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ARTICLES

Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction

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Bitter taste receptors (TAS2Rs) on the tongue probably evolved to evoke signals for avoiding ingestion of plant toxins. We found expression of TAS2Rs on human airway smooth muscle (ASM) and considered these to be avoidance receptors for inhalants that, when activated, lead to ASM contraction and bronchospasm. TAS2R agonists such as saccharin, chloroquine and denatonium evoked increased intracellular calcium ($[Ca^{2+}]_i$) in ASM in a Gβγ–, phospholipase Cβ (PLCβ)- and inositol trisphosphate (IP_3) receptor–dependent manner, which would be expected to evoke contraction. Paradoxically, bitter tastants caused relaxation of isolated ASM and dilation of airways that was threefold greater than that elicited by β-adrenergic receptor agonists. The relaxation induced by TAS2Rs is associated with a localized $[Ca^{2+}]_i$ response at the cell membrane, which opens large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, leading to ASM membrane hyperpolarization. Inhaled bitter tastants decreased airway obstruction in a mouse model of asthma. Given the need for efficacious bronchodilators for treating obstructive lung diseases, this pathway can be exploited for therapy with the thousands of known synthetic and naturally occurring bitter tastants.

Asthma and chronic obstructive pulmonary disease (COPD) together affect 300 million individuals worldwide. The major source of morbidity and mortality from both diseases is airway obstruction, which in part is due to actively constricted smooth muscle of the bronchi[1]. Although airway resistance in COPD has variable degrees of reversibility, owing to structural changes that result from smoking, therapies for COPD and asthma include antagonists directed at bronchoconstrictive receptors and agonists directed at receptors that relax ASM[2,3]. The major receptor signaling family in ASM that regulates contraction and relaxation are G protein–coupled receptors (GPCRs)[3]. There is an ongoing effort to identify GPCR pathways leading to regulation of airway tone, thereby providing new treatment strategies for asthma and COPD. This is particularly relevant because the incidence of both diseases is increasing, and at least one-half of all patients have inadequate control of the disease with currently available agents[4,5].

Unexpectedly, we recently found expression of several bitter taste receptors in isolated human ASM as part of a pan-GPCR screening effort[6]. The cognate G protein for bitter taste receptors, gustducin, is also expressed in human ASM[7,8]. Receptors for bitter tastes on the tongue are thought to have evolved for avoidance of plant-based toxins[9,10]. These GPCRs consist of at least 25 receptor subtypes, each recognizing a repertoire of agonists that usually overlaps with other bitter taste receptors, creating a redundant, broadly tuned avoidance and rejection network[9,11–13]. The finding of bitter taste receptors on ASM led to our original hypothesis that certain bronchospastic disorders, such as occupational asthma[14], might be caused by environmental inhalants acting at these airway receptors, leading to contraction and bronchoconstriction. This notion was based on the fact that bitter taste receptors couple to increases in $[Ca^{2+}]_i$ in specialized taste cells of the tongue, and this signal is also found with known bronchoconstrictive GPCRs such as those for histamine, acetylcholine and bradykinin in ASM cells[2]. By various approaches, we found that bitter tastants also increase $[Ca^{2+}]_i$ in ASM cells but unexpectedly found that bitter taste receptor agonists are bronchodilators with greater efficacy than any known therapeutic agent. These receptors transduce the relaxation response in ASM by a novel mechanism involving receptor-generated Gβγ activation of PLC and a partitioned $[Ca^{2+}]_i$ transient that opens cell surface K^+ channels, resulting in membrane hyperpolarization.

RESULTS

Human ASM expresses bitter taste receptors coupled to $[Ca^{2+}]_i$

In initial studies, we found that several known bitter taste receptor agonists (such as saccharin, chloroquine and denatonium) evoked increased $[Ca^{2+}]_i$ in cultured human ASM cells (**Fig. 1a–c**). We found the $[Ca^{2+}]_i$ responses in ASM cells to these bitter tastants to be similar in magnitude to those for known G_q-coupled bronchoconstrictive GPCR agonists such as histamine and bradykinin (**Fig. 1c**). These results prompted quantitative RT-PCR studies with primers for 25 *TAS2R* genes. Of note, the numerical designations of the *TAS2R* genes have recently changed, and here we use this new nomenclature (see

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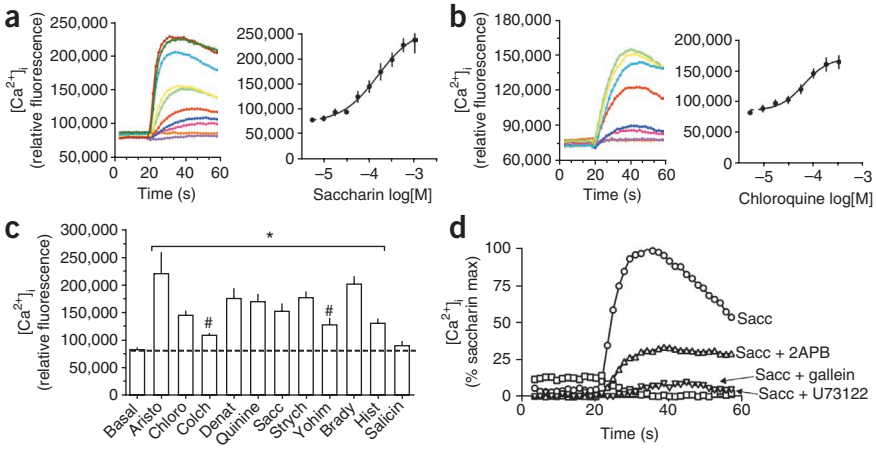


