The bitter taste receptor (TAS2R) agonists denatonium and chloroquine display distinct patterns of relaxation of the guinea pig trachea

Ville Pulkkinen,1,2 Martijn L. Manson,1 Jesper Säfholm,1 Mikael Adner,1 and Sven-Erik Dahlén1

1The Unit for Asthma and Allergy Research, the National Institute of Environmental Medicine and the Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden; 2Research Programs Unit, Department of Medical Genetics, University of Helsinki, Finland

Submitted 12 July 2012; accepted in final form 6 September 2012

The bitter taste receptor (TAS2R) agonists denatonium and chloroquine display distinct patterns of relaxation of the guinea pig trachea. Am J Physiol Lung Cell Mol Physiol 303: L956–L966, 2012. First published September 7, 2012; doi:10.1152/ajplung.00205.2012.—Activation of taste receptors (TAS2Rs) by bitter taste agonists has been reported to cause bronchodilation. The aim of this study was to extend the information on the effects of bitter taste agonists on responses induced by different contractile mediators in a standard airway physiology preparation. Isometric responses were assessed in guinea pig trachea (GPT). TAS2R agonists were administered either to segments precontracted with different agonists for contraction or given before challenge with the different contractile stimuli, including antigen in tissues from ovalbumin-sensitized animals. TAS2R mRNA expression on GPT epithelium and smooth muscle was measured with real-time PCR. Denatonium, chloroquine, thiamine, and noscapine induced concentration-dependent relaxations (Rmax: 98.3 ± 1.6, 100.0 ± 0.0, 100.0 ± 0.0, and 52.3 ± 1.1% of maximum, respectively, in the presence of indomethacin) in segments precontracted with carbachol. The receptors for denatonium (TAS2R4, TAS2R10) and chloroquine (TAS2R3, TAS2R10) were expressed in GPT. Whereas denatonium selectively inhibited contractions induced by carbachol, chloroquine uniformly inhibited contractions evoked by prostaglandin E2, the thromboxane receptor agonist U-46619, leukotriene D4, histamine, and antigen. The effects of denatonium, but not those of chloroquine, were partly inhibited by blockers of the large Ca2+-activated K+ channels and decreased by an increase of the level of precontraction. In conclusion, TAS2R agonists mediated strong relaxations and substantial inhibition of contractions in GPT. Chloroquine and denatonium had distinct patterns of activity, indicating different signaling mechanisms. The findings reinforce the hypothesis that TAS2Rs are potential targets for the development of a new class of more efficacious agonists for bronchodilation.

Bronchodilation; asthma; prostaglandins; airway smooth muscle

Bitter taste-sensing type 2 receptors (TAS2Rs) are G protein-coupled receptors (GPCRs) on cell surface that mediate gustatory taste perception on the tongue (16). The properties of TAS2Rs are different from many other GPCRs, as the receptors are promiscuous and capable of binding a wide range of compounds with relatively low specificity and affinity (16). TAS2Rs have recently been suggested to have important extracellular functions in the respiratory and gastrointestinal tracts (4, 11). In the human airway epithelium, TAS2Rs are expressed on the solitary chemosensory cells (25, 26) and ciliated epithelial cells (23), where they sense chemical irritation and promote ciliary beat frequency, respectively. Thus TAS2Rs may be protective and part of the defense against inhaled noxious compounds.

Recently airway smooth muscle cells were found to express TAS2Rs that mediated relaxations in both mouse and human isolated airway preparations (7). Stimulation of TAS2Rs increased intracellular Ca2+ signaling that was suggested to activate large conductance potassium channels (BKCa) and result in hyperpolarization of the cell membrane. However, in further studies BKCa blockers either failed to prevent (28) or only partially blocked (2) relaxation caused by the bitter tastant chloroquine, and direct recording of the channel current with patch clamp revealed that bitter tastants did not activate BKCa channels in mouse airway preparations (28). As the effects of TAS2R agonists appeared independent of the cyclic adenosine monophosphate (cAMP) and the protein kinase A (PKA) pathways (7), the mechanisms of TAS2Rs in causing airway relaxation are still unsolved. Nevertheless, the TAS2R agonists showed both greater relaxation and inhibition of airway hyperresponsiveness than a β2-adrenoceptor agonist in mouse airways (7). Given the large selection of known natural and synthetic agonists recognized by the 25 TAS2Rs (16), the findings have introduced bitter taste receptors as a potential new family of targets for asthma pharmacotherapy. Moreover, we have recently found that leukocytes of patients with severe asthma have increased expression of TAS2Rs (Orsmark-Pietras C, James A, Konradsen J, Nordlund B, Söderhäll C, Pulkkinen V, Pedroletti C, Daham K, Kupczyk M, Dahlén B, Kere J, Dahlén SE, Hedlin G, Melén E, unpublished observations), suggesting also that TAS2Rs may represent a protective endogenous mechanism upregulated in disease.

The isolated guinea pig trachea (GPT) is a commonly used model in airway pharmacology, where the receptors for agonists and mediators of mast cell activation are closely similar to those of human airways (6). Accordingly, the antigen-induced contraction in guinea pig airways is caused by the concerted action of cysteinyl-leukotrienes, histamine, and prostanooids (24), mediators also known to cause allergen-induced bronchoconstriction in human subjects with asthma (20). In the present study, we characterized the expression of TAS2Rs in guinea pig airways, as well the effects of prototype TAS2R agonists on contractions induced by the different mediators of the antigen responses. The bitter taste agonists for the study were selected on the basis of the particular receptors expressed in the guinea pig genome. We examined both the “therapeutic” effects of TAS2R agonists on tracheal segments precontracted with different contractile agonists and the “prophylactic” effects of pretreatments with TAS2R agonists on agonist-mediated contractions. Our results extend the previous findings and

Address for reprint requests and other correspondence: S.-E. Dahlén, Dept. of Experimental Asthma and Allergy Research, Institute of Environmental Medicine, Scheeles väg 1, Karolinska Institutet, SE-171 77 Stockholm, Sweden (e-mail: sven-erik.dahlen@ki.se).
provide a more detailed analysis of the mode of action of TAS2Rs in the airways. This includes assessment of interactions with different pathways for contractions and the relation between the response and the level of precontraction. As the basal tone of GPT is kept by prostaglandin (PG) E₂ (9, 21), we in particular compared the effects of several of the tested TAS2R agonists in the presence and absence of indomethacin, which inhibits spontaneous tone and biosynthesis of PGF₂α.

