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Tonic activity of Gα-gustducin regulates taste cell responsivity

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Abstract **The taste-selective G protein, α-gustducin (α-gus) is homologous to α-transducin and activates phosphodiesterase (PDE) in vitro. α-Gus-knockout mice are compromized to bitter, sweet and umami taste stimuli, suggesting a central role in taste transduction. Here, we suggest a different role for Gα-gus. In taste buds of α-gus-knockout mice, basal (unstimulated) cAMP levels are high compared to those of wild-type mice. Further, H-89, a cAMP-dependent protein kinase inhibitor, dramatically unmasks responses to the bitter tastant denatonium in gus-lineage cells of knockout mice. We propose that an important role of α-gus is to maintain cAMP levels tonically low to ensure adequate Ca^{2+} signaling.**

Keywords: Taste transduction; Calcium signaling; Phosphodiesterase; Protein kinase A

1. Introduction

Bitter, sweet, and umami (glutamate) taste stimuli are transduced by taste G protein-coupled receptors (taste GPCRs) and downstream signaling effectors. Two families of taste GPCRs exist, the T1Rs, for sweet and umami, and T2Rs, for bitter [1–3]. Taste GPCRs activate heterotrimeric G proteins that contain Gβ3 and Gγ13 [4]. The heterodimer, β3γ13, is released from tastant-bound receptors, where it stimulates phospholipase Cβ2 (PLCβ2) [5,6] to produce inositol trisphosphate (IP_3) and activate the type 3 IP_3 receptor (IP_3R3) [7,8] to release stored Ca^{2+}. Mice in which these effector genes are knocked out show taste afferent nerve and behavioral responses to sweet, bitter and umami stimuli that are either eliminated in the case of PLCβ2, [6], or compromized in the case of IP_3R3, [9]. These findings underline the central roles of PLCβ2 and IP_3R3 in taste transduction.

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Abbreviations: CMF, calcium–magnesium free; CV, circumvallate; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; Gus, gustducin; IP_3R3, inositol trisphosphate receptor type III; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PLCβ2, phospholipase C β2

Gα-gustducin (α-gus) [10] is a frequent partner of Gβ3γ13 and α-gus$^{-/-}$ mice are compromized to bitter, sweet, and umami stimuli [11]. Yet, despite its discovery over 15 years ago, the precise role of α-gus in taste transduction is still unclear. As with the closely related Gα-transducins, effector-interacting peptides derived from α-gus can activate a retinal phosphodiesterase (PDE) in vitro to decrease cyclic nucleotide levels [12,13].

We considered the possibility that α-gus in taste cells may regulate cAMP levels in a continuous fashion, in the absence of taste ligands. If this were the case, genetic ablation of α-gus should markedly alter *basal* levels of cAMP in taste cells. We directly tested this hypothesis by measuring cAMP levels in taste buds of α-gus$^{+/+}$ and α-gus$^{-/-}$ mice. We selected circumvallate (CV) taste buds for this analysis because: (a) taste buds are numerous, (b) earlier studies linking gus to bitter transduction were performed in CV, and (c) α-gus couples primarily to bitter receptors in this taste field [1]. We show that taste buds of α-gus$^{-/-}$ mice have highly elevated *basal* levels of cAMP relative to those in α-gus$^{+/+}$ mice. Further, we show in taste buds from α-gus$^{-/-}$ mice, that elevated cAMP in α-gus-lineage cells activates cAMP-dependent protein kinase A (PKA) causing a chronic inhibition of Ca^{2+} responses to bitter stimuli. Our data lead us to propose a novel explanation of the α-gus-knockout phenotype. We suggest that taste buds in α-gus$^{-/-}$ mice exist in a chronically depressed state, unable to generate robust release of stored Ca^{2+} in response to any of the taste GPCR-mediated taste qualities.

2. Materials and methods

2.1. Animals

Mouse housing and experimental procedures were approved by Colorado State University's Animal Care and Use Committee. Animals were killed by exposure to CO_2 followed by cervical dislocation before tongues were removed. Adult α-gus$^{-/-}$ mice [11] and α-gus$^{+/+}$ littermates were used for cAMP measurements. For Ca^{2+} imaging experiments, transgenic mice in which the α-gus promoter drives expression of green fluorescent protein (GFP), i.e. gus-GFP [4], were crossed with α-gus$^{-/-}$ mice. GFP-positive, α-gus-negative progeny were identified. In these mice, taste cells of the α-gus-lineage express the GFP label, while lacking α-gus itself.