Figure 1 Bitter tastants of diverse structures evoke increases in $[Ca^{2+}]_i$ in human airway smooth muscle cells. Studies were performed with cultured primary ASM cells loaded with Fluo-4 AM. (**a**,**b**) $[Ca^{2+}]_i$ transients and dose response curves to saccharin (**a**) and chloroquine (**b**). The mean ± s.e.m. dose-response curves were from five or six experiments, and the transients shown are representative of single experiments. (**c**) Maximal $[Ca^{2+}]_i$ responses to 1.0 mM of the bitter tastants aristocholic acid (aristo), chloroquine, colchicine, denatonium, quinine, saccharin, salicin, strychnine and yohimbine and the bronchoconstrictive G_q-coupled agonists bradykinin (0.01 mM) and histamine (0.1 mM). Results are means ± s.e.m. from four to six experiments. $*P < 0.01$ versus basal; $\#P < 0.05$ versus denatonium. (**d**) The $[Ca^{2+}]_i$ response to bitter tastants is ablated by the PLC inhibitor U73122 and the βγ antagonist gallein, and attenuated by the IP_3 receptor antagonist 2APB. These studies were performed in the absence of extracellular calcium. Results shown are from a single representative experiment of at least three performed.

http://www.genenames.org). We found multiple *TAS2R* transcripts to be expressed in human ASM, with the *TAS2R10*, *TAS2R14* and *TAS2R31* subtypes being the most highly expressed (**Table 1**). Further screens with additional bitter tastants revealed $[Ca^{2+}]_i$ responses in ASM to aristocholic acid, strychnine, quinine, colchicine and yohimbine (**Fig. 1c**). We found a relatively low response in ASM to colchicine, which activates TAS2R4 (expressed at intermediate levels in ASM, as determined by RT-PCR) and no response to salicin, which exclusively activates TAS2R16 (ref. 10) but was not detected in ASM by RT-PCR. The robust response to strychnine (which activates TAS2R10 and TAS2R46) is also consistent with TAS2R10 being highly expressed in ASM. Thus, in ASM, the $[Ca^{2+}]_i$ response to bitter tastants is concordant with a rank order based on agonist specificity and the bitter taste receptor subtype expression in these cells. Immunofluorescence microscopy of human ASM cells with polyclonal antisera directed against four receptors found by RT-PCR to be expressed at the mRNA level (*TAS2R10*, *TAS2R14*, *TAS2R31* and *TAS2R19*) and three receptors whose mRNAs were not detected (*TAS2R7*, *TAS2R38* and *TAS2R43*) revealed cell surface expression of the former four receptors but not of the latter three (**Supplementary Fig. 1**). These studies also revealed expression of the α subunit of gustducin in these cells (**Supplementary Fig. 1**). We found no $[Ca^{2+}]_i$ response to the sweet receptor agonists sucralose and SC45647, and the response to saccharin (which also activates sweet taste receptors) was not blocked by the sweet receptor antagonist lactisole (**Supplementary Fig. 2**).

Table 1 mRNA expression of bitter taste receptors in human ASM cells

Receptor	Ratio to *ADRB2*
TAS2R10	3.96 ± 0.893
TAS2R14	3.51 ± 0.397
TAS2R31	3.41 ± 0.498
TAS2R5	1.76 ± 0.190
TAS2R4	1.45 ± 0.271
TAS2R19	1.37 ± 0.249
TAS2R3	0.83 ± 0.079
TAS2R20	0.71 ± 0.202
TAS2R45	0.70 ± 0.118
TAS2R50	0.48 ± 0.033
TAS2R30	0.31 ± 0.060
TAS2R9	0.31 ± 0.034
TAS2R13	0.26 ± 0.037
TAS2R42	0.26 ± 0.009
TAS2R46	0.25 ± 0.041
TAS2R1	0.17 ± 0.027
TAS2R8	0.15 ± 0.007
TAS2R39	ND
TAS2R43	ND
TAS2R7	ND
TAS2R40	ND
TAS2R16	ND
TAS2R38	ND
TAS2R41	ND
TAS2R60	ND
TAS1R1	ND
TAS1R2	ND
ADRB2	1.0 (reference)
ADORA1	2.43 ± 0.446
LTB4R	0.29 ± 0.056

Results are normalized to expression of the *ADRB2*. *ADORA1* and *LTB4R* represent high- and low-expressing GPCRs as positive controls. ND, not detected. Results are from four to six experiments.

Transfection of ASM cells with siRNA directed against *TAS2R10* decreased *TAS2R10* mRNA levels by 36 ± 1.8% compared to the scrambled siRNA control. Under these conditions of *TAS2R10* knockdown, the $[Ca^{2+}]_i$ response induced by the TAS2R10 agonist strychnine was decreased by 26 ± 2.0% as compared with the scrambled siRNA control ($P < 0.05$ versus control). In additional studies, we incubated ASM cells with medium alone or polyclonal antisera directed against TAS2R10, TAS2R7 or isotype-specific IgG and then determined the strychnine-induced $[Ca^{2+}]_i$. TAS2R10 antisera decreased strychnine-induced $[Ca^{2+}]_i$ responses in a dose-dependent manner (maximal inhibition of ~77%, **Supplementary Fig. 3**), consistent with the RT-PCR and immunofluorescence results showing expression of this bitter taste receptor. In contrast, antisera against TAS2R7 (which is not expressed in ASM) at the same titers had no significant effect on $[Ca^{2+}]_i$ stimulation, nor did isotype-specific IgG (**Supplementary Fig. 3**). Taken together, these studies confirm expression of bitter taste receptors on ASM cells and link expression to bitter tastant–mediated $[Ca^{2+}]_i$ signaling. The increase in $[Ca^{2+}]_i$ in human ASM cells elicited by bitter tastants was not dependent on the presence of extracellular Ca^{2+}, the response was ablated by the Gβγ inhibitor gallein and the PLCβ inhibitor U73122, and it was partially inhibited by the IP_3 receptor antagonist 2APB (**Fig. 1d**). These results in