MATERIALS AND METHODS

Tissue preparation. The study was approved by the Swedish Animal Experimentation Ethical Review Board (N257/09 and N323/11). Male albino guinea pigs (Dunkin-Hartley; 350–600 g) were killed by an overdose of pentobarbital sodium (Apoteket, Stockholm, Sweden), followed by rapid removal of the heart-lung-package and placement in ice-cold Krebs-Henseleit (KH) buffer solution (composition in mM: 118.5 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 25 NaHCO₃, and 11.1 d-glucose). For sensitization procedure, a single dose of 100 μg of ovalbumin (OVA) grade 5 and 0.1 g of aluminum hydroxide dissolved in 0.8 ml of sterile PBS was injected peritoneally, and animals were killed 14–28 days later. Trachea was gently dissected from the surrounding connective tissue. For mRNA expression, the epithelium in the trachea was scraped off with a scalpel, and the airway smooth muscle layer was cut out. Tissue preparations were extracted and stored in RNAlater (Applied Biosystems, Foster City, CA) at room temperature until use (0–4 days later). For the functional studies, tracheal segments were cut along the cartilage into eight intact rings of equal length. For denudation experiments, epithelium was removed by mechanical scraping with a scalpel.

Selection of the TAS2R agonists used for the study. Of the 25 human TAS2Rs identified, we found guinea pig orthologs for eight TAS2Rs, namely TAS2R1, TAS2R3, TAS2R4, TAS2R10, TAS2R14, TAS2R16, TAS2R40, and TAS2R41 by queries against the Ensemble database (www.ensembl.org). We selected agonists used in a previous study (7), namely chloroquine (TAS2R3 and TAS2R10 in guinea pig), denatonium (TAS2R4 and TAS2R10), and saccharin (as a negative control because the guinea pig genome lacks TAS2R8, TAS2R43, and TAS2R44 sequences). In addition, we selected agonists that bind to TAS2R1 (thiamine) and TAS2R14 (noscapine). No suitable agonists were available for TAS2R16 and TAS2R40, as well as the orphan receptor TAS2R41.

In vitro pharmacology. Intact GPT segments were set up in 5-ml organ baths with KH buffer solution at 37°C, bubbled with carbon gas (5% CO₂ in O₂). Changes in smooth muscle force were detected using an isometric force-displacement transducer linked to a Grass polygraph. During an equilibration period of 60 min, the resting force was adjusted to 30 mN. As a control of the tracheal reactivity, histamine (0.1 mM to 0.3 mM) was cumulatively added. Before the pharmacological studies, a further 30-min equilibration period was completed. Maximal relaxation was obtained by adding a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM), and maximal contraction was obtained by adding histamine (1 mM), acetylcholine (1 mM), and KCl (40 mM) at the end of the experiments. Parts of the experiments were performed in the presence of 3 μM indomethacin to abolish the constitutive release of prostaglandins.

GPT segments were precontracted with 100 nM carbachol, in both the absence and the presence of indomethacin. Precontraction by 100 μg/mL OVA was induced in the absence of indomethacin. To dissect the effects of PGF₂α and thromboxane A₂ (TXA₂) on TAS2R agonist-mediated relaxation, the EP₁ receptor antagonist ONO-8130 (10 nM) or the TP receptor antagonist SQ-29548 (1 μM) was added to the organ bath 30 min before precontraction. Experiments with different levels of carbachol precontraction (3 μM, 100 nM, and 30 nM) were performed in the presence of indomethacin. When a stable contraction was obtained after 40 min, the segments were exposed to the bitter taste agonists or salbutamol in a cumulative manner. In addition, a similar approach was used to study the effects of chloroquine and denatonium on stable precontractions induced by 0.1 μM U46619, 0.1 μM leukotriene D₄ (LTD₄), and 3 nM 17-phenyl trinor PGF₂α, all in the presence of indomethacin.

To study the prophylactic effects of the bitter taste agonists, 300 μM denatonium or 300 μM chloroquine was given after 30-min incubation with indomethacin. The effects of 30-min denatonium and chloroquine pretreatment were studied on contractions induced by increasing concentrations of carbachol, U46619, PGF₂α, LTD₄, or histamine.

To test whether denatonium could antagonize muscarinic receptors, acetylcholine-induced dilatation of guinea pig aorta was studied. During equilibration, segments were adjusted to a basal tension of 2 mN. The tissues were tested for viability by demonstrating a contractile response to 60 mM KCl and a relaxant response to 10 μM acetylcholine when precontracted with 10 μM phenylephrine. Thereafter, the segments were washed and exposed to 300 μM denatonium, precontracted with 3 μM PGF₂α, and again exposed to 10 μM acetylcholine.

RNA preparation and real-time PCR. Guinea pig total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. The concentration and purity of the RNA was measured using a Nanodrop 3300 (Thermo Scientific, Wilmington, DE). Aliquots of RNA were reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions.

