Both chronic obstructive pulmonary disease (COPD) and asthma are severe respiratory diseases. Bitter receptor–mediated bronchodilation is a potential therapy for asthma, but the mechanism underlying the agonistic relaxation of airway smooth muscle (ASM) is not well defined. By exploring the ASM relaxation mechanism of bitter substances, we observed that pretreatment with the bitter substances nearly abolished the methacholine (MCh)-induced increase in the ASM cell (ASMC) calcium concentration, thereby suppressing the calcium-induced contraction release. The ASM relaxation was significantly inhibited by simultaneous deletion of three Gαq proteins, suggesting an interaction between Tas2R and AChR signaling cascades in the relaxation process. Biochemically, the Gαq released by Tas2R activation complexes with AChR and blocks the Gαq cycling of AChR signal transduction. More importantly, a bitter substance, kudinoside A, not only attenuates airway constriction but also significantly inhibits pulmonary inflammation and tissue remodeling in COPD rats, indicating its modulation of additional Gαq-associated pathological processes. Thus, our results suggest that Tas2R activation may be an ideal strategy for halting multiple pathological processes of COPD.

### Significance

We found that bitter substances–induced airway smooth muscle relaxation was mediated by Gαq release of Tas2R activation and the released Gαq blocked contractile responses by efficiently suppressing the Gαq cycling of AChR signaling. Given the Gαq coupled with various G protein–coupled receptors, this mechanism may be manipulated to intervene in multiple pathological processes. As an example, the bitter substance kudinoside A simultaneously attenuated airway constriction in rodent models of both asthma and chronic obstructive pulmonary disease (COPD) and decreased pulmonary inflammation and tissue remodeling in the COPD rat model.

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also elevated, but the resultant effect is relaxation. We here found that the elevated calcium by Tas2R activation was not sufficient to phosphorylate the myosin light chain. Importantly, activation of Tas2R almost abolished the calcium elevation induced by methacholine (MCh). By comparing the Gnats and Gαq sequences of helix 5 (H5, the main site of interaction with GPCRs) (21) and establishing the Gnats triple knockout (TKO) mice, we revealed a cross-talk between these cascades that was mediated by the released Gnats from activated Tas2R to interrupted Gq cycling of acetylcholine receptor (AChR) activation. To assess the role of bitter substance in multiple GPCR-associated processes, we determined the therapeutic effect of kudinoside A on chronic obstructive pulmonary disease (COPD) rats. The administration of kudinoside A not only attenuated airway constriction but also improved pulmonary inflammation and bronchial remodeling, suggesting that bitter substances such as kudinoside A have a unique advantage for asthma/COPD therapies.

Results

Tas2R Activation Prevents Acetylcholine-Induced Increases in the Calcium Level in ASM. In human ASM, Tas2R10 and Tas2R14 are considered to be critical for airway relaxation (17). As canonical chloroquine and other compounds are considered to be nonselective for Tas2R receptors, we investigated a family of bitter substances, kudinosides, for experiments. These compounds were extracted from *Ilex kudingcha* (used as bitter tea in China), and they are pentacyclic triterpenes and feature the typical structure of lactones (14). We established TAS2R10 and TAS2R14 bitter receptor reporter cells by respectively cotransfecting HEK293T cells with the expressive vectors of human TAS2R10-Gα16/gust44 and human TAS2R14-Gα16/gust44 (22, 23). Measurement for the Ca^2+ release induced by bitter substances indicated that KE-A was a potent TAS2R10 agonist (SI Appendix, Fig. S1 E and F). Note that KE-A might also be able to activate other TAS2Rs because KE-A treatment also led to a moderate increase for the control cells transfected with Gα16/gust44 only. To test whether KE-A activates Tas2R in airway smooth muscle, we isolated mouse ASMCs and primarily cultured them in dulbecco’s modified eagle medium (DMEM). Upon exposure to KE-A, the mouse ASM primary cells showed a typical increase in intracellular calcium in a similar manner as chloroquine did (Fig. 1A). However, the calcium levels were much smaller than the MCh-induced calcium release level (average ΔF/F_0: 14.56% (KE-A)/10.76% (chloroquine) vs. ΔF/F_0 of MCh: 103.9%, both P < 0.001) (Fig. 1A and B). To determine whether the elevated calcium level was sufficient to evoke smooth muscle contraction, we measured myosin regulatory light chain (RLC) phosphorylation in ASM tissues. The RLC phosphorylation in ASM strips treated