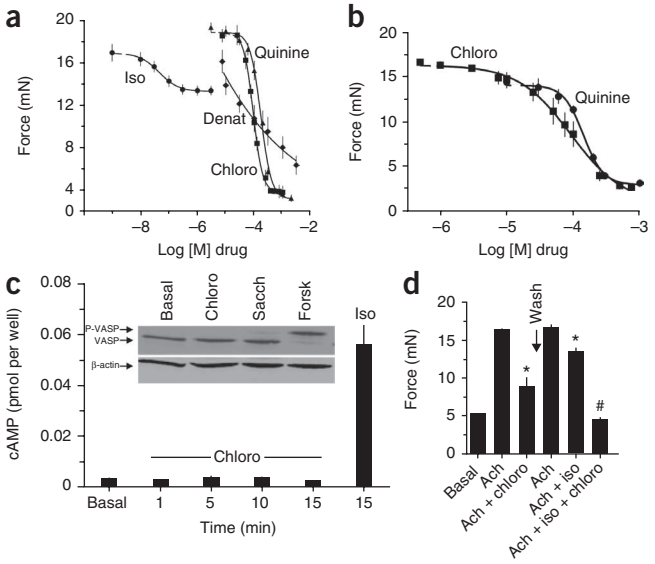


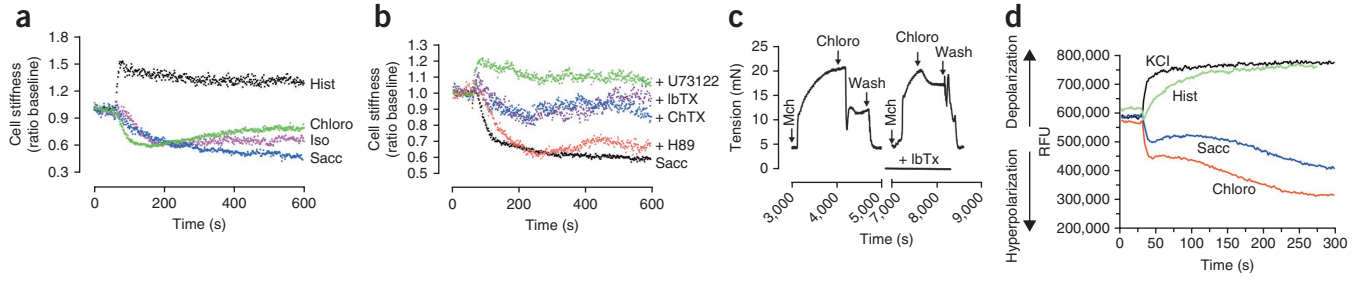
Figure 2 Bitter tastants induce relaxation of intact mouse tracheas in a non–cAMP-dependent manner. (**a**) Dose-response curves of relaxation for the β-adrenergic agonist isoproterenol (iso) and the bitter taste receptor agonists chloroquine (chloro), denatonium (denat) and quinine, derived from intact mouse tracheas contracted with 1.0 mM acetylcholine ($n = 7$ experiments). (**b**) Relaxation by chloroquine and quinine of intact mouse tracheas contracted by 1.0 mM serotonin ($n = 4$ experiments). (**c**) cAMP production in cultured human ASM cells incubated with 1.0 mM chloroquine for the indicated times, or for 15 min with 30 μM isoproterenol, as determined by radioimmunoassay. There was no evidence for chloroquine-induced cAMP accumulation ($n = 3$ experiments). Inset, immunoblot of VASP and phosphorylated VASP (P-VASP) in cultured human ASM cells exposed to 1.0 mM chloroquine or saccharin (sacc), or 10 μM forskolin (forsk). Forskolin, which stimulates cAMP production, resulted in phosphorylation of VASP as indicated by the upper band. (**d**) Bitter tastant reversibility and additivity studies with intact mouse tracheas. Intact mouse tracheas were contracted with 1.0 mM acetylcholine (ach) which was maintained in the bath when chloroquine (200 μM) or isoproterenol (30 μM), or both drugs, were added. After exposure to chloroquine alone, the rings were washed and then rechallenged with the same dose of acetylcholine. $*P < 0.05$ versus acetylcholine alone; $\#P < 0.01$ versus acetylcholine + isoproterenol, or chloroquine alone. Results are from four experiments. Data are presented as means ± s.e.m.

ASM cells are similar to signal transduction pathways for bitter taste receptors in specialized taste cells of the tongue, where the G_{gust}-associated βγ activates PLCβ, resulting in IP_3 generation, which activates the IP_3 receptor on the sarco(endo)plasmic reticulum, releasing Ca^{2+} from this intracellular store[9].

