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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

T25 digital Ultra Turrax (IKA, Wilmington, NC, USA) at medium speed during 15 s at 4 °C in 500 µl ethanol (96%). The homogenate was centrifuged in a microcentrifuge at 5,500 rpm for 15 min at 4 °C. Protein content was determined using the method by Bradford [12] to the incubation buffer (extracellular), which was used for the cAMP assay. Incubation buffers were left at 55 °C overnight and dried samples were resuspended in 50 mM acetate buffer pH 6.2. To quantify the production of cAMP in SMG, a radioimmunoassay was used as previously described [13]. Samples (100 µl) were treated with triethyleneamine: acetic anhydride (2:1) and after 10 min, 100 µl of [^{125}I]-cAMP (Specific Act: 600 Ci/mmol) and 100 µl of a rabbit polyclonal anti cAMP antibody (a generous gift from Dr. Parlow NIDDK's National Hormone and Pituitary Program, USA) were added and mixed with shaking at 4 °C overnight. Then 50 µl of 2% bovine seroalbumin in distilled H_2O and 2 ml of ethanol (96%) were added to each tube and mixed. After 10 min samples were centrifuged at 3,000 rpm and supernatants were discarded. Radioactivity in pellets was detected in a gamma counter (Wallak, Turku, Finland). The amount of cAMP produced in the unknown samples was determined by extrapolation using a cAMP standard curve. The results were expressed as pmoles of cAMP produced per milligram of protein (pmol/mg prot.).

2.6. Determination of D-myo-inositol monophosphate levels

SMG were incubated under carbogen in 500 µl stimulation buffer (10 mM Hepes,1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl pH 7.4) at 37 °C and treated with bitter agonists (during 20 min). The tissue was homogenized using a T25 digital Ultra Turrax (IKA, Wilmington, NC, USA) at medium speed during 15 s at 4 °C in 500 µl lysis buffer (1% Triton X-100). The homogenate was centrifuged at 3,000 rpm for 5 min at 4 °C and the supernatant centrifuged again at 8000 rpm for 5 min. Protein content in the resulting supernatant was measured using the method by Bradford [12] and was used to determine D-myo-inositol monophosphate (IP_1) levels with the IP-One ELISA kit from Cisbio (Bedford, MA, USA). Briefly, PLC triggers the release of D-myo-inositol 1,4,5 trisphosphate (IP_3), which in less than 30 s is transformed into D-myo-inositol biphosphate (IP_2) and IP_1. By adding LiCl, the degradation of IP_1 is inhibited, therefore it accumulates within the cell. Tissue was incubated for 3 h under shaking, together with an IP_1-Horse-Radish Peroxidase (HRP) and an anti-IP_1 monoclonal antibody. After several washes with 0.05% Tween 20, the enzymatic activity was revealed using the HRP substrate 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped after 30 min of incubation in the dark and read in an ELISA microplate reader (BioTek, Winooski, VT, USA) at 450 nm with an optical correction at 620 nm. The amount of IP_1 produced in the unknown samples was determined by extrapolation using an IP_1 standard curve. The results were expressed as nmoles of IP_1 produced per mg of protein (nmol/mg prot.).

2.7. Statistical analysis

A GraphPad Prism computer program one-way ANOVA analysis for paired samples was used to determine the significance of differences between mean values in all control and test samples. Differences between means were considered significant if $p<0.05$. Results were expressed as mean ± SEM.

