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Phenanthroline Induces Bronchodilation in Mice Via TAS2R-Gustducin Signaling

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Abstract

Bitter taste receptors (TAS2Rs) in airway smooth muscle (ASM) mediate potent bronchodilation, yet the specific G protein coupling underlying this response remains incompletely defined. Here, we investigated whether GNAT3, the α-subunit of the gustducin G protein and a hallmark of the Gi/o family, is required for phenanthroline (Phen)-induced airway relaxation. Using smooth muscle–specific Gnat3−/− mice and pharmacological approaches, we demonstrate that Phen elicits robust, dose-dependent relaxation of methacholine-contracted murine airways, with maximal efficacy comparable to the β2-agonist R-albuterol, albeit at higher concentrations. This relaxant effect was significantly attenuated in Gnat3−/− airways, indicating partial dependence on GNAT3. At the cellular level, Phen suppressed methacholine-induced global intracellular calcium elevation ($[Ca^{2+}]_i$) in ASM cells—a key mechanism driving relaxation. Critically, this inhibitory effect on $[Ca^{2+}]_i$ was abolished by pertussis toxin (PTX), a selective Gi/o inhibitor, and markedly reduced in Gnat3−/− cells. Together, these findings provide convergent genetic and pharmacological evidence that Phen induces bronchodilation via a GNAT3-dependent, PTX-sensitive Gi/o signaling pathway that dampens $[Ca^{2+}]_i$ mobilization. The incomplete blockade by Gnat3 deletion or PTX suggests contributions from additional G proteins or parallel mechanisms. Our study establishes GNAT3 as a functional component of the TAS2R–gustducin axis in ASM and advances the mechanistic understanding of bitter tastant–mediated airway relaxation with therapeutic potential for obstructive lung diseases.

Keywords

TAS2R; GNAT3; gustducin; airway smooth muscle; bronchodilation

Introduction

Gustducin is a heterotrimeric G protein initially identified in taste receptor cells and serves as a key mediator of bitter, sweet, and umami taste transduction [1-3]. Its α-subunit, GNAT3 (guanine nucleotide-binding protein, alpha-transducing 3), is the defining molecular component that confers functional specificity to gustducin [4, 5]. Heterotrimeric G proteins, composed of α, β, and γ subunits, are central to signal transduction downstream of G protein–coupled receptors (GPCRs). Based on the structural and functional properties of their α-subunits (Gα), they are classified into four canonical families: Gs, Gi/o, Gq/11, and G12/13, each engaging distinct downstream effectors [6, 7]. Among these, GNAT3-a Gα subunit belonging to the Gi/o family—remains relatively understudied in airway smooth muscle [8].

Recent studies have demonstrated that bitter compounds such as Phenanthroline induce potent relaxation of airway smooth muscle (ASM) in both mice and humans, primarily through activation of TAS2R (T2R) bitter taste receptors [9-13]. However, the identity of the G protein(s) coupling T2Rs to downstream signaling in ASM remains

controversial. Two non-canonical mechanisms have been proposed: (1) T2R-activated Gβγ directly binds to the inositol 1,4,5-trisphosphate receptor (IP3R), thereby inhibiting Gq-mediated Ca^{2+} release from sarcoplasmic stores [14]; and (2) Gβγ suppresses voltage-dependent calcium channel (VDCC)-mediated extracellular Ca^{2+} influx—essential for sustained ASM contraction—independently of large-conductance Ca^{2+}-activated potassium (BKCa) channels [15].

In both models, the Gα subunit serves as a critical nodal point, as its identity determines whether Gβγ is released from a Gi/o-type heterotrimer (e.g., gustducin/GNAT3) or another G protein family. Clarifying whether Gi/o, specifically via GNAT3, participates in phen-induced bronchodilation is therefore essential to elucidate the molecular mechanism underlying bitter tastant-mediated airway relaxation.

To address this, we employed pharmacological inhibition and generated smooth muscle-specific Gnat3 knockout mice to determine whether GNAT3, as a hallmark Gα subunit of the Gi/o family, is required for T2R-mediated airway relaxation. Our findings provide direct evidence for the involvement of GNAT3-dependent Gi/o signaling in bitter compound-induced bronchodilation, advancing our understanding of non-adrenergic airway relaxation pathways with therapeutic potential.