All PCR primers were designed toward available guinea pig sequences from the Ensembl database (www.ensembl.org) and the NCBI Genebank Sequences (www.ncbi.nih.gov) from the second scaffold of the guinea pig (Cavia porcellus) genome comprised by the Feb 2008 Cavia porcellus draft assembly (Broad Institute cavPor3) using the Primer-BLAST software. Primers for TAS2R3 were: Fwd: 5'-GGTCAAGTGCGCGACCGTGGTGTG-3' and Rev: 5'-TGAAGGAGGGCAGGGTGTTGGA-3', for TAS2R4: Fwd: 5'-TACCGACATTTGCTGTGGCTGTG-3' and Rev: 5'-ACTCCCGAGCCCACTCTTGCA-3', and for TAS2R10: Fwd: 5'-TGCCAAATTGCGATGTGAATTCTCC-3' and Rev: 5'-ACCCATTCCCCTAGAAGCCCCAGG-3'. The cDNA was synthesized at Cybergene (Stockholm, Sweden) according to standard procedures. Primers for the housekeeping gene for β-actin were adapted from previous studies (27).

Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and in MicroAmp fast optical 96-well Reaction Plates (Applied Biosystems). Each sample was analyzed in duplicate. PCR reactions (20 μl) were performed with final concentrations of 100 nM each of forward and reverse primers. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR system using default settings according to the manufacturers’ instructions. To ensure the quality of the amplification product, a melt curve analysis was performed as a final step. mRNA levels were calculated using the comparative Ct method, normalized to the housekeeping gene β-actin, and expressed as 10ⁿ mol/mol β-actin.

PGF₂α measurements. PGF₂α levels were determined by using an enzyme immunoassay (EIA) with a PGF₂α monoclonal EIA kit (Cayman Chemical, Ann Arbor, MI). Detection limit was 15 pg/mL with cross-reactivity to 8-isoprostane PGF₂α (37.4%), 8-isoprostane PGF2α (0.025%), and 12,14-dihydro-15-keto PGF₂α (0.02%). Samples for these measurements were collected directly from the organ bath between 0 and 7 min at 1-min intervals after stimulation with denatonium and chloroquine. Bradykinin was used as a positive control for PGF₂α release.

cAMP measurements. Determination of cAMP levels was performed from tracheal segments as described previously (1). The segments were equilibrated for 60 min in tissue bath conditions as described above. This was followed by 30-min incubation of indomethacin (3 μM) and a further 30-min incubation of 200 μM 3-isobu-
tyl-1-methylxanthine, a nonselective inhibitor of phosphodiesterases. Thereafter, the tracheas were precontracted with carbachol for 40 min and exposed to a single concentration of 300 μM denatonium, 300 μM chloroquine, or 300 nM salbutamol for 20 min to achieve stable and maximal effect for all three agonists. When the experiments were completed, the segments were snap-frozen in liquid nitrogen and stored in −80°C until preparation for the EIA (581002, Cayman Chemical). The tracheas were purified in accordance with the manufacturer’s instruction for the EIA; the frozen tissues were placed in 5% trichloroacetic acid and homogenized on ice. After centrifugation at 1,500 g for 10 min, the trichloroacetic acid was extracted from the supernatant by water-saturated ether. The ether was removed by heating to 70°C before analysis by EIA (detection limit = 3 pmoL/ml).

**Calculations and statistics.** All data are presented as means ± SE. The concentration-response curve values for pEC_{50}, E_{max}, and Hill slopes were calculated using nonlinear regression analysis. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple-comparison test or Mann Whitney t-test for comparisons between two groups. For the analyses, GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) was used.

**Drugs and suppliers.** Berbotoxin, charbybdotoxin, paixilline, H89, GF109203, and KT5823 were purchased from Tocris Bioscience (Bristol, UK). NaCl, KCl, CaCl2, MgSO4, NaHCO3, K3PO4, and glucose were obtained from VWR (West Chester, PA). Denatonium benzoate, dextromethorphan, chloroquine hydrochlorate, noscapine, thiamine, histamine dihydrochloride, DMSO, acetylcholine, indomethacin, papavereine, sodium nitroprusside, and salbutamol were purchased from Sigma-Aldrich (St. Louis, MO). Formoterol was a kind gift from AstraZeneca (Stockholm, Sweden). SQ-29548, PGE2, 17-phenyl trinor PGE2, U-46619, PGE2na, and LTD4 were purchased from Cayman Chemical. ONO-8130 was a kind gift from ONO Pharmaceuticals (Tokyo, Japan). Stock solutions of indomethacin and U-46619 were dissolved in 95% ethanol, whereas ONO-8130 was dissolved in DMSO. All stock solutions were stored at −20°C. The other drugs were dissolved in KH solution or deionized H2O or organic solvents on the experimental day. Dilutions of drugs were freshly made from stocks before each experiment, and parallel experiments with solvent controls were always performed.

**RESULTS**

**Bitter taste agonists are effective bronchodilators against muscarinic contraction.** To examine relaxant effects of the selected compounds, GPT was precontracted with 100 nM carbachol both in the absence and presence of indomethacin or prostanoïd receptor antagonists. The segments were either exposed to cumulative concentrations of thiamine, denatonium, chloroquine, noscapine, or saccharin or kept untreated as controls for spontaneous relaxation. The partial β2-adrenoceptor agonist salbutamol was used as a positive control for airway relaxation to allow for comparison with data previously obtained in mouse and human airways (7).