Bitter taste receptors evoke airway relaxation

We initially assumed that bitter taste receptors evoked ASM contraction, as the increase in $[Ca^{2+}]_i$ promoted by some of the bitter tastants in human ASM was similar in magnitude to other ligands acting on bronchoconstrictive GPCRs. However, in isolated intact mouse airways, chloroquine, denatonium and quinine caused dose-dependent relaxation, with a maximal response of >90% loss of the active contraction evoked by acetylcholine or serotonin (**Fig. 2a**,**b**). The maximal relaxation response to the β-adrenergic receptor agonist isoproterenol under the same experimental conditions was a 30 ± 9.2% reduction in active tension (**Fig. 2a**). We also found that bitter tastants relax baseline tracheal ring tension (**Supplementary Fig. 4**). The relaxation response to bitter tastants was fully reversible, as washing tracheal rings while maintaining the presence of the contractile agent alone resulted in a return to contraction that was equivalent in magnitude to the control condition (**Supplementary Fig. 5**). Bitter tastants also relax human ASM. In fourth-order bronchi obtained from nondiseased portions of human lung tissues, chloroquine or saccharin induced a 50–80% reduction in active tension in acetylcholine-contracted rings (**Supplementary Fig. 6**). Bitter tastant–mediated airway relaxation was not altered by the cyclooxygenase inhibitor indomethacin or the nitric oxide synthase inhibitor L-NAME (data not shown), suggesting a direct activation of ASM receptors rather than a secondary response resulting from the release of bronchoactive ligands from airway epithelial cells.

Airway relaxation observed with β-agonists is due to β_2-adrenergic receptor coupling to adenylyl cyclase, leading to an increase in cAMP and subsequent PKA activation[2]. However, we found no evidence for chloroquine-induced increases in cAMP or PKA activation in intact cultured ASM cells, as assessed by a sensitive radioimmunoassay or



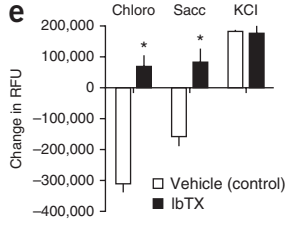


Figure 3 Isolated airway smooth muscle responses to bitter tastants as assessed by single cell mechanics and membrane potentials. (**a**) Cell stiffness of isolated ASM cells in response to 10 μM isoproterenol (iso), 1.0 mM chloroquine (chloro), 1.0 mM saccharin (sacc) or 1.0 μM histamine (hist). (**b**) Relaxation of isolated ASM cells in response to 1.0 mM saccharin in the presence of 1 μM of the PLCβ inhibitor U73122, 10 nM of the BK_{Ca} antagonists iberiotoxin (IbTx) and charybdotoxin (ChTx) or 100 nM of the PKA inhibitor H89. (**c**) The relaxation response to 1.0 mM chloroquine in isolated mouse airway contracted by 10 μM methacholine (Mch) in the absence or presence of 100 nM of the BK_{Ca} antagonist IbTx. Results are representative of five to eight experiments. (**d**) Membrane potential effects of saccharin and chloroquine. ASM cells loaded with a fluorescence-based membrane potential-sensitive dye were exposed to 1.0 mM chloroquine or saccharin, 1.0 μM histamine or 60 mM KCl (representative of four experiments). A decrease in relative fluorescence units (RFU) indicates hyperpolarization. (**e**) Effects of the BK_{Ca} antagonist IbTx (100 nM) on chloroquine and saccharin-promoted ASM hyperpolarization in intact ASM cells. Results represent the peak responses from four experiments. $*P < 0.01$ vs. vehicle control. Data are presented as means ± s.e.m.

by the PKA-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP), respectively (**Fig. 2c**). In a set of serial dosing and washout experiments with intact airways and a submaximal dose of chloroquine, we found that exposure to both chloroquine and isoproterenol resulted in relaxation that was greater than that found with either compound alone (**Fig. 2d**). These data indicate that bitter tastants evoke marked airway relaxation that is reversible, is not due to cell injury (because ASM functional contraction and relaxation are not impaired after washout), is not cAMP dependent and is additive with β-adrenergic agonists.

ASM bitter receptors signal to localized $[Ca^{2+}]_i$ and BK_{Ca}

To further ascertain the mechanism by which bitter taste receptors evoke ASM relaxation, we used magnetic twisting cytometry[15] to measure dynamic changes in stiffness of isolated human ASM cells (**Fig. 3**). In these experiments, magnetic particles attached to the cell by a peptide linker provide a highly quantitative measurement of single-cell stiffness, with isoproterenol and histamine exposure resulting in the expected relaxation and contraction from baseline, respectively (**Fig. 3a**). Chloroquine and saccharin exposure (**Fig. 3a**) resulted in ASM relaxation at the single-cell level, confirming that these bitter tastants act directly on smooth muscle cells. The relaxation response to saccharin was not blocked by the PKA inhibitor H89 (**Fig. 3b**), confirming results from cAMP and VASP phosphorylation measurements. Inhibition of PLC by U73122 eliminated the saccharin-induced relaxation of isolated ASM (**Fig. 3b**). In light of our findings with PLC inhibition (as well as βγ inhibitors and IP_3 receptor antagonists) on bitter tastant–promoted increases in $[Ca^{2+}]_i$ (**Fig. 1d**), these results suggest that the bitter tastant–mediated relaxation response in ASM is triggered by, or requires, intracellular Ca^{2+} release. Consistent with this concept, the chloroquine half-maximal effective concentration (EC_{50}) value for $[Ca^{2+}]_i$ release in cultured ASM cells (70 ± 10 μM, **Fig. 1a**) is almost identical to the EC_{50} for relaxation of intact airways (93 ± 4.3 μM, **Fig. 2a**). Depletion of sarcoplasmic reticulum Ca^{2+} stores with the sarcoplasmic reticulum Ca^{2+} reuptake inhibitor thapsigargin blocked chloroquine and other bitter tastant–mediated increases in $[Ca^{2+}]_i$ in ASM cells (**Supplementary Fig. 7**) and also inhibited chloroquine-mediated relaxation of intact airway rings (**Supplementary Fig. 8**).