3. Results

3.1. Constitutive and stimulated secretion of amylase in murine SMG

Taking into account that almost all the previous data related to the physiology of salivary amylase secretion were obtained from experiments performed on rat parotid glands, we characterized our system by studying the kinetics of amylase secretion in *ex vivo* experiments by mouse SMG. For this purpose, we measured the spontaneous activity of extracellular amylase (i.e.: constitutive secretion) as a function of time. We observed that extracellular amylase activity increases, as maltose production rises, reaching a maximum at 20 min (Fig. 1A). We confirmed that the secretion of amylase from murine SMG is under adrenergic control, since isoproterenol and methoxamine increased extracellular amylase activity in a concentration-dependent manner (Fig. 1B) being isoproterenol more potent and effective than methoxamine ($p<0.001$) because a concentration 10^{-9} M of the β adrenergic agonist increased amylase activity by 260 ± 23% while 10^{-5} M of the α adrenergic agonist only augmented the enzyme activity by 49 ± 5% (Fig. 1C). The stimulatory action of the maximal effective concentration of each adrenergic agonist was reverted by 10^{-6} M propranolol and phentolamine respectively (Fig. 1C). We also observed that carbachol stimulated amylase release from SMG and the action of its maximal effective concentration (10^{-8} M) was reduced by the preincubation of SMG with 10^{-6} M atropine, pointing to cholinergic regulation of amylase liberation by murine SMG (Fig. 1B and C).

3.2. Expression and localization of amylase in murine SMG

We also detected by immunohistochemistry amylase positive staining in SMG slices in ducts and less in serous acini (Fig. 2A and C). Control omitting the first antibody produced no staining in SMG slices (Fig. 2B). By Western blot, we observed that the intracellular content of amylase decreases by 32 ± 4% in parallel to extracellular amylase activity increment observed at 20 min (Figs. 1B and C).

3.3. Effect of bitter compounds on amylase secretion in murine SMG

Then, we analyzed the action of three different bitter compounds: theophylline, denatonium and cycloheximide on amylase secretion. Fig. 3A shows that in SMG, secreted amylase activity is reduced in a concentration-dependent manner by all compounds. Cycloheximide exerted a maximal inhibitory action on amylase secretion at 10^{-5} M in SMG, the same concentration that triggers bitter taste receptor responses in the tongue. Denatonium and cycloheximide activate two different subtypes of bitter taste receptors in murine taste cells, T2R8 and T2R5 respectively. The combination of a subthreshold concentration of denatonium (10^{-9} M) with cycloheximide, potentiated their action on amylase secretion by mouse SMG (Fig. 3B).

3.4. Signal transduction pathway involved in the action of bitter compounds

As it has been demonstrated that bitter receptors are coupled to their effectors' signaling system via Ggustducin protein in taste cells, we investigated the expression of this G protein on murine SMG. By Western blot we observed that while Gαgustducin is expressed in CV, it is absent in SMG (Fig. 4A). Taking into account that bitter agonists exerted inhibitory actions on amylase secretion, we investigated the expression of Gi protein using an anti-Gαi specific antibody. Positive immunostaining was observed either in CV or in SMG (Fig. 4A). Negative control performed by omitting the first antibodies in SMG homogenates are also shown (Fig. 4A). Moreover, the inhibitory action triggered by bitter compounds on amylase secretion, was partially reverted by preincubating SMG tissue with the anti-Gαi antibody (5 µg/ml per 30 µg/ml tissue) after permeabilization with saponin (40 µg/ml) during 15 min a 37 °C, revealing the participation of this protein in the effect of bitter compounds on amylase liberation from SMG (Fig. 4B). It has been previously reported that Ggustducin couples bitter taste receptors to PDE or adenylyl cyclase, regulating cAMP levels in taste tissue. For this reason we evaluated the action of bitter compounds on cAMP production in SMG. We observed that 10^{-6} M theoplylline and 10^{-5} M cycloheximide increased the nucleotide levels by more than 2 fold, while 10^{-5} M denatonium was less potent

