Caffeine induces gastric acid secretion via bitter taste signaling in gastric parietal cells

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Caffeine, generally known as a stimulant of gastric acid secretion (GAS), is a bitter-tasting compound that activates several taste type 2 bitter receptors (TAS2Rs). TAS2Rs are expressed in the stomach and in several extroratal sites, e.g., in the gastrointestinal tract, in which their functional role still needs to be clarified. We hypothesized that caffeine evokes effects on GAS by activation of oral and gastric TAS2Rs and demonstrate that caffeine, when administered encapsulated, stimulates GAS, whereas oral administration of a caffeine solution delays GAS in healthy human subjects. These findings demonstrate that bitter taste receptors in the stomach and the oral cavity are involved in the regulation of GAS in humans. This regulatory process can be modified by bitter-masking compounds, such as denatonium benzoate (6); hop-derived beer bitter acids; iso-\textalpha-acids (7); and catechin and procyanidin B2 (8) cause gastrin release (6) or GAS (7, 8) indicates that bitter substance-evoked chemosensory mechanisms may be involved. Chemosensory potentially plays a role at three sites to regulate GAS: (i) bitter substances could excite oral taste cells and mediate their effects through cephalic regulation of gut physiology (9) or (ii) a bitter compound could also act in the gut through induction of gastrin and/or histamine release from enteroendocrine cells and/or (iii) by modulating acid production in GAS-producing parietal cells (10).

Bitter tastants elicit bitterness through a family of oral taste type 2 bitter receptors (TAS2Rs) (11). Humans express approximately 25 TAS2Rs, of which five TAS2Rs, TAS2Rs 7, 10, 14, 43, and 46, can be activated by caffeine (12). In addition to the mouth, TAS2Rs have also been identified in nongustatory tissues, including airway epithelia (13), brain (14), intestinal cells (15, 16), and the gastric epithelia of rats and mice (17, 18). Beyond their chemosensory function, extraoral TAS2Rs are involved in nonsensory processes to expel or neutralize toxins in the upper and lower airways as well as in the gastrointestinal tract (19). Furthermore, the TAS2R pathway in the gut is involved in the regulation of food intake, digestion, and satiation (15, 16, 20, 21). Whereas, in the stomach, the endocrine effect of bitter substances on ghrelin secretion has been well described (20), a bitter compound-mediated oxytocin function on acid production in parietal cells had not yet been discovered to our knowledge. Parietal cells can be activated by histamine or acetylcholine binding to their cognate histamine H2 or acetylcholine M3 receptors (22). Activation of these receptors results, either by Gs- and adenylyl cyclase/cAMP- or by Gq- and phospholipase C (PLC)/IP\textsubscript{3}/Ca\textsuperscript{2+}-dependent pathways, in the activation of the H+K+-ATPase, which pumps protons into the stomach lumen (22).

In taste cells located on the tongue, the signaling cascade of TAS2Rs also includes a cAMP-dependent and a PLC\textsubscript{2}/IP\textsubscript{3}/Ca\textsuperscript{2+}-dependent pathway (23). Initiation of the latter major pathway leads to calcium release from intracellular compartments, which in turn activates transient receptor potential M5 ion channels. These channels mediate an influx of sodium ions and membrane depolarization (23), leading to ATP release and bitter perception. The \alpha-subunit of gustducin has been described to stimulate

gastric acid secretion | caffeine | homoeriodictyol | bitter taste receptors | TAS2Rs

\textbf{Significance}

This study shows that caffeine’s effect on gastric acid secretion (GAS) is more complex than has been previously thought. Oral and gastric bitter taste receptors are involved in the regulation of GAS in humans. This regulatory process can be modified by the bitter-masking compound homoeriodictyol. Practical applications of the results may include treatment of gastroesophageal reflux disease or peptic ulcer by manipulating gastric pH by means of bitter tastants and inhibitors.


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PDEs, resulting in low cAMP levels and PKA activities, which keep the IP3 type 3 receptor hypophosphorylated and sensitzed (24). Therefore, the tonic activity of α-gustducin regulates taste cell responsivity. Transducin, a similar G protein also present in taste cells, can replace the function of α-gustducin (25, 26). TAS2R-expressing cells in the gastrointenstinal tract have been reported to coexpress the downstream taste signaling components, suggesting that similar signal transduction pathways could also mediate gastrointestinal physiology (27). However, the detailed signal transduction pathways in extraoral chemosensitive cells are yet unknown.