2.2. Physiological solutions and reagents

Tyrode's solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 glucose, and 1 pyruvate (pH 7.4 with NaOH). Calcium–magnesium free (CMF) Tyrode's solution was similar to the above except that $MgCl_2$ and $CaCl_2$ were omitted (i.e. nominally

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CMF) or were replaced with 1 mM BAPTA for isolating taste buds. The PKA inhibitor, H-89, and the protein kinase C (PKC) inhibitor, bisindolymaleimide I (Bis, Calbiochem; San Diego, CA, USA) were diluted from dimethyl sulfoxide stocks into Tyrode's solution before use.

2.3. cAMP measurements

Taste bud-enriched CV epithelia from α-gus$^{-/-}$ and α-gus$^{+/+}$ mice were enzymatically delaminated, dissected free of adjacent non-taste epithelium, and were processed in parallel as we described previously [14,15]. Tissues were lysed to extract cAMP into a soluble supernatant and total cAMP in each tissue extract was measured using enzyme immunoassay (Amersham Biosciences, Piscataway, NJ, USA) [14,15]. Total protein in each tissue piece was quantified using a Nano-Orange Kit (Invitrogen, Carlsbad, CA, USA). In all cases, cAMP titer is presented normalized to total protein in the tissue.

2.4. Immunocytochemistry

α-Gus was immunodetected in paraformaldehyde fixed cryosections, using a rabbit polyclonal anti-α-gus (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-395; 1:500) and Cy5 goat anti-rabbit secondary (Jackson ImmunoResearch Laboratories) [16]. Controls for antibody specificity included omitting primary antibody and the lack of staining in taste buds of the α-gus$^{-/-}$ mouse.

2.5. Taste cell isolation

CV taste epithelia were isolated and placed in CMF-Tyrode's solution for 5 min. Taste buds were removed by gentle suction with a fire-polished pipet and plated onto cover slips coated with poly-L-lysine (Sigma, St. Louis, MO, USA) [17].

2.6. Ca^{2+} imaging

Intracellular Ca^{2+} measurements were obtained from fura-2-loaded taste cells as described previously [16]. Images were acquired with the CCD Sensicam QE camera (Cooke Co., Romulus, MI, USA) using a 40× oil immersion objective lens. Emission at ~510 nm was captured following sequential excitation at 350 nm and 380 nm. Calcium levels are reported as a ratio of fluorescence emissions, F350/F380, captured every 1–5 s using Imaging Workbench 5.2 (Indec Biosystems Inc.). Denatonium (Sigma), H-89 (10 μM, Calbiochem), and bisindolymaleimide I (0.15 μM, Calbiochem) were bath applied using gravity flow perfusion. In most experiments, denatonium was applied at 10 mM, the lowest concentration that elicits significant afferent nerve responses and behavioral rejection in α-gus$^{-/-}$ mice [11], and is a sub-maximal concentration for nerve recordings in wild-type mice [18].

2.7. Statistical analyses

Paired and unpaired two-tailed *t*-test and Bonferroni corrections were performed using Prism v5.00 (GraphPad, San Diego, CA, USA).

3. Results

α-Gus activates a retinal PDE in vitro [12,13]. If a similar activity occurs in taste cells, chronic absence of α-gus might result in altered cAMP levels. Hence, we measured total cAMP in CV taste bud-enriched epithelium from α-gus$^{-/-}$ and α-gus$^{+/+}$ mice. We also carried out parallel measurements of cAMP levels in adjacent non-taste lingual epithelium as a control. The resting level of cAMP in α-gus$^{+/+}$ circumvallate epithelium was 2.65 ± 0.35 (mean ± S.E.M.) pmole cAMP/μg protein. The value is similar to that measured from rat CV epithelium [14,15]. The basal level of cAMP was approximately 3.8 fold higher in CV epithelium from α-gus$^{-/-}$ mice as compared to α-gus$^{+/+}$, a highly significant difference ($P = 0.0005$; $n = 6$; *t*-test; Fig. 1). We attributed this difference to taste buds because adjacent regions of non-taste epithelium had much lower resting levels of cAMP, and these did not differ between genotypes ($P = 0.504$; $n = 6$; *t*-test). The data suggest that cAMP levels in taste buds are controlled by a PDE that is primarily regulated by α-gus, and may be active in the absence of taste stimulation.