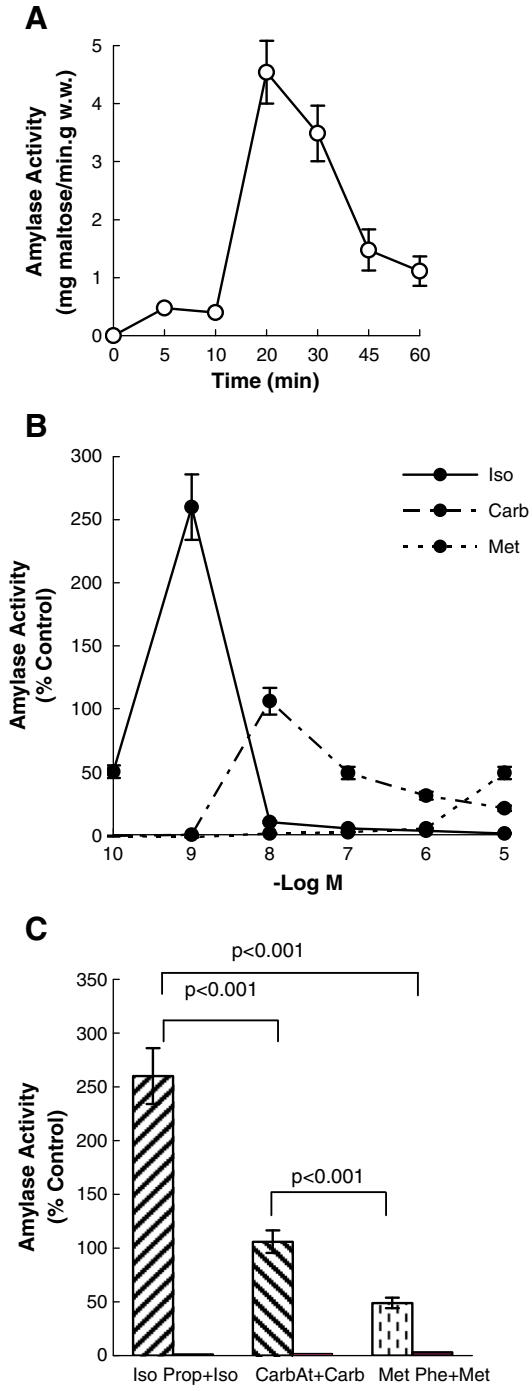

Fig. 1. Amylase secretion in murine submandibular gland (SMG). A) Time-dependence of amylase secretion. Amylase activity was measured in the incubation solution (extracellular) as it was stated in Materials and methods. Values are mean ± SEM of n = 4 experiments. Results were expressed as milligrams of maltose per minute and per gram of wet weighed tissue (mg maltose/min. g w.w.). B) Concentration–response curves of isoproterenol (Iso), carbachol (Carb) and methoxamine (Met) on amylase activity determined in the secreted fraction as it was stated in Materials and methods. Values are mean ± SEM of n = 3 experiments. Results were expressed as percentage of control (untreated SMG). C) Action of the maximal effective concentration of isoproterenol (Iso) (10^{-9} M), carbachol (Carb) (10^{-8} M) and methoxamine (Met) (10^{-5} M) on amylase activity determined in the secreted fraction of the glands, in the absence or presence of (10^{-6} M) propranolol (Prop), atropine (At) or phentolamine (Phe). Values are mean ± SEM of n = 3 experiments. Results were expressed as percentage of control (untreated SMG).

to increase cAMP levels in SMG (~63%) (Fig. 4C). The preincubation of SMG with an anti-Gαi antibody (5 μg/ml per 30 mg/ml tissue) after permeabilization with saponin (40 μg/ml) during 15 min a 37 °C, significantly reduced the effect of bitter compounds on cAMP production (Fig. 4C).

By Western blot we observed that PLCβ2, the effector enzyme involved in bitter taste signaling pathway, is expressed either in CV or in SMG (Fig. 4A). In addition, the inhibitory action on amylase secretion exerted by denatonium and cycloheximide (56.8 ± 4.4% and 44.0 ± 3.2%, respectively, considering control as 100%) was partially reverted by adding 10^{-5} M 2-nitro-4-carboxyphenyl N,N diphenyl-carbamate (NCDC), a non selective PLC inhibitor, before the bitter agonists (71.2 ± 2.3% and 72.0 ± 1.9%; $p<0.001$ vs. control), pointing to the participation of this enzyme in the secretion of amylase from SMG. Moreover, IP turnover, measured as IP_1 production by SMG, was significantly inhibited by 10^{-6} M theophylline or by 10^{-5} M denatonium and cycloheximide (Fig. 4D). This inhibitory action on IP_1 levels, was not modified by adding the anti-Gαi antibody before bitter agonists (data not shown).