This study investigated whether gastric and oral TAS2Rs contribute to the regulation of caffeine-induced mechanisms of GAS in humans. To study this hypothesis, the effect of caffeine on GAS was investigated in a human intervention trial, taking into account taste receptor activation in the mouth and the stomach. The underlying gastric mechanisms were studied by TAS2R expression analysis and by means of the validated HGT-1 cell culture model, which maintains the relevant characteristics of human parietal cells (28, 29).

Results
Oral Bitter Perception Reduces GAS in Human Subjects. Real-time gastric pH measurements were performed after caffeine administration in human subjects by means of Heidelberg pH diagnostic capsules (29–32). Heidelberg pH capsules are used to determine gastric acid secretory ability under conditions simulating the ingestion of food or beverages by means of radiotelemetry. For the measurements, overnight-fasted subjects swallow the pH capsule, followed by a saturated sodium bicarbonate solution. Ingestion of the bicarbonate solution triggers an increase in stomach pH and a subsequent attempt by the parietal cells to reestablish acidity. The impact of foods or beverages on the reacidification time can be analyzed by administration of the test material before or after the pH challenge. In this study, subjects swallowed a caffeine solution with or without a bitter-masking compound, homoeiodicyctol (HED) (33, 34) 5 min after or 25 min before the bicarbonate challenge. Reacidification time was measured for three distinct delivery protocols (1–3), each of which assesses different sites of TAS2R activation (Fig. 1A). The subjects underwent the following interventions in 11 consecutive study site visits: the first 8 visits were performed using protocol 1, subjects drank 125 mL water, a caffeine solution (37.5, 75, or 150 mg caffeine in 125 mL water) with or without 30 mg HED, or an HED solution (30 mg HED in 125 mL water), thereby stimulating oral and gastric TAS2Rs (Fig. 1B). For delivery protocols 2 and 3, a dose of 150 mg caffeine was administered along with 125 mL water, either encapsulated to selectively stimulate gastric TAS2Rs, or as a sip-and-spit solution to activate only oral TAS2Rs, respectively (Fig. 1B).

During the final three visits, subjects were asked to drink 125 mL water or 150 mg caffeine with or without 30 mg HED in 125 mL water (delivery protocol 2) 25 min before the bicarbonate challenge to evaluate the effect of administration time. The intervention time of 25 min was chosen according to previous publications that demonstrated that caffeine starts to stimulate gastric acid after 30 min (2, 5).

Drinking the volume water control solution 5 min after the bicarbonate challenge resulted in a mean reacidification time of 23 ± 1 min (individual representative gastrogram shown in Fig. 1C). Oral application of caffeine by sip-and-spit or drinking led to prolongations (P < 0.05) of reacidification time by delta reacidification time values (reacidification timecompound − reacidification timecontrol) of 20 ± 6 min and 8 ± 2 min, respectively, compared with administration of a volume water control solution, indicating a delay of GAS (Fig. 1D). Stimulation of gastric sites only by encapsulated caffeine resulted in a shorter delta reacidification time of 5 ± 3 min relative to sip-and-sip administration (P < 0.05; Fig. 1 C and D). Individual gastrograms were quantified by determining the slope after the onset of reacidification. A higher slope indicates that, when reacidification has started, the gastric pH returns to its initial pH faster. The slope of the gastrogram (relative to water control) obtained after administration of encapsulated caffeine was higher (0.20 ± 0.16 pH units per min) compared with the slope calculated after drinking (−0.20 ± 0.10 pH units per min) and sip-and-sip intervention (−0.39 ± 0.05 pH units per min), thereby stimulation of oral receptors occurred (Fig. 1E). To extend the time period over which the effect of caffeine on GAS could be measured, we repeated the experiments with encapsulated caffeine administered with 125 mL water 25 min before the alkaline challenge. This intervention allows gastric pH changes to be recorded over a time period of 25 min to approximately 85 min after caffeine administration and revealed a stimulation of GAS, indicated by a reduced delta reacidification time of −23 ± 4 min by caffeine (Fig. 2B) compared with control treatment (empty capsule plus 125 mL water reacidification time, 41 ± 4 min; P < 0.01).