![Fig. 1. Bitter substances affect increases in the cytosolic Ca^{2+} concentration. ASMCs were isolated from mouse airways and cultured in vitro. The primary cells were then subjected to the measurement of intracellular calcium. (A) Ca^{2+} release upon stimulation with 10 μM MCh, 30 μM chloroquine, and 30 μM KE-A. The calcium release induced by MCh was significantly inhibited by pretreatment with chloroquine or KE-A. (B) Quantification of the peak values of the released calcium, as shown in A. (C) After respective stimulation with MCh or bitter substances, the airway smooth muscle tissues were applied to measure the phosphorylation level of RLC. (D and E) Treatment with chloroquine or KE-A did not initiate ASM contraction. ****P < 0.0001, n > 5.](https://doi.org/10.1073/pnas.2121513119)
with MCh alone was significantly increased by 20 s, but not after treatment with KE-A or chloroquine (Fig. 1.C). Consistently, KE-A and chloroquine treatment did not induce ASM contraction (Fig. 1.D and E).

Because cytosolic calcium and its resultant RLC phosphorylation are required for smooth muscle contraction, we speculated that Tas2R activation plays a critical role in the release of calcium from MCh-stimulated mouse ASMCs. We incubated ASMCs with bitter substances (30 μM chloroquine and 30 μM KE-A) and then stimulated the cells with MCh, revealing that the calcium elevation was significantly inhibited ($P < 0.001$) (Fig. 1.A and B). Collectively, our results showed that Tas2R activation induced a slight increase in the intracellular calcium levels that was insufficient to induce RLC phosphorylation, whereas it significantly suppressed MCh-mediated calcium release.

**Gnats Are Necessary for the Inhibitory Effect of Tas2R Activation on Acetylcholine-Induced Calcium Elevation.** The suppressive effect of Tas2R activation on MCh-mediated calcium release clearly implied an interaction between the Tas2R and AChR pathways. To confirm this interaction, we deleted gustducin and two α-transducins by establishing compound Gnat1/2/3 KO mice (Gnat1, Gnat2, and Gnat3) using CRISPR-Cas9 technology (SI Appendix, Figs. S2A and S3). All of the mice with Gnat KO appeared normal in terms of their physiological activities, body growth, fur appearance, etc. The smooth muscle and epithelium of the mutant airways appeared normal also (SI Appendix, Fig. S2B–D). To measure the inhibitory effect of bitter substances on MCh-induced calcium release, we then prepared primary smooth muscle cells from wild-type (WT) and mutant airways. Upon stimulation with MCh, the KE-A- and chloroquine-pretreated ASMCs with individual deletions of Gnat1, Gnat2, or Gnat3 exhibited significantly increased cytosolic calcium levels to an extent similar to that in the control cells (Fig. 2.A and B). The cytosolic calcium level was not increased in the muscle cells with double KO of Gnat2/3, but the calcium release was slightly increased in muscle cells with double KO of Gnat1 and Gnat3 in the KE-A group (Fig. 2.B, C, G, and H). However, when Gnat1, Gnat2, and Gnat3 were deleted simultaneously (TKO), the calcium levels in KE-A- and chloroquine-pretreated cells were increased to a significantly greater extent than that in control cells (ΔF/ΔF0 of the fluo-4 intensity: TKO = 50.39% vs. WT = 20.17% for chloroquine, and TKO = 44.15% vs. WT = 21.26% for KE-A) (Fig. 2.D, E, I, and J). This result indicates that the three Gnat members are necessary for the counteractive effect of Tas2R activation on calcium elevation.