Taking into account that protein kinase A (PKA) has been pointed as a cross talk enzyme between cAMP and PLC metabolic pathways, we tested the action of a PKA inhibitor on inositol phosphate turnover. The preincubation of SMG with 10^{-5} M H-89 returned denatonium inhibitory effect to control value and reversed the inhibition produced by theophylline and cycloheximide to stimulation, doubling the control value (Fig. 4D).

3.5. Expression of T2R6 in murine SMG

Considering that T2R receptors were identified in the gastrointestinal tract, these receptors could also be expressed in SMG. By immunohistochemistry, we identified the presence of the T2R6 subtype and Fig. 5A and C show positive and specific staining in the ducts and less in the serous acini of SMG. Fig. 5B shows the image of the negative control of T2R6 immunohistochemistry in SMG slices, obtained by omitting the specific anti T2R6 antibody. By Western blot we detected significant amounts of T2R6 protein on murine salivary glands, using mouse CV as a positive control for the expression of these receptors. Negative control performed without the primary antibody is also shown (Fig. 5D).

4. Discussion

As accessory exocrine glands of the digestive tract, the salivary glands supply a variety of proteins, fluids and electrolytes that play a key role in facilitating the onset of the digestive process [3]. In major salivary glands, the components of saliva are mainly produced by acinar cells and are conveyed to the oral cavity by a cell-lined duct system [1]. Mammalian salivary glands are mainly composed of two epithelial cell types: the acinar cells, which secrete the salivary fluid as well as most of the salivary proteins, and the ductal system, which secretes some proteins and modify the ionic composition of saliva as they convey it to the mouth [14]. It is well known that SMG, major salivary glands, secrete amylase, an important digestive enzyme, and produce saliva that is isotonic with plasma [15]. Regarding amylase, here we demonstrate in *ex vivo* experiments that the spontaneous activity of amylase in the extracellular compartment is an increasing function of time until 20 min, and then decreases. This effect is simultaneously accompanied by a moderate decrease in the amount of protein in the gland. We confirmed that the secretion of amylase to external media is under autonomic nervous control, since all adrenergic and cholinergic agonists increased amylase activity in this media, being the β adrenergic agonist, isoproterenol, the most potent stimulant. Precisely, Quissell [16] reported that noradrenaline activates β adrenergic receptors leading to increased cAMP levels that is essential for exocytotic secretion. It has also been documented that stimulation of α adrenergic receptors or muscarinic receptors also elicits amylase release but at levels that are significantly lower than those observed from β adrenergic receptor-mediated response in rat