HED Reduces the Caffeine-Evoked Effects on GAS in Human Subjects. To determine if TAS2R bitter-taste receptors mediate the effect of caffeine on GAS, 125 mL water containing 150 mg caffeine and/or 30 mg of the bitter-masking compound HED (33, 34) were swallowed 5 min after the alkaline challenge (delivery protocol 1). Administration of HED alone resulted in a reacidification time of 21 ± 2 min, comparable to that of water (24 ± 1 min) as volume control.

Unexpectedly, concomitant administration of HED and caffeine resulted in accelerated gastric emptying in 4 of 10 subjects, as indicated by passing of the Heidelberg capsule into the duodenum before complete reacidification. The same effect was observed in 2 of 10 subjects after drinking a solution of 30 mg HED dissolved in 125 mL water. When HED and caffeine were administered encapsulated (delivery protocol 2), reacidification times could be analyzed in only six subjects, as four subjects demonstrated accelerated gastric emptying as seen after oral and gastric delivery (protocol 1). These results raised the question whether the bitter-masking compound HED promotes gastric motility by stimulating gastric relaxation. Experiments using strips of dissections of human stomach biopsy specimens revealed that treatment with 1 mM HED in an organ bath induced a maximum relaxation after 40 min, with mean tension values of 45.4 ± 6.7%, compared with water control values of 107 ± 5.7% (Fig. S1 A and B).

In those subjects who were subjected to delivery protocol 1 and did not respond with accelerated gastric emptying, HED largely reversed the effects of caffeine on reacidification time; whereas drinking of the caffeine solution 5 min after alkaline challenge resulted in a delta reacidification time of 8 ± 2 min, concomitant caffeine and HED administration revealed a mean value of 1 ± 1 min (Fig. 2A and B), but showed no effect on the slope of the gastrogram (Fig. 2C). In contrast, gastric administration of encapsulated caffeine 25 min before alkaline challenge (delivery protocol 2) induced GAS compared with administration of water, resulting in a delta reacidification time of −23 ± 4 min (Fig. 2 D and E). Although the reversing effect of HED on the caffeine-mediated reacidification shown in Fig. 2D did not reach statistical significance in terms of reacidification time (P = 0.087; Fig. 2E), concomitant application of HED and caffeine reduced the slope of the gastrogram compared with caffeine administration, with mean respective values of 0.18 ± 0.13 pH units per min and 0.64 ± 0.26 pH units per min (P < 0.05; Fig. 2F).

The potent attenuation of caffeine's effects on GAS by the bitter-masking agent HED suggests that TAS2Rs are critically involved in caffeine's action in the mouth and the stomach.
Sensory Evaluation. To verify that the subjects were capable of sensing caffeine bitterness, the bitter recognition threshold of the same subjects who underwent the gastric pH measurements was determined by means of a threshold test, which yielded a result of 117 ± 44 mg/L for caffeine. In addition, the subjects rated the bitterness of 1.200 mg/L caffeine in the absence or presence of 240 mg/L HED in a blinded duo sensory test and confirmed the bitter-masking effect of HED reported by Ley et al. (33): Whereas the mean bitterness rating (±SD) for the caffeine solution was 7.5 ± 1.7, ratings for caffeine plus HED revealed mean values of 5.8 ± 1.9, corresponding to a ∼20% ± 8% reduction of caffeine-mediated bitterness by HED (Fig. S2A). The subjects’ bitterness scores correlated with reacidification time (correlation coefficient, 0.66; \( P = 0.03 \); \( n = 10 \); Fig. S2B and C) after caffeine administration by drinking (delivery protocol 1, 5 min after alkaline challenge), as well as with reacidification time after caffeine plus HED administered by drinking (correlation coefficient, 0.89; \( P < 0.05 \); \( n = 6 \); 5 min after alkaline challenge). No statistically significant correlation between bitter intensity rating and reacidification time was calculated after administration of encapsulated caffeine (delivery protocol 1; \( P > 0.05 \)).