**Gnats Are Necessary for ASM Relaxation Induced by Tas2R Activation.** We then measured the relaxation responses of Gnat-deficient ASMs to bitter substances. The responses of all muscles with single or double KO of the Gnat 1/2/3 genes were comparable to those of the control, except that the muscle with Gnat1/3 double KO showed a reduced relaxation response to KE-A (Fig. 3.A–D and SI Appendix, Fig. S4). In the triple KO muscle, however, the relaxation responses to KE-A and chloroquine were significantly inhibited. In particular, when the mutant muscle was treated with 10 μM KE-A, only ~25% of the force evoked by MCh was relaxed, while ~80% was relaxed in the control muscle. The half maximal inhibitory concentration (IC50) values of KE-A increased by ~200% (from 5.85 ± 0.22 μM to 12.36 ± 1.42 μM), while the IC50 of chloroquine increased by ~125% (from 24.43 ± 0.64 μM to 29.46 ± 0.76 μM). This result indicated that the deletion of Gnats suppressed Tas2R activation–mediated relaxation. We also measured the contractile responses of these smooth muscles and found that the maximum contraction force induced by MCh had no difference between WT and Gnat knockout mice. This indicated that the suppression effect of Gnats deletion on relaxation was unlikely contributed by contractile alteration after gene deletion.

![Graphs](https://example.com/graphs.png)  
**Fig. 2.** The suppressive effects of KE-A and chloroquine on MCh-induced Ca²⁺ elevation in Gnat1/2/3 knockout mouse ASMCs. (A) Pretreatment with chloroquine did not suppress the MCh-induced calcium release in ASMCs deficient for Gnat1, Gnat2, or Gnat3. (B) Pretreatment with chloroquine did not suppress the MCh-induced calcium release in ASMCs with double knockout of Gnat2/Gnat3 or Gnat1/Gnat3, although the levels in the cells with Gnat1/Gnat3 double knockout were slightly increased. (C) Quantification of A and B. (D) Triple knockout of Gnat1/2/3 counteracted the suppressive effects of chloroquine, quantification of the peak calcium levels in E. (F) KE-A did not suppress the MCh-induced calcium release in ASMCs deficient for Gnat1, Gnat2, or Gnat3. (G) KE-A did not suppress the MCh-induced calcium release in ASMCs with double knockout of Gnat2/Gnat3, but the levels were slightly increased in ASMCs with Gnat1/Gnat3 double knockout. (H) Quantification of the peak calcium levels in F and G. (I) Triple knockout of Gnat1/2/3 counteracted the suppressive effects of KE-A. (J) Quantification of the peak calcium levels in I. Cytosolic calcium levels are expressed as ΔF/ΔF0 (%) . *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$; unpaired Student’s t test and two-way ANOVA.
To measure this effect in vivo, we sensitized wild-type and Gnat1/2/3 triple KO mice with ovalbumin (OVA) and established an asthmatic animal model as previously reported (9, 24). While all of the animals had comparable airway hyperresponsiveness, as evidenced by a three- to fourfold increase in respiratory system resistance (Rrs) values after MCh challenge ([SI Appendix](https://doi.org/10.1073/pnas.2121513119)), the asthmatic animals exhibited more acidic mucin protein and inflammatory infiltration of cells particularly of eosinophils around the airways, greater impairment, and swelling of epithelial goblet cells ([SI Appendix](https://doi.org/10.1073/pnas.2121513119), Figs. S5 A–D). The amount of eosinophil cells in the bronchoalveolar lavage fluid (BALF) and eosinophilic inflammation–related cytokines were also increased after OVA challenge ([SI Appendix](https://doi.org/10.1073/pnas.2121513119), Figs. S5 E–K). Upon treating each asthmatic animal with 150 μg chloroquine, the wild-type mice showed significantly reduced Rrs values during the observation period, while the TKO mice showed no reduction in Rrs or even moderately increased Rrs values at ~5 min after treatment (Fig. 3 E–G). Similarly, when the mice were treated with KE-A (3 μg for each animal), the TKO mice showed no apparent reduction in Rrs values, which were significantly higher than those of the wild-type mice (Fig. 3 H–J) (P < 0.01). We also measured central airway resistance (Rn) of these animals, but no difference was observed between WT and TKO groups after bitter substances treatment, indicating that Tas2R activation by the substances had an effect predominantly on bronchioles ([SI Appendix](https://doi.org/10.1073/pnas.2121513119), Figs. S6 B and C). This observation showed that the deletion of Gnat1/2/3 also impaired the relaxation response under asthmatic conditions.