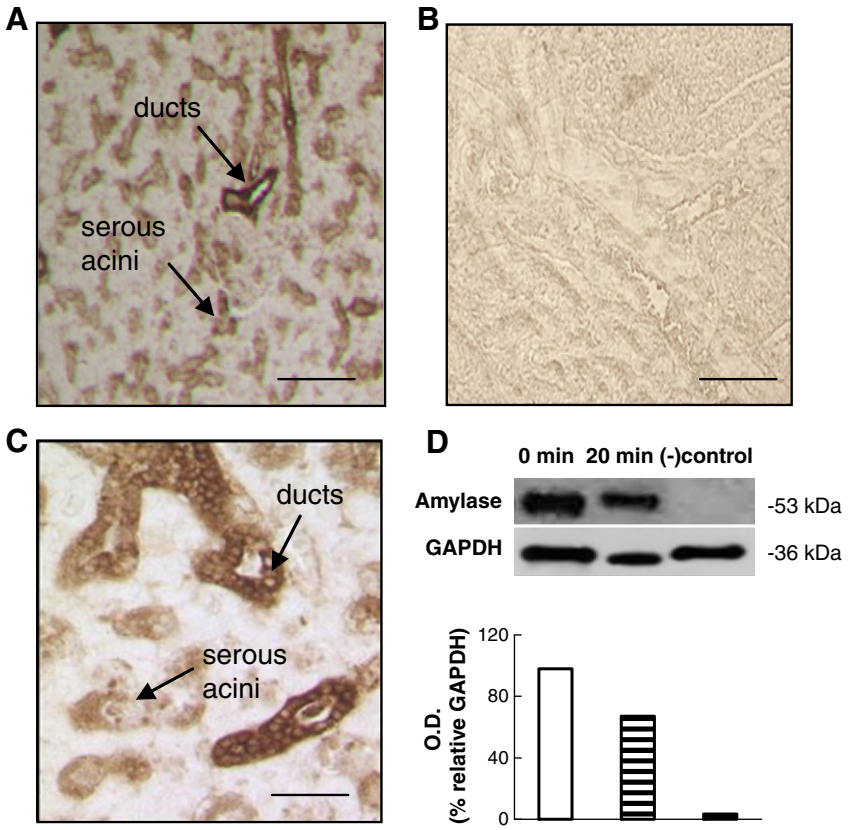

Fig. 2. Amylase expression in murine submandibular glands (SMG). Immunohistochemistry using a specific amylase antibody in SMG. Immunostaining is seen mainly in serous cells and ductal cells. A) Scale bar = 60 μm, C) scale bar = 15 μm, and B) negative control, omitting primary antibody. Scale bar = 60 μm. D) Western blot assay to detect amylase was performed in homogenates of murine SMG. Molecular weights of proteins are indicated on the right. Optical density of the bands was calculated by densitometric analysis, and values are plotted as histograms. Values were relativized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as loading control. One representative experiment of three is shown.

parotid glands [14]. These results are concordant with ours obtained with methoxamine and carbachol on the release of amylase from mouse SMG. It was reported that amylase is localized in the cells of the GCT from the ductal system, since these cells show abundant granules by histology images [17,18]. Complementing these previous works, using a specific anti-amylase antibody, our results indicate that amylase is localized in cells from the ductal system and also in acinar cells.

The addition of three bitter-tasting compounds which differ in their chemical structure: denatonium, a quaternary amine, cycloheximide, a

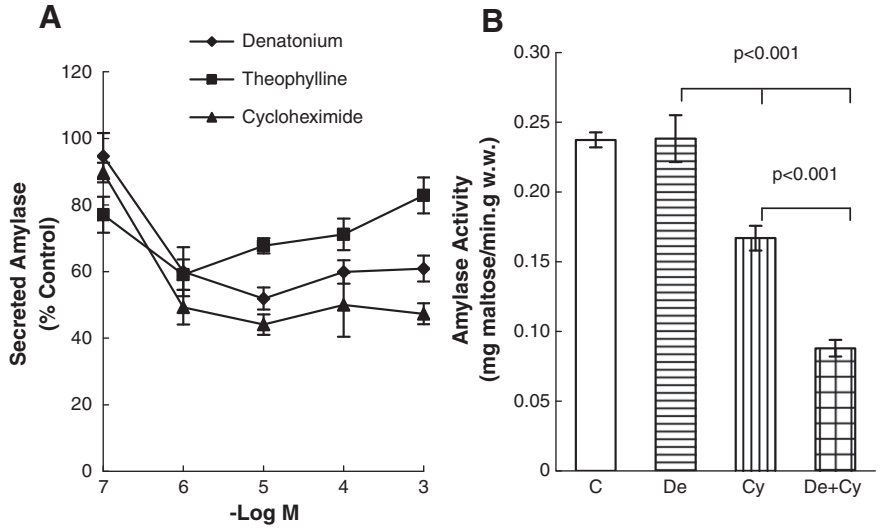

Fig. 3. Effect of bitter compounds on amylase secretion in submandibular glands (SMG). A) Concentration–response curves of theophylline, denatonium and cycloheximide. Values were expressed as percentage of control (untreated SMG) considered as 100% and are mean ± SEM of n = 4 experiments. B) Effect of the combination of a subthreshold concentration of denatonium (Den) (10^{-9} M) with a threshold concentration of cycloheximide (Cy) (10^{-9} M). Values were expressed as milligram of maltose produced per min and per gram of wet weighed tissue (mg maltose/min. g w.w.).