1. Methods

1.1 Animals and ethics statement

The GNAT3-/- mice were obtained from Jackson Laboratory. Age matched Wild types were used in this study. 3-4-month-old mice were utilized in this study. Prior to enrollment in the study, all animals were genotyped using standard PCR methods to confirm genotypes. All experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Shandong First Medical University & Shandong Academy of Medical Science.

1.2 Immunofluorescence

Mouse bronchi were harvested immediately after euthanasia by CO_2 asphyxiation. Tissues were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, cryoprotected, and sectioned at a thickness of 10 μm using a cryostat. Sections were blocked for 1 h at room temperature with blocking buffer containing 10% bovine serum albumin (BSA) and 0.3% Triton X-100 in phosphate-buffered saline (PBS). To check for GNAT3 immunoreactivities and label the smooth muscles, the sections were incubated with Rabbit anti-GNAT3 antibody (1:1000, Abcam) overnight at 4°C, respectively, or co-incubated with mouse anti-α-actin antibody (1:1000, Santa Cruz). The sections were incubated with goat anti-rabbit antibody (1:1000, Alexa Fluor 488) or the mixture of goat anti-rabbit antibody (1:1000, Alexa Fluor 488) and goat anti-mouse antibody (1:1000, Alexa Fluor 555) for 1 h at room temperature, followed by nucleic acid dye DAPI. Pictures were acquired by a confocal laser-scanning microscope (ZEISS Celldiscoverer 7 Confocal Microscope).

1.3 Isometric contraction measurement

Mouse extrapulmonary bronchi were carefully dissected from the lungs and freed from the surrounding connective and adipose tissues in ice-cold Krebs solution (composition in mM: 118 NaCl, 4.69 KCl, 2.52 $CaCl_2$, 1.16 $MgSO_4$, 1.01 NaH_2PO_4, 25 $NaHCO_3$, and 11.1 glucose). Bronchial rings approximately 2.5 mm in length were prepared and mounted on a wire myograph (Model 610M, Danish Myo Technology). Isometric tension was recorded using a PowerLab data acquisition system (AD Instruments). Tissues were bathed in oxygenated Krebs solution (95% O_2 / 5% CO_2) maintained at 37°C. After a 20-minute equilibrium, a 2 mN stretch was applied three times to the rings. Then, stimulation with 60 mM K^+ was performed twice with a 10 - minute interval prior to assessing the response to methacholine.

1.4 Isolation of airway smooth muscle cells

The trachea and main bronchi were rapidly dissected from mouse lungs and immediately placed in ice-cold dissociation medium (DM; in mM: 136 NaCl, 5.36 KCl, 0.44 KH_2PO_4, 4.16 $NaHCO_3$, 0.34 Na_2HPO_4, 5 $MgCl_2$, 20 HEPES, and 10 glucose, pH 7.1). Surrounding connective and adipose tissues were carefully removed under a stereomicroscope using fine surgical scissors. The cleaned airway segments were then incubated at room temperature for 30 min in Mg^{2+}-free DM supplemented with 30 U/mL papain (Sigma - Aldrich), 0.2 mM dithiothreitol (DTT), and 0.02 mM EDTA.

Following enzymatic pre-digestion, tissues were transferred to a second digestion solution (Mg^{2+}-free DM) containing 3 U/mL collagenase IA (Sigma - Aldrich), 1 mg/mL bovine serum albumin (BSA), and 0.1 mg/mL

deoxyribonuclease I (Worthington Biochemicals). Digestion was performed sequentially at 34°C for 6 min, followed by two additional 4 - minute intervals. After digestion, the tissue fragments were gently triturated using a fire-polished glass Pasteur pipette to generate a single cell suspension. The dispersed cells were resuspended in DM containing 1 mM Ca^{2+} and stored on ice for functional experiments within 6 hours.