One potential mechanism for Ca^{2+}-mediated relaxation in ASM is hyperpolarization due to stimulation of BK_{ca} channels. These channels are known to be expressed on human ASM[16] (which we confirmed; **Supplementary Fig. 1**) and have been reported to regulate airway tone[17]. To test whether the observed relaxation is due to bitter tastant–triggered Ca^{2+} activation of BK_{Ca}, we pretreated human ASM cells with medium alone, the Ca^{2+}-dependent K^+ channel antagonist charybdotoxin or the specific BK_{Ca} channel antagonist iberiotoxin. Pretreatment with both antagonists ablated saccharin-mediated ASM relaxation, as assessed in isolated cells (**Fig. 3b**). Similar results were found with chloroquine (data not shown). Pretreatment with iberiotoxin also attenuated chloroquine-induced relaxation of isolated mouse tracheal rings (**Fig. 3c**). Relaxation of ASM following BK_{Ca} activation would be expected to be from hyperpolarization of the cell membrane[18]. We loaded ASM cells with a membrane potential–sensitive fluorescent dye[19], and incubation with KCl and histamine resulted in the anticipated procontractile membrane depolarization (**Fig. 3d**). Incubation with the bitter tastants chloroquine and saccharin resulted

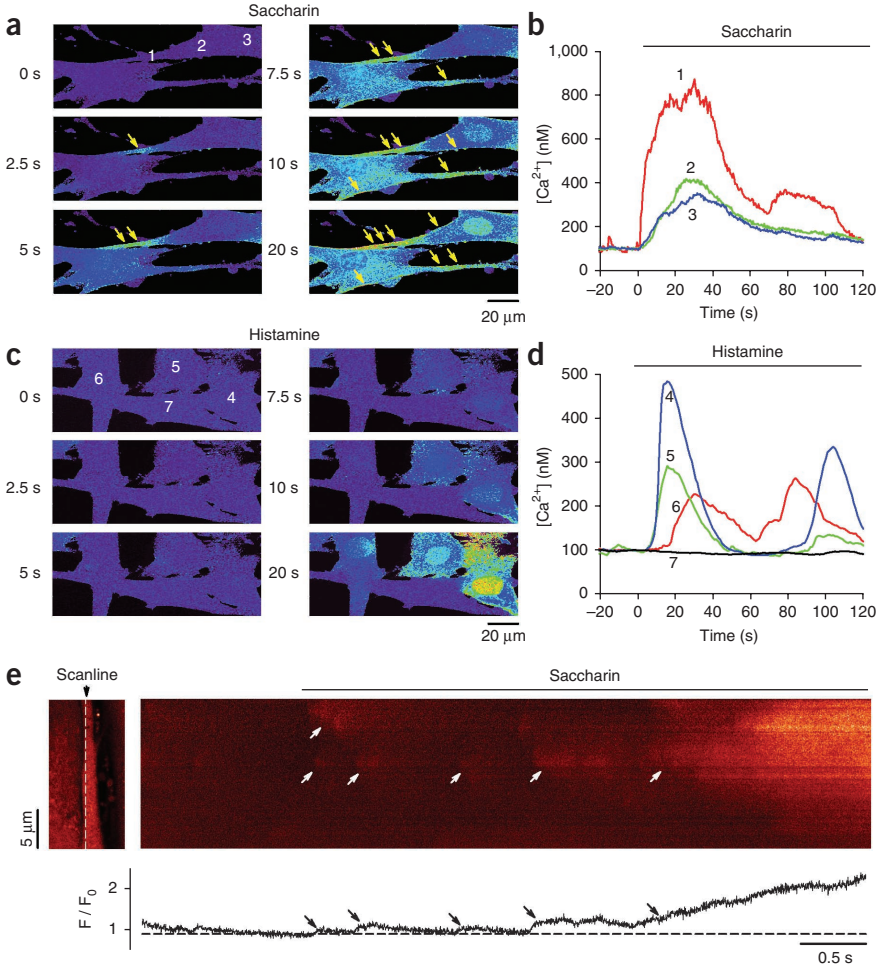


Figure 4 Saccharin preferentially triggers localized $[Ca^{2+}]_i$ responses in ASM cells. (**a**,**b**) Sequential confocal images of Fluo-3–loaded cells showing localized $[Ca^{2+}]_i$ increases in the cell upon exposure of ASM cells to 0.3 mM saccharin (**a**). The images represent Fluo-3 fluorescence after background subtraction and baseline normalization (F / F_0) with intensity encoded by pseudocolor. The arrows highlight local $[Ca^{2+}]_i$ 'hot-spots'. The numbers over areas of the cells represent regions of interest (ROI) which correspond to the numbered intensity tracings (**b**). (**c**,**d**) $[Ca^{2+}]_i$ images (**c**) and intensity tracings of ROIs (**d**), in ASM cells (loaded as in **a** and **b**) in response to exposure to 1.0 μM histamine. (**e**) Confocal line-scan imaging showing spatially and temporally resolved local $[Ca^{2+}]_i$ events activated by saccharin in a peripheral site. The scan line (white dashed line) was placed within 1 μm parallel to the cell membrane at one end of an elongated ASM cell, as shown at left. Arrows indicate several local $[Ca^{2+}]_i$ events that occurred before the more defined increase within the isolated region. At the bottom is the spatially averaged normalized fluorescence signal (F / F_0) generated from the line scan. Results are from single experiments representative of five performed.

in membrane hyperpolarization (**Fig. 3d**), which is inhibited by iberiotoxin (**Fig. 3e**). Thus, the bronchodilatory effect of bitter tastants is due to $[Ca^{2+}]_i$-dependent activation of BK_{Ca}. This is distinct from histamine-induced increases in $[Ca^{2+}]_I$, which cause depolarization and contraction, and suggests that the intracellular distribution of the Ca^{2+} responses to histamine and bitter tastants are different.