**Fig. 1.** Results of the gastric pH measurements demonstrate that the effect of caffeine (CAF) on reacidification time is influenced by the type of administration. (A) Overview of the different administration types in the human intervention trial. (B) Overview of the study procedure. (C) Gastrograms of different Heidelberg capsule measurements from one test subject combined in one graphic show that 150 mg caffeine diluted or administered with 125 mL water (blue line) administered by sip and spit (3) prolongs the reacidification time (i.e., time until the original pH is reached again) more than administration via drinking (2) or in encapsulated form (1). (D) Delta reacidification time of gastrograms show that sip-and-spit administration resulted in the highest prolongation of reacidification time compared with gastric and gastric plus oral administration. (E) Delta slope of the gastrograms indicate that encapsulated administration (gastric delivery) strongly stimulate GAS when reacidification has started. Data are displayed as mean ± SEM, \( n = 5–10 \); one-way ANOVA with Holm–Sidak post hoc test; significant (\( P < 0.05 \)) differences are indicated by distinct letters [\(^* \), \(^{**} \), \(^{***} \)] relative to that receptor of 0.76 ± 0.039, 0.97 ± 0.190, 1.16 ± 0.025, 0.62 ± 0.017, and 0.83 ± 0.071, respectively.

The presence of the broadly tuned, caffeine-sensitive TAS2R10 receptor (12) in the gastric epithelium was confirmed by immunohistochemical staining of stomach surgical specimens from the antrum and fundus/corpus region. The specificity of the TAS2R10 antibody was verified in transiently transfected HEK-293T cells (Fig. S3). In gastric mucosa, cell types were identified by H&E staining (Figs. S4 and S5). Parietal cells are localized in the glands of gastric fundus and body, and are scattered in the middle and, to a lesser extent, in the bottom part of the mucosa (Fig. S4). They are characterized by broad pink cytoplasm. Chief cells stain with basophilic cytoplasm and are mainly located in the bottom parts of the mucosa (Fig. 3A and Fig. S4). Localization of TAS2R10 staining was confined to parietal cells and to gastric chief cells in the fundus/corpus, showing strong cytoplasmic granular reactivity (Fig. 3A, a and b). Staining of glandular cells in the gastric antrum was faint, consisting of very weak cytoplasmic and focal intermediate membranous reaction (Fig. 3A, e and f). In contrast, mucus-producing foveolar cells in the fundus/corpus (Fig. 3A, a and b) and antrum (Fig. 3A, e and f) did not show
expression of TAS2R10. Blocking experiments showed a clear staining reduction (Fig. 3 A, c, d, g, and h), supporting the epitope specificity of the antiserum. Like TAS2R10, the downstream signaling molecule transducin was localized in parietal and chief cells of the corpus/fundus, indicating that TAS2R10 and transducin are coexpressed in a substantial fraction of these cells. In addition, transducin immunoreactivity is present in the membranes of foveolar cells in gastric fundus/corpus (Fig. 3 A, i and j), but not in the antrum (Fig. 3 A, m and n). TAS2R10 and transducin expression was also detected in almost all HGT-1 cells (Fig. 3B), indicating that both proteins are coexpressed. Together, our data show that the mRNAs for caffeine’s cognate TAS2Rs and at least one bitter receptor polypeptide are present in the human stomach and HGT-1 cells.

Effect of Bitter and Bitter-Masking Compounds on Proton Secretion in HGT-1 Cells. Following our hypothesis that bitter compounds induce mechanisms of GAS via TAS2Rs, various bitter compounds, such as theobromine, tannic acid, yohimbine, denatonium benzoate, sodium benzoate, and aristolochic acid were tested and verified for their stimulating effects on proton secretion in HGT-1 cells (Fig. 4A). The concentrations of the tested compounds were chosen based on preliminary experiments to elicit the strongest effect on proton secretion without impairing cellular viability (>90% compared with nontreated controls (100%)). The responses were similar in magnitude, or even more pronounced, compared with those elicited by histamine, a major activator of proton secretion in parietal cells (10). These responses to bitter compounds indicate that several TAS2Rs could be activated in HGT-1 cells. Treatment of HGT-1 cells with 0.3–3,000 μM caffeine increased proton secretion, with 3,000 μM caffeine showing the highest effect (Fig. 4A and B). The bitter-masking compounds HED and eriodictyol (ED), which have been described to reduce the bitter taste of caffeine in human sensory panels (33, 34), also reduced the caffeine-evoked proton secretion in HGT-1 cells (Fig. 4B and Fig. S6C).