Although indomethacin prevented the spontaneous increase of basal tone in naïve preparations, carbachol induced a similar stable magnitude of precontraction in the presence or absence of indomethacin [precontraction levels: 73.5 ± 3.8% (n = 19) and 75.2 ± 4.2% (n = 18) of E_{max}, respectively]. Thiamine, chloroquine, denatonium, and noscapine evoked concentration-dependent relaxations, whereas saccharin had no effect (Fig. 1, Table 1). Indomethacin had no effects on the potency of the TAS2R agonists (Table 1). However, the magnitudes (R_{max}) of the relaxations induced by thiamine (72.7 ± 6.6%) and denatonium (57.5 ± 5.2%) were enhanced by indomethacin (100.0 ± 0.0 and 98.3 ± 1.6%, respectively). In contrast, chloroquine induced an almost complete relaxation also in the naïve preparation (98.2 ± 1.1%; n = 6) that was unaffected by indomethacin (100.0 ± 0.0%). Compared with denatonium, chloroquine was eightfold less potent (pEC_{50}: 3.8 ± 0.1 and 4.5 ± 0.1, respectively) and had a Hill slope that was significantly steeper (−5.5 ± 0.7 and −1.5 ± 0.1, respectively). The magnitude of the noscapine-induced relaxation was somewhat decreased by indomethacin (Fig. 1, Table 1).

Because cyclooxygenase inhibition with indomethacin enhanced airway relaxation for denatonium and thiamine, we tested the hypothesis that these two TAS2R agonists were opposed by PGE2, the contractile prostanoïd that is known to regulate tone in the preparation (9, 18, 21). The magnitude of denatonium-induced relaxation was thus indeed enhanced by the EP1 receptor antagonist ONO-8130 (99.3 ± 0.7%) (Fig. 1E, Table 1) but not by the TP receptor antagonist SQ-29,648. Similar results were obtained with thiamine (Fig. 1C). Neither the EP1 nor the TP receptor antagonist, however, had any influence on the relaxation induced by noscapine (Fig. 1F).

**Expression of functional TAS2Rs in GPT.** There was significant real-time PCR expression of receptors for TAS2R3, TAS2R4, and TAS2R10 in smooth muscle and epithelium of the trachea (Fig. 2A). The rank order of bitter taste receptor expression was TAS2R3 > TAS2R10 > TAS2R4. The expression was generally higher in the tracheal epithelium than in the smooth muscle cells.

In addition, we screened the expression pattern for other TAS2Rs, namely TAS2R1, TAS2R14, and TAS2R16 (data not shown). For TAS2R1, the lack of complete whole genome sequences for guinea hampered our efforts, and we were unable to design properly working primers. The expression levels of TAS2R14 and TAS2R16 were near the detection limit, and we did not receive consistent data. In further functional experiments, we, however, found that dextromethorphan (agonist for TAS2R1 and TAS2R10) induced almost complete relaxations (94.7 ± 1.5%, pEC50 4.4 ± 0.1, n = 6) of GPT segments precontracted by 100 nM carbachol in the presence of 3 μM indomethacin. The response to dextromethorphan closely resembled that of denatonium.

On the basis of these patterns of relaxation responses and expression of mRNA, we chose denatonium and chloroquine for the extended analysis of pharmacological activities.

**Role of epithelium and smooth muscle in the relaxation.** Both denatonium and chloroquine act on receptors that were found to be expressed (denatonium TAS2R4 and TAS2R10; chloroquine TAS2R3 and TAS2R10). In view of the expression data, we next determined whether the epithelial TAS2R expression was necessary for relaxation mediated by denatonium or chloroquine. The potency of chloroquine on airway relaxation was, however, only marginally changed and, in fact, enhanced by epithelial removal (pEC_{50} 3.8 ± 0.1 and 4.2 ± 0.1, respectively, P = 0.04), and there was no significant effect compared with the intact trachea for denatonium (Fig. 2, B and C).

**Differential effects of denatonium and chloroquine on precontractions induced by agonists other than carbachol.** First, because the cyclooxygenase inhibitor indomethacin as well as the EP1 receptor antagonist ONO-8130 enhanced denatonium-mediated airway relaxation, the results suggested that either denatonium was unable to reduce the component of tone regulated by PGE2 (18) or that denatonium stimulated release of PGE2 that opposed its relaxant effect. To assess the latter possibility, PGE2 was measured by EIA in serial samples from
the organ bath fluid after addition of denatonium to preparations precontracted with carbachol. Whereas the positive control bradykinin caused a marked increase of PGE2 in the bath, there was no release of PGE2 after exposure to denatonium or chloroquine (Fig. 3A). In contrast, when the preparation was precontracted with the EP1/EP3 receptor agonist 17-phenyl trinor PGE2, denatonium failed to relax the preparation also in the presence of indomethacin (Fig. 3B). On the other hand, chloroquine induced complete relaxation of GPT precontracted with the EP1/EP3 receptor agonist 17-phenyl trinor PGE2 (Fig. 3B).

A differential effect of the two tested TAS2R agonists was confirmed in subsequent experiments using several different means of producing a precontraction. Thus, whereas denatonium was unable to reverse the antigen-induced contraction in preparations from sensitized animals challenged with OVA, chloroquine induced complete smooth muscle relaxation also in this setting (Fig. 4A). Likewise, chloroquine was able to relax preparations precontracted with the thromboxane receptor (TP) agonist U-46619, LTD4, or histamine, whereas denatonium was unable to relax contractions induced by any of these three agonists (Fig. 4, B–D).

Effects of pretreatment with denatonium or chloroquine on responses to contractile agonists. In the presence of indomethacin, the preparation was preincubated for 30 min with denatonium (n = 4) (B), and bitter taste agonists thiamine (n = 7; acting on TAS2R1 (C), chloroquine (n = 6–7; TAS2R3, TAS2R10) (D), denatonium (n = 5–9; TAS2R4, TAS2R10) (E), noscapine (n = 6–8; TAS2R14) (F), and saccharin (n = 4; no TAS2Rs in guinea pig) (G) on airway relaxation in GPT precontracted with 100 nM carbachol in the presence and absence of 3 μM indomethacin, 10 nM prostaglandin E2 (PGE2) receptor (EP1) antagonist ONO-8310 and 1 μM thromboxane TP receptor antagonist SQ29648. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (SNP) (0.1 mM) at the end of each experiment. ACh, acetylcholine.