A highly localized increase in $[Ca^{2+}]_i$ is associated with BK_{Ca} channel activation[20]. To define the characteristics of saccharin-induced $[Ca^{2+}]_i$ increases in ASM cells as compared to those from histamine, we performed high-resolution real-time confocal imaging in Fluo-3–loaded cells. Localized $[Ca^{2+}]_i$ signals were detected at the slender ends and sarcolemmal regions of ASM upon exposure to saccharin (**Fig. 4a**). This response was rapid (observed within 2.5 s in the cross-sectional studies, **Fig. 4a**,**b**), and the magnitude was greater than at the central region of the myocytes (**Fig. 4a**). In contrast, the response to histamine in ASM cells caused a relatively delayed rise in $[Ca^{2+}]_i$ throughout the cell (**Fig. 4c**,**d**), without the localized features observed with saccharin. When using the line-scan mode at regions within 1 μm and parallel to the cell membrane of ASM cells, we detected spatially and temporally discernible $[Ca^{2+}]_i$ events very early after the application of saccharin, before the subsequent sustained localized rise in $[Ca^{2+}]_i$ (**Fig. 4e**). These results confirm the notion that saccharin promotes localized $[Ca^{2+}]_i$ signals in ASM cells.

Bitter tastants counteract asthmatic bronchoconstriction

Collectively, the above results all pointed to a previously unknown ASM relaxation pathway that might be used for treating reversible obstructive lung diseases such as asthma. To assess this potential, we administered bitter tastants by inhalation in the context of a mouse model of allergic airway inflammation and bronchial hyper-responsiveness. We sensitized mice to ovalbumin and then repetitively challenged them with inhaled ovalbumin, which resulted in acute airway inflammation (**Fig. 5a**,**b**). We measured airway resistance in these intact, sedated, intubated mice at baseline, after inhalation of the bronchoconstrictor methacholine, and then after inhalation of bitter tastants during the bronchoconstrictive phase. The positive control for these studies was inhaled albuterol, a β-agonist that is the most commonly used bronchodilator for treating asthma. In the ovalbumin-sensitized mice, the concentration of inhaled methacholine required to increase baseline airway resistance by four- to fivefold was 8 mg ml^{-1}, compared to ≥16 mg ml^{-1} for nonsensitized mice, confirming the airway hyperreactivity phenotype. Inhaled aerosolized quinine (150 μg) decreased airway resistance in normal and sensitized mice by 53 ± 3% and 50 ± 8%, respectively (**Fig. 5c**,**d**). Bronchodilatory effects were also found with denatonium (200 μg), which decreased airway resistance by 44 ± 6% in the normal mice and 57 ± 4% in the sensitized mice. Albuterol (3 μg) showed less reduction of airway resistance compared to quinine in the ovalbumin-challenged mice (**Fig. 5d**), suggesting that bitter tastants may be more effective than β-agonists in an inflammatory hyper-responsive state such as asthma.

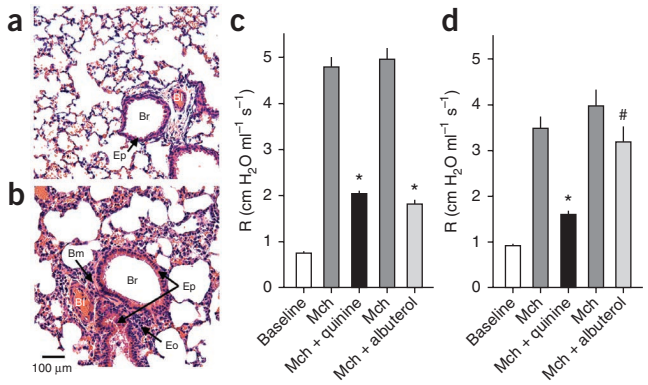


Figure 5 Bitter taste receptor agonists attenuate bronchoconstriction in a mouse model of asthma. (**a**,**b**) Photomicrographs from sections of control (**a**) and ovalbumin-challenged (**b**) mouse lungs showing eosinophilic inflammation of the airway, epithelial hyperplasia and basement membrane thickening in ovalbumin challenged airways (H&E stain). Br, bronchus; Bm, basement membrane; Eo, eosinophil; Ep, epithelium; Bl, blood vessel. (**c**,**d**) Airway resistance in control (**c**) and ovalbumin-challenged (**d**) mice measured at baseline, in response to aerosolized methacholine (mch) and in response to single doses of quinine 150 μg or the β-agonist albuterol (3 μg) given during the bronchoconstrictive phase ($n = 5$ experiments). The studies were carried out with a dose of methacholine that resulted in a four- to five-fold increase in airway resistance over baseline (≥16 mg ml^{-1} in control mice and 8 mg ml^{-1} in ovalbumin-challenged mice). *$P < 0.01$ versus methacholine; #$P < 0.05$ versus methacholine. Data are presented as means ± s.e.m.