Because α-gus$^{-/-}$ mice have elevated basal levels of cAMP, phosphorylation by PKA could also be chronically elevated and this could underlie the decreased bitter sensitivity observed in α-gus$^{-/-}$ mice [19]. In that earlier study, it could not be determined whether residual responses to bitter tastants were in cells of the α-gus-lineage. To resolve this uncertainty, we used mice in which GFP was expressed in α-gus-lineage taste cells of α-gus$^{-/-}$ mice (Fig. 2). Taste cells, loaded with fura-2, were stimulated with denatonium (10 mM). Denatonium elicited little or no increase in intracellular Ca^{2+} in the GFP-labeled taste cells of the α-gus$^{-/-}$ mice. To determine if elevated cAMP and PKA-dependent phosphorylation were responsible for this loss of sensitivity, we also examined bitter responses in the presence of the membrane permeant PKA inhibitor, H-89 (10 μM). After treatment with H-89, Ca^{2+} responses in GFP-labeled α-gus$^{-/-}$ cells were larger (Fig. 3A). Responses to denatonium were unmasked by H-89 in 7 of 21 GFP-labeled α-gus$^{-/-}$ taste cells that were previously unresponsive to denatonium (e.g. Fig. 3A). On average, responses to denatonium in α-gus$^{-/-}$ cells were significantly enhanced, 6.5-fold in the presence of H-89 (Fig. 3A and C). We noted that H-89 caused a slight elevation of the baseline Ca^{2+} in many taste cells, even prior to taste stimulation. This suggests that both basal Ca^{2+} levels and taste-evoked Ca^{2+} signals are regulated by PKA activity.

Next, we asked if H-89 had a similar enhancing effect on Ca^{2+} responses to denatonium in gus-expressing taste cells of α-gus$^{+/+}$ mice. Surprisingly, we found that H-89 also significantly increased Ca^{2+} responses to denatonium in α-gus$^{+/+}$ mice (2.9-fold; Fig. 3B and C). After H-89 treatment, responses to denatonium were unmasked in 5 of 26 previously unresponsive Gus-GFP-positive cells.

The effect of H-89 on individual taste cells was reversible and repeatable (Fig. 3D) in both knockout and wildtype mice. The vehicle, DMSO, had no effect on either resting Ca^{2+} level or denatonium responses ($n = 4$; data not shown). Although 10 μM H-89 is selective for PKA, it may also block PKC slightly. Thus, we applied a robust, membrane permeant inhib-

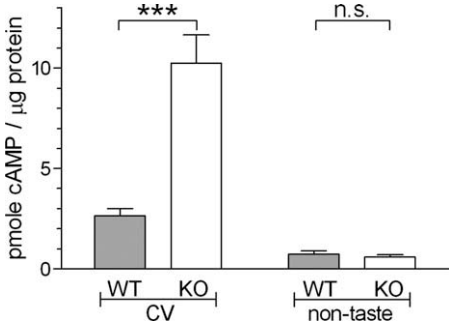

Fig. 1. Taste buds in gus$^{-/-}$ mice have elevated resting levels of cAMP. Taste epithelium delaminated from CV papillae, and adjacent non-taste (NT) lingual epithelium from gus$^{+/+}$ and gus$^{-/-}$ mice were analyzed for cellular cAMP and total protein. Mean (±S.E.M.) basal values in pmole cAMP per μg protein were: 2.65 ± 0.35 (α-gus$^{+/+}$ taste), 10.25 ± 1.40 (α-gus$^{-/-}$ taste); 0.73 ± 0.17 (α-gus$^{+/+}$ non-taste) and 0.58 ± 0.12 (α-gus$^{-/-}$ non-taste). Taste samples were significantly different across the genotypes ($P = 0.0005$; $n = 6$; unpaired *t*-test) while the non-taste samples were not different ($P = 0.50$; $n = 6$; unpaired *t*-test).