Fig. 1. A: a trace depicting denatonium-mediated relaxation in guinea pig trachea (GPT) precontracted with 100 nM carbachol in the presence of 3 μM indomethacin. Effects of salbutamol (n = 4) (B), and bitter taste agonists thiamine (n = 7; acting on TAS2R1 (C), chloroquine (n = 6–7; TAS2R3, TAS2R10) (D), denatonium (n = 5–9; TAS2R4, TAS2R10) (E), noscapine (n = 6–8; TAS2R14) (F), and saccharin (n = 4; no TAS2Rs in guinea pig) (G) on airway relaxation in GPT precontracted with 100 nM carbachol in the presence and absence of 3 μM indomethacin, 10 nM prostaglandin E2 (PGE2) receptor (EP1) antagonist ONO-8310 and 1 μM thromboxane TP receptor antagonist SQ29648. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (SNP) (0.1 mM) at the end of each experiment. ACh, acetylcholine.
Table 1. Effects of TAS2R agonists and salbutamol on guinea pig tracheal segments precontracted with 0.1 μM carbachol with and without 3 μM indomethacin

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Saccharin</th>
<th>Thiamine</th>
<th>Chloroquine</th>
<th>Denatonium</th>
<th>Noscapine</th>
<th>Salbutamol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rmax</td>
<td>pEC50</td>
<td>Hill slope</td>
<td>Rmax</td>
<td>pEC50</td>
<td>Hill slope</td>
<td>Rmax</td>
</tr>
<tr>
<td>No indomethacin</td>
<td>10.7 ± 2.5</td>
<td>9.5 ± 2.1</td>
<td>72.7 ± 6.6</td>
<td>98.2 ± 1.1</td>
<td>57.5 ± 5.2</td>
<td>72.9 ± 4.4</td>
<td>98.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>pEC50 5.3</td>
<td>100.0 ± 0.0*</td>
<td>100.0 ± 0.0</td>
<td>98.3 ± 1.6*</td>
<td>52.3 ± 1.1†</td>
<td>98.7 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>rmax 13.1 ± 4.3</td>
<td>14.7 ± 5.3</td>
<td>3.59 ± 0.10</td>
<td>3.82 ± 0.10</td>
<td>4.48 ± 0.10</td>
<td>4.17 ± 0.04†</td>
<td>6.85 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>pEC50 5.51</td>
<td>0.91 ± 0.28</td>
<td>0.06 ± 0.03</td>
<td>0.19 ± 0.02</td>
<td>0.71 ± 0.08</td>
<td>2.12 ± 0.15</td>
<td>1.74 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Hill slope 0.13</td>
<td>0.05</td>
<td>0.09</td>
<td>0.12</td>
<td>0.08</td>
<td>0.15</td>
<td>0.12</td>
</tr>
</tbody>
</table>

The effects of denatonium, thiamine, and noscapine on precontracted segments were studied in the presence of 10 nM prostaglandin E2 receptor (EP1) antagonist ONO-8130 and 1 μM thromboxane A2 receptor TP antagonist SQ29548. *P < 0.0001, †P < 0.005 in comparison to the segments with no indomethacin.

chloroquine substantially inhibited the concentration-response relations for all other tested agonists (U-46619, PGE2, LTD4, and histamine), whereas denatonium had no significant effect (Fig. 5, C–F).

The effect of denatonium, but not that of chloroquine, is partly mediated through the Ca2+-induced large-conductance potassium channels. To further investigate the mechanisms of the relaxation induced by denatonium and chloroquine, blockers of Ca2+-induced large-conductance potassium channels (BKCa also known as MaxiK and Slo) were used. These included the scorpion venoms iberiotoxin and charybdotoxin that are blockers of the BKCa channels at 100 nM concentration (12) as well as paxilline, an indole alkaloid mycotoxin from Penicillium paxilli that is a potent BKCa channel blocker at 1 μM (14). None of the BKCa channel blockers altered the maximal relaxation to the two TAS2R agonists in preparations precontracted by carbachol. However, the potency of denatonium (pEC50 4.5 ± 0.1), but not that of chloroquine, was decreased by iberiotoxin, charybdotoxin, and paxilline (pEC50: 3.5 ± 0.2, 3.7 ± 0.1, and 3.5 ± 0.2, respectively; Fig. 6, A and B). For all three BKCa blockers, there was thus approximately one log order of magnitude of shift in the concentration-response relation for the denatonium-mediated airway relaxation. In line with previous observations (12, 17), it was confirmed that the potency of salbutamol similarly was decreased by iberiotoxin without effects on the maximal relaxation (Fig. 6C).

To investigate the involvement of other pathways important for relaxation, the effects of kinase inhibitors for PKA, PKC, and PKG on denatonium-induced airway relaxation were examined. However, 30-min pretreatment with 10 μM H89 (PKA inhibitor), 1 μM GF109203 (a selective inhibitor of PKC), and...
Both denatonium and chloroquine were able to induce maximal relaxations irrespective of the level of precontraction (Fig. 7, A and B; note that all levels of precontraction have been set to 100% to clearly illustrate the effect). The potency of denatonium, but not that of chloroquine, was, however, gradually decreased with increasing levels of precontraction. In contrast to both bitter taste agonists, salbutamol caused only a weak relaxation at the highest level of precontraction (Fig. 7C). Moreover, for the two lower levels of precontraction, where maximum relaxation still could be reached with salbutamol, the loss of potency with increasing level of precontraction was significantly greater for salbutamol than for denatonium (12 ± 2-fold and 2 ± 1-fold, respectively). In addition, we studied the effects of formoterol, which is a stronger agonist for β2-adrenoceptors than salbutamol. As shown in Fig. 7C, formoterol failed to relax the highest level of precontraction. However, the loss of potency with the two lowest precontraction levels was similar to that of denatonium (Fig. 7D).