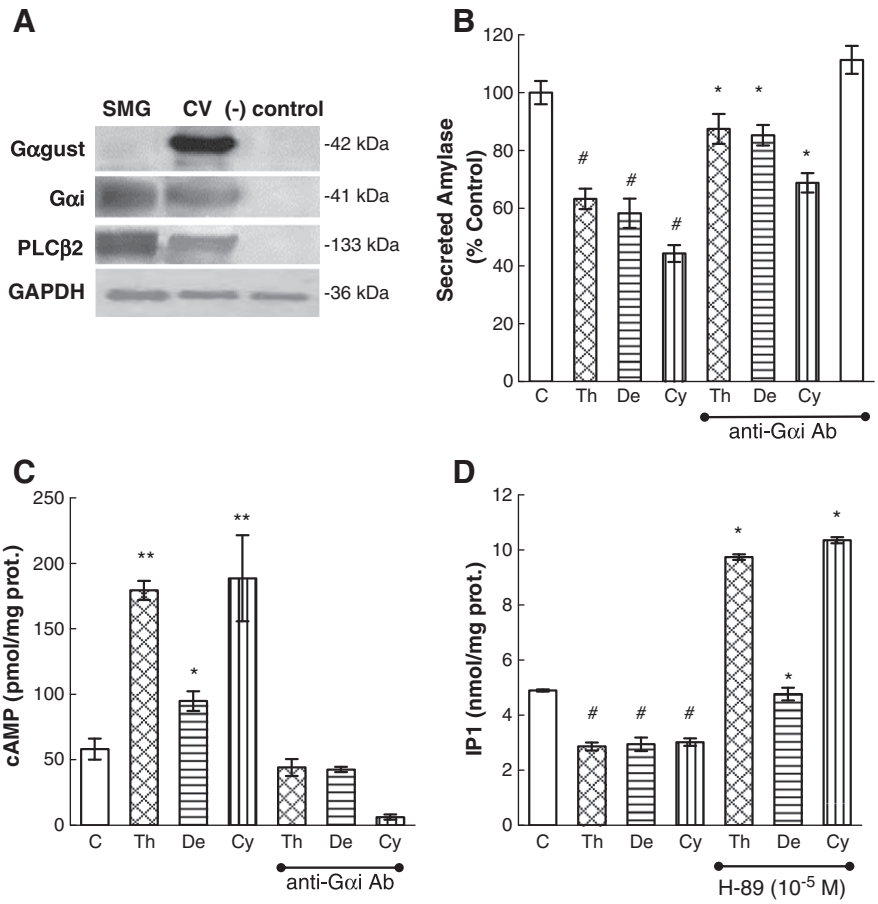

Fig. 4. Participation of G protein and signaling molecules in the effect of bitter compounds on amylase secretion from murine submandibular gland (SMG). A) Western blot to detect Gαgustducin (Gαgust), Gαi and phospholipase Cβ2 (PLCβ2) was performed in homogenates from SMG as it was stated in Material and methods. Molecular weights of the proteins were indicated on the right. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. One representative experiment of three is shown. Positive control was performed in murine circumvallate papillae (CV) of the tongue. Negative (−) control was performed in SMG omitting the primary antibody. B) Effect of anti-Gαi antibody (5 μg/ml per 30 mg/ml) added before the maximal effective concentration of theophylline (Th) (10^{-6} M), denatonium (De) (10^{-5} M) or cycloheximide (Cy) (10^{-5} M) on amylase secretion. SMG tissue was permeabilized with saponin (40 μg/ml) for 15 min at 37 °C. Values are mean ± SEM of n = 3 experiments. Results were expressed as percent of control (C) (untreated SMG) considered as 100%. #p<0.001 vs. control; *p<0.001 vs. their respective bitter compound. c) Effect of anti Gαi antibody (5 μg/ml per 30 mg/ml) added before the maximal effective concentration of theophylline (Th) (10^{-6} M), denatonium (De) (10^{-5} M) or cycloheximide (Cy) (10^{-5} M) on cAMP production by SMG. SMG tissue was permeabilized with saponin (40 μg/ml) for 15 min at 37 °C. Values are mean ± SEM of n = 3 experiments. Results were expressed as picomoles per milligram of protein (pmol/mg prot.) Control (C) (untreated SMG) *p<0.05; **p<0.001 vs. C. D) Effect of H-89 (10^{-5} M) added before the maximal effective concentration of theophylline (Th) (10^{-6} M), denatonium (De) (10^{-5} M) or cycloheximide (Cy) (10^{-5} M) on IP1 production by SMG. Values are mean ± SEM of n = 3 experiments. Results were expressed as nanomoles per milligram of protein (nmol mg/prot.). Control (C) (untreated SMG). #p<0.001 vs. control; *p<0.001 vs. their respective bitter compound.