**Bitter Substances Also Relax the Depolarization-Induced Contraction, which Is Dependent on AChR Signaling.** Smooth muscle contraction may be also initiated by membrane depolarization through L-type calcium channels, which is distinct from AChR signaling cascade. Previous reports have shown that bitter substances also relax depolarization-induced ASM contraction (8). We herein observed that KE-A effectively relaxed KCl-evoked ASM (Fig. 4 A, C, and G) but did not relax other smooth muscle (Fig. 4 B). Given that agonistic relaxation is mediated by Gnats, as observed above, we wanted to elucidate the mechanism underlying the relaxation of depolarization-induced contraction.
Before addressing this, we pretreated ASM with atropine, an antagonist of M-type AChRs and found more than 80% of the force induced by 60 mM KCl was reduced (Fig. 4D and H); however, this inhibition was not observed in other tissues such as the aorta and jejunum (Fig. 4D–F and H). This observation indicated that in ASM, depolarization-induced contraction was dependent on AChR signaling. Although the mechanistic formation of this dependency effect has to be explored, our result provides convincing evidence that bitter substances relax depolarization-induced contraction through AChR signaling also.

**Tas2R Activation Inhibits AChR Signaling via the Interaction of Gnats with AChR.**

The suppressive effects of Gnats on MCh-induced contraction and calcium release prompted us to assess whether an interaction exists between the Tas2R and AChR signaling cascades. As Gnats are highly similar to Gα, we hypothesized that this interaction might be mediated by the binding of Gnats to AChR at the Gα binding site. To test this hypothesis, we first stained the MACHr M3 and Gnats simultaneously in primary ASMCs, revealing that the M3 receptor overlapped well with Gnat1 (Fig. 5A). However, Gα, another important G protein coupled with GPCR, did not overlap with the M3 receptor (SI Appendix, Fig. S7F), suggesting that the overlapping pattern of the M3 receptor varied with different G proteins. We then constructed MACHr M3 and Gnats plasmids to transfet HEK293T cells for coimmunoprecipitation assay (SI Appendix, Fig. S7A–C). Expectedly, the Gnat1/2/3 and M3 receptors simultaneously appeared in the precipitated fraction (Fig. 5B–D). To assess this interaction in vivo, we precipitated the lysate of fresh mouse ASM tissues with an anti-MACHr M3 antibody. Gnat1, Gnat2, and Gnat3 signals were clearly detected in the precipitated pellet (Fig. 5G).

We next wanted to determine which structural features of Gnats are responsible for this interaction. The C-terminal H5 region of Gα was the main binding site with GPCRs, and the Gnat1/2/3 proteins were shown to share an identical H5 sequence (IYSHMTCATDTQNVKKFVFDAVTDIIIKENLKDCGLF), which showed a 61.11% identity with the Gα H5 sequence (SI Appendix, Fig. S7D and E). We also detected the EGFP-H5Gnat protein in the MACHr M3 precipitated fraction (Fig. 5E), but the truncated Gnat1 without H5 just showed a weak interaction (Fig. 5F). To functionally confirm this H5 interaction, we overexpressed Gnat1 and H5Gnat in HEK293T cells and then assessed the suppressive effect of Tas2R activation on calcium release. The calcium evoked by MCh was significantly inhibited when Gnat1 and H5Gnat were overexpressed (Fig. 5H–J), while overexpressing Gnat1 without H5 slightly counteracted the inhibition of calcium release. Altogether, our observations strongly suggest that bitter substances inhibit AChR signaling through the physical interaction of Gαs with AChR via the H5 region.