1.5 Measurement of global $[Ca2+]_i$

To monitor global $[Ca^{2+}]_i$, fura-3-AM (Molecular Probes™, ThermoFisher) fluorescence was measured using the ImageXpress Micro Confocal Imaging System (MOLECULAR DEVICES, USA). Fresh isolated airway smooth muscle cells were incubated in DM solution containing 1 μM fluo-3-AM for 1hours in room temperature. The cells were then placed on the glass-bottomed chamber, and the medium was changed to bath solution (in mM) (130 NaCl, 5.5 KCl, 1 $MgCl_2$, 10 Hepes, 2.2 $CaCl_2$, and 5 glucose, pH 7.4). Before imaging, changed the medium into normal DM without fluo-3-AM. The 488 nm line of an Argon Ion laser provided fluorescence excitation. The images were acquired at a speed of 1 Hz for global $[Ca^{2+}]_i$ measurement. Image J (1.54j version) was utilized for the analysis. $[Ca^{2+}]_i$ was represented as $\Delta F/F_0 \times 100$, with F calculated by integrating fluo-3 fluorescence over the entire cells for global $[Ca^{2+}]_i$ after background correction with areas free of cells.

1.6 Statistical analysis

Data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM), with n denoting the number of cells or bronchial segments analyzed. Statistical comparisons were performed using one-way analysis of variance (ANOVA) or unpaired two-tailed t-tests, as appropriate. Differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

2. Results

2.1 Expression of GNAT3 in mouse airway smooth muscles

In this study, α-smooth muscle actin (α-SMA) antibody was used to identify smooth muscle tissue. As shown in Figure 1, DAPI staining (blue) revealed the characteristic ring-like architecture of the airway, composed of both airway smooth muscle and cartilage. The smooth muscle layer was specifically labeled by α-SMA immunostaining (green), clearly distinct from the adjacent cartilage. Notably, robust GNAT3 immunoreactivity (red fluorescence) was observed and colocalized with the α-SMA-positive smooth muscle layer. These results demonstrate that GNAT3 is expressed in mouse airway smooth muscle cells.

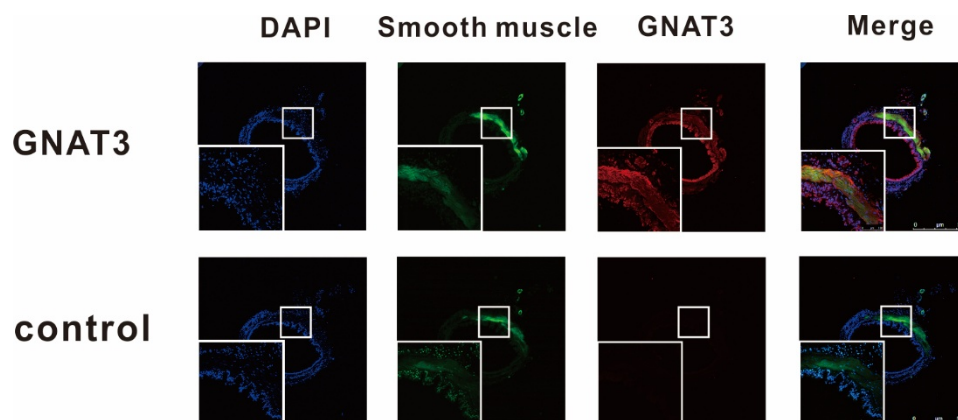

Figure 1. Expression of GNAT3 in mouse airway smooth muscle.

Notes. Immunofluorescence staining (red) for GNAT3 in frozen sections of mouse airway smooth muscle. Nuclei were counterstained with DAPI (blue), and smooth muscle cells were labeled with an α-actin antibody (green). Control sections were processed identically but with primary antibodies omitted (incubated in PBS only). Scale bar = 500 μm.

2.2 Phen reversed methacholine-induced airway smooth muscle contraction through the GNAT3 pathway

To investigate the bronchodilatory potential of Phen, a bitter-tasting compound, we assessed its ability to relax methacholine-induced contraction in isolated mouse airways. Isometric tension recordings demonstrated that 100 μM

methacholine elicited robust and sustained contraction of airway rings (Figure 2A), confirming their functional viability.

As a positive control, the β_2-adrenergic agonist R-albuterol potently reversed methacholine-induced tone—consistent with its clinical use as a first-line bronchodilator. Strikingly, Phen also induced significant relaxation, supporting the emerging paradigm that bitter compounds can act as potent bronchodilators.