dimethyl glutarimide antibiotic and the methylxantine theophylline, inhibit amylase secretion with similar maximal effective concentrations (10^{-5} M–10^{-6} M). It has been reported that when different bitter compounds enter the oral cavity, they stimulate bitter taste receptors [5]. Ozeck et al. [19] have reported that cycloheximide, a selective agonist for mouse T2R5 subtype, can elicit biological responses due to receptor activation at a range between 10^{-7} M and 10^{-4} M when this bitter receptor is expressed in HEK293 cells, being 10^{-5} M the maximal effective concentration for promoting this action. Moreover, denatonium, is known to activate mouse T2R8 and it has been recently demonstrated that it is sensed as bitter in the mouth of three different species of non human primates at 10^{-5} M concentration [5,20]. Concordantly, we observed a maximal effective response on the inhibition of amylase secretion at 10^{-5} M, for denatonium and cycloheximide. A combination of subthreshold/threshold concentrations of both bitter agonists exerts a synergistic effect on the inhibition of amylase secretion; probably revealing that their action on different bitter taste receptors at the same time leads to a greater effect than that of both compounds added separately [21].

In taste cells, bitter receptors are coupled via Ggustducin, to their signaling effector system. Activated Gαgustducin stimulates PDE to hydrolyze cAMP and Gβγ dimmer released from activated Ggustducin, stimulates PLCβ2 to generate IP_3, which leads to the release of Ca^{2+} from internal stores [22,25]. However, Ggustducin protein is not expressed in SMG, but Gαi protein is, and its activation is involved in the inhibitory effect triggered by bitter compounds on amylase secretion as it is reverted by adding an anti-Gαi antibody to SMG before the bitter agonists. Evidence indicates that bitter taste receptors coupled to other G proteins than Ggustducin either in transfected cells or in taste cells [21,23]. Particularly, Ueda et al. [24] concluded that T2R can couple to G proteins other that αgustducin that are found in taste cells, including $G\alpha_{i2}$ and thus this protein may be involved in bitter taste perception in mice. The absence of Gαgustducin in murine SMG would allow bitter compounds coupling Gαi to PDE leading to the inhibition of PDE activity and as a consequence up-regulating cAMP levels. The latter was confirmed when we measured bitter compounds induced-cAMP levels in the presence of an anti-Gαi antibody.

PLCβ2 isoform is present in SMG and bitter compounds reduced IP_1 production. This effect was not modified in the presence of an anti-Gαi antibody (data not shown) revealing that this action was not directly mediated via an αi protein subunit. Turner and Sugiya [14] have studied the mechanism involved in the secretion of amylase in rat parotid acinar