Antagonistic or Agonistic Effect of HED and ED on TAS2Rs-Induced Ca2+ Mobilization in HEK-293T Cells. To identify the TAS2Rs that are targeted by HED and its structural analog ED, Ca2+-mobilization in the presence of these compounds by transiently transfected HEK-293T cells was analyzed with or without costimulation with specific agonists of TAS2Rs (12). HED and ED were identified as agonists for TAS2R14 and as antagonists for TAS2Rs 43, 20, and 50 (Table S1). HED is also an antagonist for TAS2R31 (Table S1). As TAS2R43 can be activated by caffeine (12), the effect of caffeine and HED was further investigated in HEK-293T cells transiently transfected with TAS2R43. TAS2R43 in these cells was then activated by aristolochic acid or caffeine for the performance of calcium imaging experiments in the presence of increasing concentrations (0.03–30 μM) of HED and ED. Both compounds reduced TAS2R43 responses to aristolochic acid or caffeine (Fig. 4C).

Caffeine-Induced Proton Secretion Is Reduced in TAS2R43 KO HGT-1 Cells. To determine whether TAS2R43 is involved in mechanisms of caffeine-induced GAS, a homozygous 13-bp deletion in the TAS2R43 gene (Fig. S7) of HGT-1 cells was induced by using a CRISPR-Cas9 CD4-vector (i.e., TAS2R43-KO). As negative control (NC), HGT-1 cells were treated in parallel with the same vector containing a nontargeting scrambled guide RNA (gRNA). Off-target effects of the transfected gRNA were excluded by a whole-genome sequencing analysis. The stimulating effect of caffeine and the TAS2R43 agonist aristolochic acid on proton secretion in HGT-1 cells was substantially reduced in TAS2R43-KO cells compared with NC cells (Fig. 4D). These data demonstrate that TAS2R43 is involved in caffeine’s action on proton secretion in HGT-1 cells. Considering that caffeine’s effect on proton secretion in HGT-1 cells is sensitive to TAS2R43 and HED, and that TAS2R43 is blocked by HED in TAS2R43-transfected HEK-293T cells, the data strongly suggest that caffeine mediates its effect through at least one TAS2Rs (TAS2R43) if not more.
Table 1. mRNA expression of TAS2Rs in HGT-1 cells normalized to the expression of the acetylcholine receptor (CHRM3).

<table>
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<th>Receptor/gene</th>
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<th>SEM</th>
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<tr>
<td>CHRM3</td>
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<tr>
<td>TAS2R1</td>
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Data are shown as mean ± SEM; n = 3–4 biological replicates, tr = 3 technical replicates. The mRNA of TAS2Rs is similarly or even more highly expressed compared with the mRNA of CHRM3 in HGT-1 cells.

Effect of Pharmacological Blockers on Caffeine-Induced Proton Secretion.

Caffeine-evoked proton secretion was reduced by neither the PLCβ inhibitor U73122 nor the IP3 inhibitor neomycin (Fig. 4E). In contrast, it was reduced by the adenyl cyclase inhibitor NKY80 (22.5 ± 7.4%; P < 0.01), suggesting that cAMP but not Ca2+ signaling is involved in TAS2R-mediated regulation of acid secretion in HGT-1 cells.

Effect of Caffeine and HED on cAMP Levels in HGT-1 Cells. To confirm that adenyl cyclase mediates proton secretion in HGT-1 cells via caffeine-dependent TAS2R stimulation, intracellular cAMP levels were determined in response to treatment with caffeine and HED (Fig. 4F). Treatment of HGT-1 cells for 10 min with 3.0 mM caffeine increased cAMP levels by 12 ± 4.6% (P < 0.05) in comparison with the treatment with DMEM (control, 100 ± 20%). However, coapplication of caffeine and HED (83.3 ± 2.7%; P < 0.01) and HED alone (84.9 ± 5.1%; P < 0.05) reduced cAMP levels in HGT-1 cells. Treatment with forskolin, a stimulator of adenyl cyclase, increased cAMP levels to 131 ± 10.3% (P < 0.05) in HGT-1 cells in comparison with treatment with the solvent control ethanol (i.e., EtOH). These observations confirm that caffeine activates TAS2Rs signaling through changes in cAMP levels.

Discussion

Acid secretion in the stomach is a fundamental process that is finely regulated at different levels. Initial activation of GAS is regulated by the CNS when food is smelled and tasted (10, 35).