DISCUSSION

Our findings confirm and extend the concept that bitter taste receptors may represent a new target for bronchodilation in asthma and obstructive airway diseases. We show that bitter taste receptors are expressed in the GPT and that agonists for these receptors can induce strong relaxations. In fact, in carbachol-contracted preparations, denatonium and chloroquine relaxed maximally contracted preparations that were resistant to the β-adrenergic agonist salbutamol. Taken together, our results in another species confirm several of the key observations on the potential of agonists of TAS2Rs as bronchodilators (7). On the other hand, in our extended analysis, we found that the properties of the most potent agonist denatonium were distinct from those of chloroquine. Whereas denatonium only reduced the effects of cholinergic contractions, chloroquine uniformly inhibited contractions evoked also by histamine, contractile prostanoids, and cysteinyl-leukotrienes. This was observed when the mediators were given alone as agonists or when they were released together endogenously by the antigen challenge. In addition, although relaxations induced by denatonium were sensitive to inhibitors of BKCa activation, the effect was only partial and similar to the effect of that class of drugs on the β-adrenergic agonist salbutamol.

We identified expression of guinea pig TAS2Rs for denatonium (TAS2R4 and TAS2R10) and chloroquine (TAS2R3 and TAS2R10), consistent with the functional effects of these two agonists. In addition, we observed relaxations to thiamine (TAS2R1), dextramethorphan (TAS2R1 and TAS2R10), and noscapine (TAS2R14). All these agonists induced strong relaxation of GPT precontracted with carbachol. For denatonium, dextramethorphan, chloroquine, and thiamine, the magnitude of relaxation was comparable to those of salbutamol or formoterol, however, with markedly lower potencies. The low potency is expected and in accordance with the existing literature on agonists for bitter taste receptors (16). Furthermore, the potency values of salbutamol and formoterol obtained in this study are comparable with the results from previous studies in isolated GPT (pEC50: 6.91 ± 0.19 and 6.89 ± 0.10 for salbutamol, respectively, and 9.32 ± 0.08 and 8.76 ± 0.09 for formoterol, respectively) (13). One of the challenges for
and 173 of chloroquine in GPT. The potency of thiamine was higher than that of its agonist saccharin. Although we could not study expression of TAS2R1 in mice airways, which also express its receptor (7), the fact that saccharin was active in mice airways, which also express its receptor, suggests that SACCHIN is active in mice airways. However, it is possible that the activity of SACCHIN in mice airways is achieved through a different mechanism.

The effects of denatonium and chloroquine differed though. Whereas relaxations induced by denatonium were enhanced by treatment with either indomethacin or the EP1 receptor antagonist ONO-8130, the response to chloroquine was the same irrespective of whether spontaneous tone was abolished or not. The observations suggested that denatonium was unable to inhibit responses to PGE2, which is established to maintain the spontaneous tone of the preparation (18, 21). This mode of action was confirmed because denatonium failed to inhibit the contraction response both to PGE2 itself and the analog 17-phenyl trinor PGE2. The finding that the TP receptor antagonist SQ-29548 did not mimic the effects of indomethacin or the EP1 antagonist provided further strength to this conclusion. As we also found that denatonium did not induce release of PGE2 from the GPT, we can attribute the reduced response to denatonium in naïve preparations solely to its inability to counteract the part of the preload contributed by constitutive release of PGE2, and the data support that thiamine induces relaxation by the same PGE2-sensitive mechanism. A similar

Fig. 4. A: effects of denatonium and chloroquine on airway relaxation of ovalbumin-sensitized guinea pig tracheal segments preconstricted with ovalbumin. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM) at the end of each experiment (n = 4–6). Effects of denatonium and chloroquine on 100 nM U46619 preconstriction (n = 4–6) (B), 100 nM LTD4 preconstriction (n = 4) (C), and 10 μM histamine (n = 3) (D) in the presence of 3 μM indomethacin.

Any future drug development is obviously to identify more potent ligands for TAS2Rs.

Another need is to define the TAS2Rs that are most suitable targets. Despite the lack of available antagonists for TAS2Rs, the effects of the tested agonists in the guinea pig tissues already provide relatively clear insight into which receptors have been activated. Thus the potency of denatonium, known to selectively activate TAS2Rs, was approximately fivefold higher in GPT compared with the potency reported for the same compound against hTAS2R10 in transfected human embryonic kidney (HEK)-293T cells (EC50: 25 μM and 120 μM, respectively) (16). Because denatonium is 100-fold more potent for hTAS2R10 than for hTAS2R4, the data indicate that the relaxant effect of denatonium is mediated through the TAS2R10. This interpretation is further supported by the very similar potency and pattern of activity of dextromethorphan and denatonium. Dextromethorphan is known to activate both TAS2R1 and R10 (16), but, in absence of confirmed expression of TASR1 in GPT, we interpret our data to support that denatonium and dextromethorphan mainly evoke the observed relaxations via TASR10. One explanation for the observed higher potency of denatonium compared with the transfected human cells might be an interaction between the TAS2R10 and a concomitant activation of TAS2R4. Although the potency of chloroquine was low, it was strikingly similar to that obtained for hTAS2R3 in transfected HEK-293T cells (EC50: 178 μM and 173 μM, respectively) (16). Because chloroquine has a 1,000-fold lower potency for hTAS2R10 (16), it is assumed that the relaxant effect of chloroquine solely was mediated through TAS2R3.