The minimum effective concentration (MEC) of Phen was ~10^{-5} M, higher than that of R-albuterol (10^{-8} M) (Figure 2B), suggesting that greater doses may be needed for therapeutic efficacy in vivo. However, at 10^{-3} M, Phen nearly abolished contractions even against methacholine challenges (Figure 2B), exhibiting greater maximal relaxant efficacy than R-albuterol—highlighting its potential as a novel bronchodilator class.

Given that GNAT3 (gustducin α-subunit) is a critical signaling component downstream of TAS2Rs in chemosensory cells, we hypothesized that it may mediate Phen's effect in airway smooth muscle and Phen is bitter. Indeed, in wild-type (WT) airways, Phen significantly relaxed methacholine-induced contraction. In contrast, this response was markedly blunted in Gnat3−/− airways (Figure 2C, D), indicating that GNAT3-dependent bitter taste signaling contributes to Phen-induced bronchodilation.

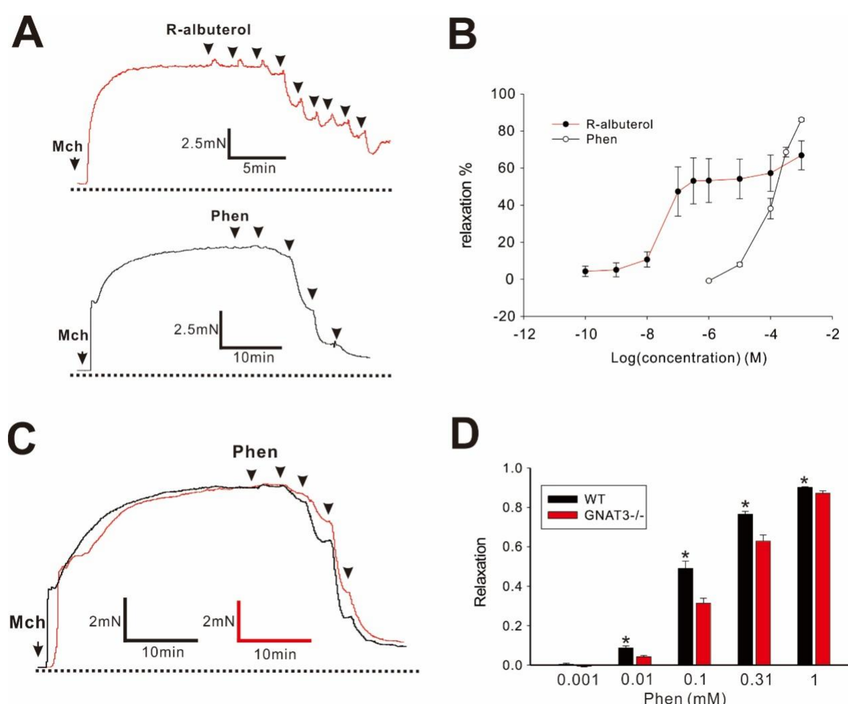

Figure 2. Phen reverses methacholine-induced airway smooth muscle contraction via the Tas2R–GNAT3 signaling pathway.

(A) Representative traces showing the effects of R-albuterol and Phen on the 100 μM methacholine induced mice airway smooth muscle contractions. The concentrations of R-albuterol were 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 3.1×10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} (M). The concentrations of Phen were 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} (M). **(B)** Summary of the relaxation responses to (R)-albuterol and Phen, expressed as a percentage of the maximal contraction induced by 100 μM methacholine (n = 6 for (R)-albuterol; n = 8 for Phen). Data are presented as mean ± SEM. **(C)** Representative cumulative concentration–response traces showing the effects of Phen (0.001, 0.01, 0.1, 0.31, and 1 mM) on methacholine-induced contractions in airway smooth muscle from wild-type (WT, black) and GNAT3-/- (red) mice in an accumulative method. **(D)** The difference in relaxation responses between wild-type (WT) and Gnat3−/− mice to 100 μM methacholine-induced airway smooth muscle contraction was assessed at relaxant concentrations of 0.01, 0.1, 0.31, and 1 mM. The differences were calculated as the ratio of (mean $value_{WT}$ – mean $value_{GNAT3-/-}$) and mean $value_{WT}$ at certain concentrations. The data are expressed as mean ± SEM.