**Fig. 4.** Kudinoside A relaxed the KCl-evoked contraction of the airway but not of the aorta or jejunum smooth muscle. (A) KE-A (30 μM) relaxed the ASM contraction evoked by 10 μM MCh or 64 mM KCl. (B and C) KE-A did not relax the tension force of the aorta and jejunum smooth muscle evoked by agonists and KCl depolarization. (D) The depolarization-evoked contraction of ASM was significantly induced by atropine. (E) The depolarization-evoked aortic contraction was partially inhibited by atropine. (F) The depolarization-evoked contraction of jejunum smooth muscle was not relaxed by atropine. (G) Quantitation of the data in A–C. (H) Quantitation of the data in D–F. *P < 0.05, **P < 0.01, unpaired Student’s t test; ***P < 0.001, unpaired Student’s t test. NE: norepinephrine.
Inhalation of Kudinoside A Attenuates COPD in Rats. In addition to bronchoconstriction, pulmonary remodeling and inflammation are typical pathological features of COPD and are caused by multiple factors such as inflammatory mediators, cytokines, and the overproduction of ACh (25–27). Most of these factors are agonists of Gq-coupled GPCRs that are also expressed in various nonmuscle cells. We thus assessed the therapeutic efficacy of bit- ter substances in COPD rats. We first established COPD rats, which exhibited increased airway resistance, inflammatory infiltration, and mucus secretion (Fig. 6A and D). The administration of KE-A could increase the ratio of forced expiratory volume in 0.2 second to forced vital capacity (FEV0.2/FVC) compared with COPD rats (Fig. 6B). Importantly, after treatment with KE-A, the lung inflammation scores of rats with COPD were significantly reduced (P < 0.05) (Fig. 6C and D). Especially, the macrophage level in pulmonary alveoli was reverted to that in control rats (Fig. 6E and F). Movat’s staining of the lung tissues showed that the smooth muscle layers of the bronchi and arteries of rats with COPD were significantly thicker than those of the controls and that KE-A treatment ameliorated this effect (Fig. 6G–H). Furthermore, the extracellular matrix layers around the intrapulmonary bronchi and arteries of rats with COPD were significantly thicker than those of the controls but were restored significantly after KE-A treatment (Fig. 6I). In conclusion, KE-A effectively attenuates COPD inflammation and remodeling and shows therapeutic potential for the disease.

To further investigate the effect of Tas2R activation on inflammation, we established lipopolysaccharide (LPS)-induced acute pulmonary inflammation mouse model (28, 29). Forty-eight hours after treatment with LPS inhalation, pulmonary inflammation was significantly evoked as evidenced by apparent inflammatory infiltration of immune cells and significantly elevated expression of cytokines (Fig. 7 and SI Appendix, Fig. S8). When these mice were simultaneously treated with KE-A, the inflammatory infiltration of the cells was comparable to the control (Fig. 7A–C), whereas the expressions of IL-1β, CSF-3 (colony stimulating factor 3), MCP-1 (monocyte chemoattractant protein-1), IL-6, and TNF-α were significantly attenuated (Fig. 7D–M). However, this suppressing effect was significantly inhibited by Gna10 deletion. This result convincingly showed an essential role of Tas2R activation in acute pulmonary inflammation.