Furthermore, the observation that the guinea pig does not have sequences for the known saccharin TAS2Rs is consistent with the observation that saccharin failed to relax GPT. Saccharin was active in mice airways, which also express its receptor (7). Although we could not study expression of TAS2R1, the potency of its agonist thiamine was similar to that of chloroquine in GPT. The potency of thiamine was higher than expected from data in transfected HEK-293T cells (16). For noscapine, which was as potent as denatonium but did not induce the same strong relaxation as salbutamol in GPT, the potency is in accordance with the effect on hTAS2R14 in transfected HEK-293T cells. The low efficacy may thus relate to limited receptor expression. Taken together, the analysis comparing our current data with those on human TAS2Rs supports that denatonium, chloroquine, dextromethorphan, thiamine, and noscapine cause relaxations of GPT through activation of specific TAS2Rs.

In this study, TAS2R3, TAS2R4, and TAS2R10 transcripts were detected both in epithelial and smooth muscle cells of GPT. Although denudation of the tracheal epithelium caused small shifts of the concentration response curves for the bitter taste agonists, it did not remove the relaxant effects. Thus these results support that the relaxations to denatonium and chloroquine are mediated almost exclusively through a direct effect on the airway smooth muscle.

The effects of denatonium and chloroquine differed though. Whereas relaxations induced by denatonium were enhanced by treatment with either indomethacin or the EP1 receptor antagonist ONO-8130, the response to chloroquine was the same irrespective of whether spontaneous tone was abolished or not. The observations suggested that denatonium was unable to inhibit responses to PGE2, which is established to maintain the spontaneous tone of the preparation (18, 21). This mode of action was confirmed because denatonium failed to inhibit the contraction response both to PGE2 itself and the analog 17-phenyl trinor PGE2. The finding that the TP receptor antagonist SQ-29548 did not mimic the effects of indomethacin or the EP1 antagonist provided further strength to this conclusion. As we also found that denatonium did not induce release of PGE2 from the GPT, we can attribute the reduced response to denatonium in naïve preparations solely to its inability to counteract the part of the preload contributed by constitutive release of PGE2, and the data support that thiamine induces relaxation by the same PGE2-sensitive mechanism. A similar
A decrease in relaxation has been shown for the β2-adrenoceptor when the EP1 receptor is activated (15).

Our data furthermore indeed implicate that the TAS2R agonists denatonium and chloroquine mediated relaxations through different signaling pathways. Whereas denatonium failed to reverse on-going antigen-induced contractions in the OVA-model, as well as the contractions induced by any of the mediators of that response (histamine, U-46619 for PGD2/TXA2 and LTD4) (24), chloroquine was able to cause relaxations irrespective of contractile agonist. The difference in the effects of the two agonists was also shown when GPT was contracted with the different agonists after pretreatment with either denatonium or chloroquine. In these experiments, denatonium could again only reduce the effect of carbachol, whereas chloroquine decreased the effects of all contractile agonist used in the studies. The results further indicated that the denatonium-induced relaxation is dependent on the type of intracellular signaling induced by the particular agonist, whereas chloroquine-mediated relaxation responses appear to be signaling-independent, or at least via pathways that are able to counteract a broader range of signaling mechanisms.

As BKCa channels are shown to be involved in the relaxation mediated by β2-adrenoceptors (12, 17) and TAS2Rs, we further investigated the importance of this mechanism in GPT by the use of three different BKCa channel blockers. The effects of chloroquine were not affected by iberiotoxin, charybdotoxin, and paxilline. With denatonium, roughly a one-log shift change was obtained with all channel blockers, but the maximal effect

**Fig. 5.** A: trace depicting the effects of denatonium, and chloroquine prestimulation on contractions induced by increasing concentrations of carbachol. GPT was prestimulated with 300 μM denatonium or 300 μM chloroquine for 30 min in the presence of 3 μM indomethacin followed by increasing concentrations of carbachol (n = 5–6) (B), U46619 (n = 4) (C), PGE₂ (n = 5) (D), leukotriene D4 (LTD₄) (n = 5–6) (E), and histamine (n = 5–6) (F). Data are expressed as percentages of maximal contraction obtained with histamine (1 mM), acetylcholine (1 mM), and KCl (60 mM), which were added to the bath at the end of each experiment. In histamine experiments, the first histamine peak used as a control of the guinea pig trachea reactivity was used as a reference to avoid the effects of receptor desensitization.
was not altered. Similar results have been reported for the channel blockers against salbutamol (12). However, when the toxins were added, the level of the carbachol precontraction was increased, which per se was shown to induce a rightward shift of the concentration-response curve to denatonium (discussed below). This suggests that bitter taste agonists induce smooth muscle relaxation by more mechanisms than activation of BKCa channels. Our findings appear to be in line with the

Fig. 6. GPT was treated with 3 µM indomethacin in the presence and absence of Ca2+ induced large K+ channel blockers iberiotoxin (100 nM), charybdotoxin (100 nM), and paxilline (1 µM), and precontracted with 100 nM carbachol. The effects of denatonium (A), chloroquine (B), and salbutamol (C) on airway relaxation are expressed as percentages in relation to maximal relaxation. D: effects of kinase inhibitors on denatonium-induced airway relaxation. KT5823 = selective inhibitor of protein kinase G (1 µM), GF109203 = selective inhibitor of protein kinase C (1 µM), H89 = protein kinase A inhibitor (10 µM). E: samples were collected from the organ bath fluid 20 min after 300 nM salbutamol (n = 4), 300 µM denatonium (n = 4), 300 µM chloroquine (n = 4) and stimulation of GPT precontracted with 100 nM carbachol for 40 min followed by 30 min incubation of 200 µM 3-isobutyl-1-methylxanthine, a nonspecific inhibitor of phosphodiesterases. The absolute cAMP release (pg/ml/mg) was measured with enzyme immunoassay. **P < 0.01, ***P < 0.001. F: guinea pig aorta was precontracted with 10 µM phenylephrine and exposed to 10 µM ACh. Thereafter, the segments were washed and exposed to 300 µM denatonium, precontracted with 3 µM PGF2α, and again exposed to 10 µM ACh.