2.3 Phen suppressed the methacholine-induced intracellular global calcium elevation

To elucidate the mechanism by which Phen relaxes airway smooth muscle, we examined its effect on intracellular calcium level in freshly isolated airway smooth muscle cells (ASMCs). Stimulation with 100 μM methacholine induced a robust elevation in global intracellular calcium concentration ($[Ca^{2+}]_i$) (Figure 3A). Notably, application of Phen at 10 μM or 100 μM significantly attenuated this methacholine-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner. These results indicate that Phen suppresses global $[Ca^{2+}]_i$ elevation, which likely underlies its bronchodilatory effect.

Given that bitter taste signaling in airway smooth muscle involves G protein–coupled taste receptors (TAS2Rs) and the gustducin α-subunit GNAT3, we next investigated whether GNAT3 mediates Phen's action. Pretreatment with pertussis toxin (PTX)—a selective inhibitor of $G\alpha_i/G\alpha_o$ proteins, significantly blunted Phen-induced relaxation (Figures 3A, B). Furthermore, in airways from Gnat3−/− mice, the relaxant response to Phen was markedly reduced compared to wild-type controls (Figure 3C, D).

Collectively, these findings demonstrate that Phen inhibits methacholine-induced $[Ca^{2+}]_i$ elevation to promote airway relaxation, and this effect is mediated through a GNAT3-dependent, PTX-sensitive G protein signaling pathway.

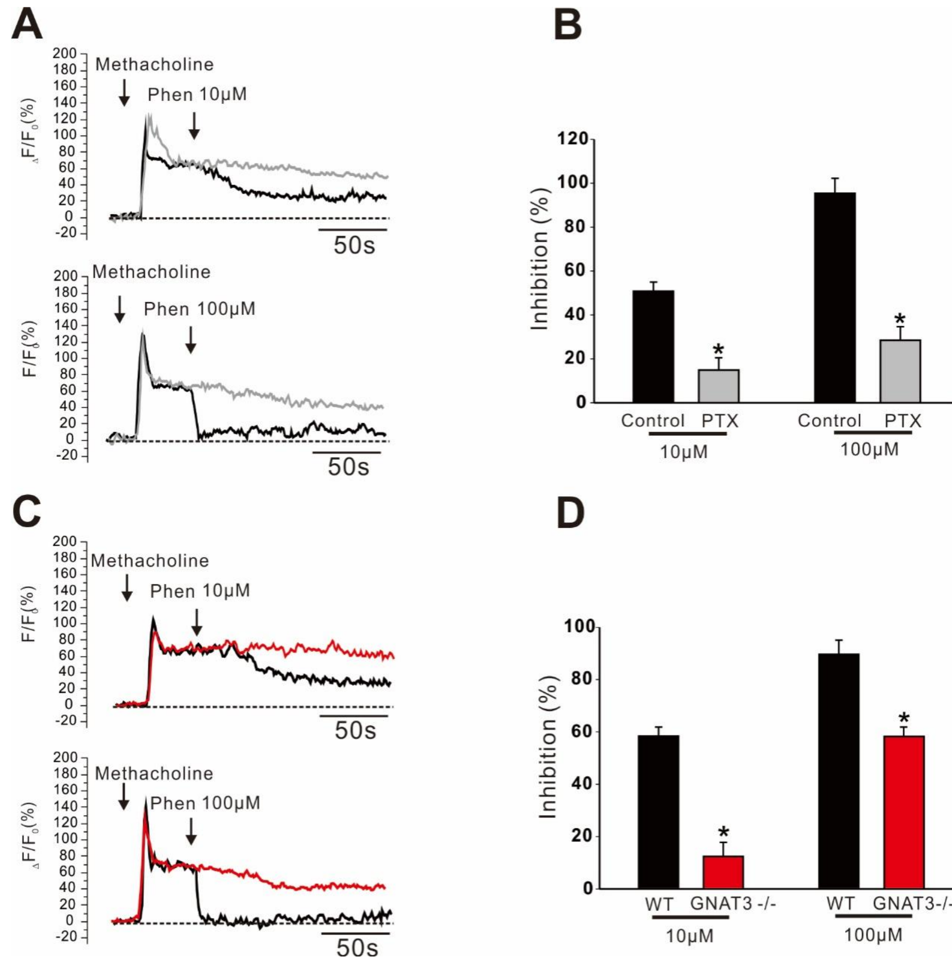

Figure 3. Phen reverses methacholine-induced increases in intracellular calcium concentration ($[Ca^{2+}]_i$) via the Tas2R–GNAT3 pathway.

