer (Bio-Rad, Hercules, CA, USA). The amount of maltose produced in the unknown samples was determined by extrapolation using a maltose standard curve. The results were expressed as milligrams of maltose released per minute and per gram of wet weighed tissue (mg maltose/min. g w.w.) and as a percentage of control (untreated glands), considered as 100%.

2.3.1. Immunohistochemistry

SMG were fixed by immersion in formalin solution and then dehydrated, cleared, embedded in paraffin wax and cut (7-µm thickness) on a rotating microtome. Paraffin sections containing the SMG were deparaffinized and rehydrated through xylene and descending ethanol series, then blocked with peroxidase blocking reagent (DAKO, Milan, Italy). After several washes in PBS, sections were incubated with primary antibodies. For amylase detection, sections were first incubated 1 h at room temperature in a mouse on mouse (M.O.M.) IgG blocking reagent (Vector, Burlingame, CA, USA), prepared according to the manufacturer's instructions. A mouse monoclonal anti-amylase antibody (1:800) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was then incubated 30 min at room temperature in a humidified chamber. When using goat polyclonal anti-T2R6 (1:50) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking solution, it was incubated overnight at room temperature in a humidified chamber. After washes, sections were incubated 1 h at room temperature with biotinylated donkey anti-goat (1:400) (DAKO, Milan, Italy), 10 min with M.O.M. biotinylated anti-mouse (Vector, Burlingame, CA, USA) immunoglobulins or biotinylated swine anti-rabbit (1:400) (DAKO, Milan, Italy). The immune reaction was detected using a Vectastain Elite ABC kit (Vector, Burlingame, CA, USA) and visualized with 3, 3′-diaminobenzidine tetrahydrochloride (DAKO) within 5–10 min. Control sections were prepared by omitting the primary antibody. Controls did not show immunolabelling. An Olympus BX51 photomicroscope equipped with a KY-F58 CCD camera (JVC, Japan) was used for the observation of sections. The images obtained were analyzed and stored using Image-ProPlus software (Media Cybernetics, Silver Springs, MD, USA).

2.4. Western blotting

SMG and CV were homogenized at 4 °C in a RIPA lysis buffer containing 10 mM Tris–HCl (pH 7.0) 50 mM NaCl, 1% Triton X-100, 1 mM EDTA and EGTA, 5 mM PMSF, 10 µg/ml of aprotinin, leupeptin and trypsin inhibitor, 0.1 mM Na_3VO_4 and 50 mM NaF. Using an Eppendorf microcentrifuge, the homogenate was then centrifuged 15 min at 3000 rpm at 4 °C. The supernatant was centrifuged again at 10,000 rpm for 30 min at 4 °C. The last supernatant was kept at −80 °C.

Protein concentration of the supernatants obtained was determined using the method by Bradford [12]. Using a Mini-PROTEAN electrophoresis system (Bio-Rad Hercules, CA, USA) samples were run on a 10% SDS-polyacrilamide gel, loading 80 to 150 µg of protein per lane, followed by electrotransference into nitrocellulose membranes. Blotted proteins were then stained in a saturated solution of Ponceau 2R to corroborate transfer efficiency, washed briefly in distilled water and incubated for 1 h at room temperature in TBS–Tween 20 0.05% with 5% skim milk. Membranes were then incubated overnight under agitation at 4 °C with the following antibodies: mouse monoclonal anti-amylase (1:200), rabbit polyclonal anti-Gαgustducin (1:250), goat anti-Gαi-1/2/3 (1:250), rabbit anti-PLCβ2 (1:400) or goat polyclonal anti-T2R6 (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted in TBS–Tween 20 0.05% with 5% skim milk. After washes in TBS–Tween 0.05%, membranes were incubated under agitation for 1 h at room temperature with anti-mouse, anti-rabbit or anti-goat secondary antibodies (1:8000) conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA), diluted in TBS–Tween 20 0.05% with 5% skim milk. After 3 washes in TBS–Tween 20 0.05%, bands were revealed by ECL using 250 mM luminol, 90 mM p-coumaric acid, 1 M Tris–HCl (pH 8.5) in distilled water and 30% H_2O_2. Bands were quantified by densitometry and the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Controls were prepared by omitting the primary antibody and did not show immunolabelling. The molecular weight of the bands was identified comparing their Rf with the corresponding molecular weight markers (Bio-Rad, Hercules, CA, USA).

2.5. Determination of cAMP levels

SMG were incubated under carbogen in 500 µl KRB buffer containing 10^{-3} M 3-isobutyl-1-methylxanthine (IBMX) at 37 °C and treated with bitter agonists (during 20 min). The tissue was homogenized using a