Fig. 7. Effects of denatonium (n = 5–9) (A), and chloroquine (n = 4–9) (B), salbutamol (n = 4) (C), and formoterol (n = 6) (D) on airway relaxation in GPT precontracted with 3 µM, 100 nM, and 30 nM carbachol (CCh) in the presence of 3 µM indomethacin. Data are expressed as percentages of maximal relaxation obtained with a combination of papaverine (0.1 mM) and sodium nitroprusside (0.1 mM) at the end of each experiment.
recent results of Zhang et al. (28), who did not find evidence of specific contribution of the BK$_{Ca}$ channels to the relaxation caused by chloroquine in isolated mouse airways. However, An et al. (2) found the chloroquine-induced relaxation of mouse trachea and human airway smooth muscle to be partially sensitive to BK$_{Ca}$ channel blockers. It is likely that methodological issues and species differences in bitter taste receptor homolog expression could contribute to different results in different systems. It is possible that BK$_{Ca}$ channel activation may be a general process in smooth muscle regulation and not specific for responses mediated by TAS2Rs.

As discussed by others (5), the level of precontraction can influence both the magnitude and the potency of the agonist-induced relaxation. Indeed, when the preload was increased to supramaximal levels, both denatonium and chloroquine induced maximal relaxations, whereas neither of the β$_2$-adrenoceptor agonists did. This confirmed the observation that bitter taste agonists induced a stronger effect than β$_2$-adrenoceptor agonists (7). Furthermore, whereas the potency for denatonium increased with decreasing preload, the potency for chloroquine was unaffected. When the preload was decreased, β$_2$-adrenoceptor agonists, both with an increase of potency, induced a maximal relaxation similar to that of the bitter taste agonists. However, the increase in potency for salbutamol was greater than for formoterol, which probably is related to salbutamol being a partial agonist and more sensitive to the level of preload than formoterol, which is a full agonist. The finding that neither β$_2$-adrenoceptor agonists produced maximum relaxation at the highest preload indicates that the bitter taste agonists have a greater efficacy than the β$_2$-adrenoceptor agonists and induce smooth muscle relaxation by other mechanisms. These distinct mechanisms between bitter taste agonists and β$_2$-adrenoceptor agonists were recently shown also by the lack of interference in desensitization experiments (3).

In preliminary attempts to identify the signaling pathways for the denatonium-mediated relaxation, we used kinase inhibitors for PKA, PKC, and PKG, but none of the used inhibitors had any effects. We also measured the levels of cAMP, which is a central second messenger for the β$_2$-adrenoceptor-mediated relaxation that can also activate other pathways than PKA (8). However, we did not find any increase in intracellular cAMP after maximal stimulation of GPT with denatonium and chloroquine. Thus we could not identify the signaling mechanisms for the bitter taste agonist-mediated airway relaxations, and further search for this is currently hampered by the lack of molecular tools in the guinea pig.

The selective effect of denatonium on cholinergic responses in the GPT was obviously not due to muscarinic antagonism because acetylcholine-mediated relaxation in aorta was not inhibited by denatonium. Although the Hill slope values for denatonium suggest a GPCR-mediated mechanism for this agonist in our studies, it should be kept in mind that denatonium is able to activate both G protein-dependent and-independent pathways in the taste buds (22). In another study, intragastric administration of TAS2R agonists induced release of ghrelin and inhibited gastric emptying. However, only the former effect was abolished in mice lacking the α$_L$-gustducin G protein (11). For chloroquine, the Hill slope was consistently steep, which suggests an involvement of ion channel activation (10), which can either be linked through a TAS2R or due to other mechanisms. Because both agonists induce strong airway smooth muscle relaxation, it should be of importance to delineate the pathways further.

In conclusion, our results demonstrate that, in this particular preparation, denatonium may completely relax contractions induced by muscarinic agonists, but it does not inhibit responses induced by other contractile agonists. The selective effect of denatonium on muscarinic responses is most likely due to TAS2R10 activation, whereas chloroquine via activation of TAS2R3 and other yet unidentified additional actions display a pattern of global inhibition of different contractile responses in GPT. Although we could not find a specific G protein-coupled pathway in this first description of relaxation of guinea pig airways induced by activation of bitter taste receptors, the potency of the bitter taste agonists showed clear similarities to human expression systems (16). Taken together with previous findings in other species and models (3, 5, 7, 19), our findings reinforce that TAS2Rs are potential new targets for development of a novel class of bronchodilators. Priority should now be given to define the signaling mechanisms and to identify agonists, which are more potent and selective.

ACKNOWLEDGMENTS

The authors thank Ingrid Delin for excellent technical assistance.

GRANTS

This work was supported by the Swedish Medical Research Council, the Swedish Heart-Lung foundation, Vinnova Chronic inflammation - diagnostic and therapy (CIDAIt), Swedish Foundation for Strategic Research (SSF), the Stockholm County Council Research Funds (ALF), Karolinska Institutet, the Swedish Society of Medicine, the Bernard Osher Initiative for Severe Asthma Research, and the Centre for Allergy Research at Karolinska Institutet. V. Pulkkinen is supported by the Academy of Finland (post-doctoral researcher’s project no 126701), Research Funds of the University of Helsinki, the Finnish Anti-tuberculosis Association Foundation, the Väinö and Laina Kivi Foundation, and the Wenner-Gren Foundations.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