DISCUSSION

GPCRs expressed on ASM represent the major family of signaling receptors that regulate airway tone and diameter. In asthma, multiple locally generated ligands act on these receptors leading to bronchoconstriction. The GPCRs mediating bronchoconstriction couple to $G_{\alpha q}$, increase $[Ca^{2+}]_i$ and trigger ASM contraction. In contrast, GPCRs coupled to $G_{\alpha s}$ increase cAMP, relax ASM and bronchodilate, with the β_2-adrenergic receptor being the target for β-agonists, the most commonly used therapeutic for bronchospasm. Identification of new ASM receptors that lead to bronchoconstriction and dilation further refines our understanding of the signaling network at play in asthma, leading to potential new therapeutic approaches. Here we found that several GPCRs belonging to the bitter taste receptor family are expressed on human ASM. A recent report[21] also identified bitter taste receptors on motile cilia of airway epithelial cells that increase beat frequency, which may represent a mechanical defense against noxious inhalants. These receptors are also found in the anterior nasal cavity, where they promote sneezing and regulate respiratory rate[22]. Binding to bitter taste receptors increases $[Ca^{2+}]_i$ in ASM via a Gβγ-, PLCβ- and IP_3 receptor–dependent manner. Although increased $[Ca^{2+}]_i$ would be expected to cause ASM constriction, such as that observed with $G_{\alpha q}$-coupled receptor activation, we observed marked relaxation in intact airways of mice and humans, and in isolated human ASM cells.

The dose-response curves for $[Ca^{2+}]_i$ stimulation and ASM relaxation by a given bitter tastant showed equivalent EC_{50} values, suggesting a connection between this intracellular signal and the physiological response. Indeed, depletion of sarcoplasmic reticulum Ca^{2+} resulted in the loss of bitter tastant–mediated increases in $[Ca^{2+}]_I$ and the relaxation effect in intact airway rings. Spatially restricted increases in $[Ca^{2+}]_i$ were observed in response to bitter tastants, which promoted BK_{Ca}-channel opening and membrane hyperpolarization. The basis for the restricted $[Ca^{2+}]_i$ response from bitter tastants remains to be defined. Notably, we observed a small degree of depolarization from bitter tastants in the context of BK_{Ca} blockade, suggesting that the two calcium pools (histamine receptor–induced versus TAS2R-induced) may have some overlap. Nevertheless, the net effect of the histamine-mediated $[Ca^{2+}]_i$ increase is depolarization and contraction, whereas for bitter tastants it is hyperpolarization and relaxation.

Lastly, aerosolized administration of bitter tastants relaxed the airways in a mouse model of allergic inflammation, indicating that

the pathway has therapeutic relevance in a diseased state. The evolutionary basis for bronchodilating bitter taste receptors on ASM remains speculative. They may act in a compensatory manner to acyl-homoserine lactones, which are agonists of these receptors[23] and are secreted by Gram-negative bacteria[24] during bronchitis or pneumonia. This action may provide protection against bronchospasm or airway closure, which might otherwise lead to worsening disease.

There is an unmet need for additional therapeutic options in the treatment of obstructive airway diseases such as asthma. Here we show that agents that bind bitter taste receptors cause marked bronchodilation of intact airways greater than that promoted by β-agonists. Furthermore, the effect of bitter taste receptors seems to be additive with that of β-agonists, which is consistent with their different mechanisms of action and suggests further studies on combination therapy could be considered. The choice of compounds that could be developed for this purpose is extensive given the marked structural diversity between bitter tastants. Indeed, there are many known synthetic agents, developed for other purposes, that activate bitter taste receptors[10] and are nontoxic. Furthermore, there are thousands of plant-derived bitter tastants and their metabolites that could have favorable therapeutic profiles[10].

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

D.A.D., single-cell mechanics and imaging, data analysis and manuscript preparation; W.C.H.W., expression studies, gene knockdown, airway physiology, data analysis and manuscript preparation; E.L.M., calcium signaling and data analysis; K.S.R., intact airway studies, expression studies, data analysis and manuscript preparation; R.M.S., airway physiology; S.S.A., single cell mechanics, data analysis, manuscript preparation; J.S.K.S., confocal calcium imaging, data analysis, manuscript preparation; S.B.L. directed all studies, data analysis and interpretation and is the primary author of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

$[Ca^{2+}]_i$, cAMP and membrane potential measurements. Primary human ASM cells were obtained from a commercial source (Clonetics) and maintained as previously described[25], with experiments done in cells at passages 3–8. To detect changes in $[Ca^{2+}]_i$, we loaded attached cells (80,000 cells per well) with Fluo-4 AM (BD Biosciences) and probenecid for 1 h; agonists were added by an automated pipetting system in triplicate, and the 525 nm signals were generated by excitation at 485 nm with a Flex Station II (Molecular Devices). The doses were as follows: 0, 10, 18, 32, 56, 100, 178, 320, 560 and 1,000 μM saccharin; 0, 10, 18, 32, 56, 100, 178 and 320 μM chloroquine. Unless otherwise stated, the medium for these experiments contained 1.5 mM calcium. In some studies, ASM cells were transfected with 4 μg *TAS2R10*-targeting siRNA (Invitrogen) or 4 μg of a scrambled-sequence siRNA control by electroporation (Nucleofector, Lonza). cAMP was measured by a ^{125}I-cAMP based radioimmunoassay[26]. The effects of bitter tastants on membrane potential of whole ASM cells was measured with a membrane potential–sensitive fluorescent dye (Molecular Devices) as previously described[19] in a 96-well plate format.