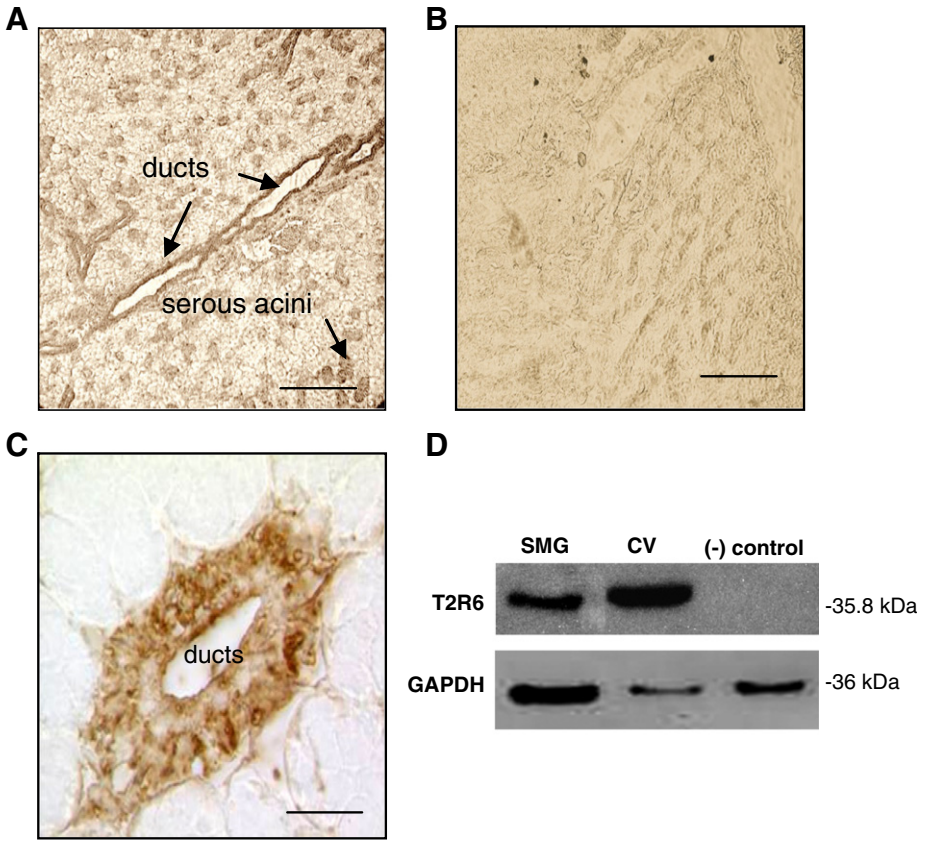

Fig. 5. Expression of T2R6 receptor in murine submandibular gland (SMG). Immunohistochemistry using a specific anti-T2R6 antibody in SMG. Immunostaining is seen mainly in ductal cells. A) Scale bar = 60 μm and C) scale bar = 9 μm. B) Negative control, omitting the primary antibody. Scale bar = 60 μm. D) Western blot assay to detect T2R6 was performed in homogenates of murine SMG. Circumvallate papillae (CV) were used as positive control. Negative (−) control, without primary antibody is shown on the third lane. Molecular weights of proteins were indicated on the right. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. One representative experiment of three is shown.

cells. They postulated that this process is composed by three steps: (1) docking of vesicles to the plasma membrane (2) a "priming" event accompanied by ATP hydrolysis and promoted by cAMP and (3) fusion/secretion triggered by the elevation of intracellular Ca^{2+} concentrations. Without the last step, amylase secretion is reduced. On line with our results, other studies indicate that elevated cAMP levels inhibit phosphoinositide hydrolysis and IP_3 generation in a variety of cell types [26,27]. The interaction between elevated cAMP and intracellular Ca^{2+} release could also occur by the modulation of PLC and/or IP_3 receptors by cAMP dependent-PKA activation that could turn down the PLC pathway [28]. Concordantly we observed that the addition of the PKA inhibitor H-89 previous to bitter agonists reverted the inhibitory action on IP_1 levels exerted by denatonium and turned to stimulatory the effect of theophylline and cycloheximide.

The signaling pathways activated in taste cells by methylxantines like caffeine and theophylline have not been studied in detail. Regarding theophylline, our results indicate that besides its known direct action on PDE activity [29] it could be also acting through bitter GPCR, since its actions on amylase secretion and cAMP levels were reverted in the presence of an anti-Gαi antibody.

Besides being present in the taste buds of the oral cavity, some members of the T2R receptor family were also found in the gastrointestinal tract of rodents and it has been suggested that they could modulate intestinal incretin secretion in humans [30]. SMG are part of the digestive tract and we demonstrated for the first time that the T2R6 subtype is expressed in murine SMG, predominantly in serous cells and interlobular and striated ducts, a location that is coincident with amylase expression. It must be taken into account that the anti-T2R6 antibody is one of the few commercially developed for immunodetection purposes.

In conclusion, three bitter compounds that differ in their structure inhibited amylase secretion in murine SMG. This action was exerted via a Gi protein, expressed in these glands. The Gαi subunit was involved in the up-regulation of cAMP, which in turn activates a PKA protein, inhibiting the PLC-IP_1 metabolic pathway.

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