Discussion

In this report, we investigated the mechanism of ASM relaxation induced by Tas2R activation. Our results showed that, chloroquine and KE-A slightly increased the intracellular calcium levels in ASMCs through canonical Tas2R signaling. Pretreating the ASM with these substances nearly abolished the calcium elevation evoked by MCh. This observation suggests that the inhibition of calcium signaling during ASM contraction underlies Tas2R activation–mediated relaxation. Because the deletion of Gna10 counteracted the inhibitory effect and Gna10 had the capacity to bind the AChR, we suggest that the direct interaction of Gna10 with AChR also underlies the inhibitory effect in addition to ASM relaxation. Based on our
observations, we proposed a working model for ASM relaxation induced by Tas2R activation ([SI Appendix, Fig. S9]). When Tas2Rs and AChR are activated simultaneously within ASMCs, the released Gnats ectopically bind AChR and thus block Gq cycling, leading to the inhibition of signal transduction required for ASM contraction.

According to our model, ASM relaxation is mediated by released Gnats rather than by calcium elevation. This result together with the result that the elevated calcium level induced by Tas2R activation did not induce RLC phosphorylation suggests that the calcium elevation induced by bitter substances is irrelevant to the development of force tension. Another paradox is that bitter substances can relax depolarization-evoked ASM ([8]). Here, we observed that atropine significantly inhibited the depolarization-evoked force, suggesting that the depolarization pathway depends on the AChR, at least in ASM; however, the underlying mechanism remains unknown. Thus, the relaxation effect of bitter substances on the depolarization-evoked force of ASM is also mediated by ACh signaling. Note that this is not true for other smooth muscles because the depolarization-evoked force of these muscles did not depend on ACh signaling. Thus, this model not only explains why Tas2R activation can relax depolarization-evoked force but also why bitter substances display differential relaxation in different muscles.

As several GPCR receptors couple with Gq, Tas2R activation theoretically influences numerous GPCR-mediated signaling pathways other than the muscarinic pathway. In asthma and COPD, a vast number of acute inflammatory mediators (e.g., bradykinin, leukotrienes, and histamine) and ACh are overproduced and exert their effects on pulmonary inflammation and remodeling through GPCRs present in ASM, epithelial, and immune cells ([30–35]). Here, we showed that repeated treatments with kudinoside A not only significantly attenuated airway constriction but also inhibited pulmonary inflammation and remodeling. We also used an acute lung injury mice model to further examine the effect of KE-A to inhibit pulmonary inflammation. As Tas2R signaling serves as an
intrinsic physiological inhibitor of various GPCRs, Tas2R agonists have a unique advantage over current COPD/asthma medicines in terms of their diverse beneficial effects. In addition, since Tas2R activation has a greater effect on relaxing small airways rather than the central airway as we observed in this report, the bitter substances could have a better effect to treat COPD in contrast to asthma.

Kudinosides are isolated from kudingcha and are primarily made of ilex plants, and kudingcha may have various pharmaceutical activities in different systems. For example, in the vascular system, crude extracts of kudingcha may improve blood circulation in the heart and brain (36). In the immune system, kudingcha extracts and saponins may effectively modulate the functions of macrophages and lymphocytes, thereby inhibiting different inflammatory reactions. These diverse pharmacological effects of kudingcha also support the role of kudinoside A in altering COPD pathological processes.

Materials and Methods
All animal manipulations in this study were conducted in accordance with the criteria of the Institutional Animal Care and Use Committee of the Model Animal Research Center of Nanjing University (Nanjing, China). We established Gnat knockout mice by CRISPR-Cas9 technology, and animals used for experiments were 8 to 10 weeks of age. ASM isometric contraction was recorded by a PowerLab recording device (AD Instruments). A Fluo-4 Direct Calcium assay kit (Invitrogen, F10472) was used to measure the calcium signal at an excitation wavelength of 494 nm and an emission wavelength of 516 nm with a microplate reader (BioTek, Synergic H1). The asthma mice model was sensitized with 100 μg of OVA (Sigma-Aldrich) by intraperitoneal injection, and the COPD rat model was administered SO2 via inhalation. Respiratory function was measured with a flexivent forced concussion pulmonary function detector with a negative pressure-driven forced expiration module (SCIREQ, FX-4).

Data Availability. All study data are included in the article and/or SI Appendix.

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