***Ex vivo* intact airway physiology.** All mouse studies were approved by the Animal Care and Use Committee, and human tissue studies were approved by the Institutional Review Board of the University of Maryland–Baltimore. We excised 5-mm sections of trachea from FVB/N mice (Taconic) and studied them in an isometric myograph system (Radnoti) as previously described[27]. A passive tension of 5 mN was applied for each ring for a baseline. For relaxation studies, rings were contracted with acetylcholine (1.0 mM) or serotonin (1.0 mM), which were maintained during addition of multiple doses of isoproterenol or bitter tastants. For studies where acetylcholine was used to contract the rings, the doses were as follows: 0, 0.01, 0.03, 0.10, 0.30, 1.0 and 3.0 μM isoproterenol; 0, 25, 50, 75, 100, 125, 250, 300, 375, 500 and 1,000 μM chloroquine; 0, 10, 30, 60, 100, 200, 300, 1,000 and 2,000 μM quinine; and 0, 10, 30, 100, 300, 1,000 and 3,000 μM denatonium. For studies where serotonin was used to contract the rings, the doses were as follows: 0, 1, 3, 7.5, 10, 25, 50, 75, 100, 250, 500 and 750 μM chloroquine; and 0, 30, 60, 100, 200, 300 and 1,000 μM quinine. Fourth-order bronchi from human lungs were obtained from extracted tissue specimens at the time of therapeutic or diagnostic surgical procedures. Bronchi were dissected from regions without gross pathology, and the rings were prepared and studied in a similar manner to those of the mice.

Magnetic twisting cytometry. Dynamic changes in baseline cell stiffness were measured as an indicator of contraction and relaxation of isolated human ASM cells by magnetic twisting cytometry as described previously[15]. For each individual ASM cell, baseline stiffness was measured for the first 60 s, then, after addition of the drug, stiffness was measured continuously for the next 540 s. In some experiments, cells were pre-exposed to vehicle or inhibitors for 10 min before addition of GPCR agonists.

Confocal imaging of regional and local $[Ca^{2+}]_i$ signals. Regional and local $[Ca^{2+}]_i$ signals were visualized as previously described[28,29] with the membrane-permeable $[Ca^{2+}]_i$-sensitive fluorescent dye Fluo-3 AM (Molecular Probes). Cultured human ASM cells were loaded with 5 μM Fluo-3 AM for 30–45 min at ~22 °C. Cells were washed with Tyrode solution (Sigma) containing extracellular Fluo-3 AM and incubated for 15–30 min to allow complete de-esterification of cytosolic dye. Excitation was at 488 nm and fluorescence was measured at >505 nm. Two-dimensional images were scanned at 0.22 μm/pixel, 512 pixels/line, 256 lines/image once every 0.5 s. Line scan images were collected at 0.075 μm per pixel, 512 pixels per line at 2-ms intervals for 10,000 lines per image. The amplitudes of $[Ca^{2+}]_i$ signals were calibrated to absolute $[Ca^{2+}]_i$ by a pseudo-ratio method[30].

Bitter taste receptor expression. Total RNA was extracted from human ASM cells, and reverse transcription was carried out with 2 μg RNA and oligo-dT primers (Invitrogen). Real-time PCR was carried out with an Applied Biosystems 7300 Real Time PCR system with methods previously described in detail[31]. Specific primers for the 25 *TAS2R* genes, *TAS1R1*, *TAS1R2*, *ADRB2*, *ADORA1*, *LTB4R* and *GAPDH* were obtained from Applied Biosystems. Data were analyzed with the $\Delta\Delta C_t$ method, with *ADRB2* as the reference[32]. The PCR products derived from primers for *TAS2R10*, *TAS2R14*, *TAS2R31*, *TAS2R5*, *TAS2R4* and *TAS2R19* were sequenced and verified to be from the respective human bitter taste receptor. Immunofluorescence microscopy was carried out on fixed cells as described[25].

Ovalbumin sensitization and pulmonary function testing. Sensitization of 6-week-old BALB/c mice (Taconic) was carried out by intraperitoneal injections of 100 μg ovalbumin (Sigma) in 200 μl alum, or alum alone (control) on days 0 and 14. Mice were then challenged with 1.0% aerosolized ovalbumin on days 19, 21 and 24. Twenty-four hours after the last challenge, mice were sedated and intubated, then ventilated, and measurements of airway resistance were taken as previously described[33]. Mice were challenged with sequential doses of 2.0, 4.0, 8.0, 16, 32 and 64 mg ml^{-1} of methacholine (Methapharm) in the nebulizer until a dose resulted in a sustained airway resistance of approximately four- to fivefold greater than baseline. Three minutes after the last methacholine inhalation, the bitter tastants quinine (150 μg) or denatonium (200 μg, both from Sigma), or the β-agonist albuterol (3.0 μg; Letco Medical) were administered by aerosol over 10 s. Resistance measurements (Raw, cm H_2O ml^{-1} s^{-1}) were taken every 30 s throughout the experiment.

Statistical analyses. Dose-response curves for $[Ca^{2+}]_i$ and *ex vivo* tracheal ring studies were analyzed by iterative nonlinear (sigmoidal) least-squares fitting. Results from all studies were compared with paired or unpaired two-way *t* tests (depending on study design), with $P < 0.05$ considered significant. When multiple comparisons were sought, analysis of variance with *post hoc t* tests was used with a correction for multiple comparisons. Data are presented as means ± s.e.m.

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