(A) Representative traces showing that Phen (10 μM and 100 μM) reversed the $[Ca^{2+}]_i$ elevation induced by methacholine (100 μM) in mouse airway smooth muscle cells under control conditions (black traces) and after pertussis toxin (PTX) pretreatment (gray traces). **(B)** Summary graph of the inhibitory effects of Phen (10 μM and 100 μM) on methacholine-induced $[Ca^{2+}]_i$ responses, expressed as $_{\Delta}F/F_0$ (n = 16–23 cells; $P < 0.01$ vs. control). **(C)** Representative traces illustrating the reversal by Phen (10 μM and 100 μM) of methacholine (100 μM)-induced $[Ca^{2+}]_i$ increases in airway smooth muscle cells from wild-type (WT) and GNAT3-/- mice. **(D)** Summary graph showing the effects of Phen (10 μM and 100 μM) on methacholine-induced $[Ca^{2+}]_i$ responses in WT versus Gnat3-/- mice, expressed as $_{\Delta}F/F_0$ (n = 15–27 cells; ****$P < 0.05$ vs. WT).

3. Discussion

In this study, we employed Gnat3−/− mice and pharmacological approaches to demonstrate that GNAT3 partially mediates the airway relaxation induced by Phen, a bitter-tasting compound. We found that Phen dose-dependently reversed acetylcholine (ACh)-induced contraction in isolated airways, with a maximal relaxant efficacy comparable to that of R-albuterol, a first-line β2-adrenergic bronchodilator—though Phen required higher concentrations to achieve similar effects. This functional response was significantly attenuated in airways from Gnat3−/− mice, indicating partial dependence on GNAT3.

At the cellular level, Phen suppressed the ACh-induced elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) in airway smooth muscle cells. This inhibitory effect on $[Ca^{2+}]_i$ was abolished by pretreatment with PTX, a selective inhibitor of Gi/o proteins, and was also markedly reduced in Gnat3−/− cells. These findings provide convergent evidence—from both pharmacological inhibition and genetic ablation—that GNAT3, as the α-subunit of the gustducin G protein, contributes to Phen-mediated suppression of $[Ca^{2+}]_i$ and subsequent airway relaxation.

Our results align with prior reports that bitter compounds like Phen can relax airway and uterine smooth muscle [10, 16]. Phen has also been reported to exert anti-cancer effects in head and neck squamous cell carcinoma through TAS2R5-mediated calcium signaling [17]. Here, we confirm a dose-dependent bronchodilatory effect of Phen in murine airways, consistent with its proposed action via bitter taste receptors (TAS2Rs). The ability of Phen to rapidly inhibit ACh-triggered $[Ca^{2+}]_i$ surges further supports the existence of a functional TAS2R-gustducin signaling axis in airway smooth muscle, through which bitter agonists reduce contractile tone.

Notably, neither PTX treatment nor Gnat3 deletion completely abolished Phen's relaxant or calcium-inhibitory effects, suggesting the involvement of additional signaling pathways. Given that TAS2Rs (including T2R5, a putative target of Phen) may couple to multiple G protein families beyond gustducin [17]—such as other Gi/o subtypes or even Gq in certain contexts—the residual Phen activity in Gnat3−/− tissues likely reflects redundant or alternative G protein coupling. Moreover, Phen might influence calcium dynamics through non-TAS2R mechanisms, such as direct modulation of ion channels or mitochondrial calcium handling.

In summary, we provide functional and mechanistic evidence that Phen induces airway relaxation via a GNAT3-dependent pathway that suppresses intracellular calcium elevation. However, the incomplete blockade by GNAT3 loss or PTX implies that multiple parallel mechanisms may contribute to the bronchodilatory action of bitter compounds—a feature that could be leveraged for therapeutic development in obstructive airway diseases.

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