When food enters the stomach, mechanical or chemical receptors (i) initiate GAS via activation of afferent/efferent fibers connected to the CNS, (ii) stimulate the gastrin-producing G cells or the histamine-producing enterochromaffin-like cells, or (iii) directly stimulate the HCl-producing parietal cells. Caffeine stimulates GAS, and, so far, it has been assumed that it acts via inhibition of PDE or by antagonizing adenosine receptors in gastric parietal cells (1). As caffeine activates five of the 25 human TAS2Rs (12), we hypothesized that its bitterness also contributes to its stimulating effect on GAS via activation of TAS2Rs.

First, we found that oral consumption of caffeine delayed GAS in healthy subjects, whereas caffeine that was administered encapsulated, being released in the stomach, accelerated this process compared with oral administration. The delay induced by oral caffeine presentation might be explained by findings reported by McMullen et al. (36). They demonstrated that caffeine in a coffee drink accelerated the heart rate without increasing the vascular tonic in comparison with caffeine administered encapsulated concomitantly to a decaffeinated coffee drink. The increase in heart rate was likely induced by vagal withdrawal instead of sympathetic activation. This finding is important, considering that the cephalic-phase response during digestion is thought to activate the vagus nerve to enhance digestion (36). The study of McMullen et al. (36) also agrees with the present results suggesting that orally sensed caffeine elicits vagal withdrawal that would reduce rather than enhance the digestive capacity, for example by delaying GAS. An explanation why the delaying effect of caffeine has not been discussed earlier might be that most of the previous studies investigating the effect of caffeine on GAS used gavages to bypass oral cavity receptors (2, 3, 5) and therefore did not take into account an inhibitory effect of caffeine on GAS by oral perception. Furthermore, we demonstrated that TAS2R bitter receptors in the stomach are involved in the caffeine-induced secretion of gastric acid. This conclusion is based on the observation that the bitter masking compound HED similarly reduced caffeine’s bitterness as well its effects on GAS evoked by combined oral/gastric or gastric-only caffeine application. The finding that concomitant oral ingestion of caffeine and HED accelerated passing of the Heidelberg capsule into the duodenum in 4 of 10 subjects compared with caffeine administration indicates that HED might induce gastric emptying. This hypothesis has been verified by measuring the effect of HED on gastric motility in strips of stomach dissections in an organ bath. Avau et al. (37) demonstrated that bitter compounds such as denatonium benzoate increased contractility in gastric strips of mice and caused an impairment of gastric relaxation after intragastric infusion. Whether a bitter masking compound has opposite effects by causing gastric relaxation is an open question. The data presented here did not show any effects of HED on gastric secretion in humans, nor on proton secretion in HGT-1 cells at the concentrations tested. However, HED induced gastric relaxation, indicating physiological targets other than GAS.

To confirm that caffeine induces GAS via gastric TAS2Rs, we demonstrated that the mRNA of 22 of 25 TAS2Rs as well as transducin is present in HGT-1 cells, and that the five TAS2Rs that can be activated by caffeine are present in the stomach mucosa. These findings were corroborated by immunohistochemical detection of TAS2R10 and transducin in different gastric cell types for which useful antisera were available. So far, to our knowledge, only one validated antibody against human TAS2R38 (38) is known. Neither caffeine nor HED bind to TAS2R38. An antibody against TAS2R43 (OSR00171W; Thermo Fisher Scientific) was tested. Unfortunately, during validation in transfected HEK-239T cells, this antibody turned out to be unspecific. Nevertheless, we could demonstrate data for the validation of an antibody targeting TAS2R10 (Fig. S3) and show the expression of TAS2R10 on a protein level in gastric mucosa and HGT-1 cells.
As TAS2R10 was highly expressed in parietal cells, as detected by immunohistological staining, we focused on the cellular mechanisms in HGT-1 cells, which exhibit the characteristics of parietal cells (28, 29). Nevertheless, we cannot exclude that other cell types or gastrointestinal hormones may have contributed to the detected effects in the human intervention study. We demonstrated here that the bitter-masking agent HED reduced the stimulatory effect of caffeine on proton secretion in healthy subjects and in HGT-1 cells. As TAS2R43 is the only one of the five TAS2Rs that can be activated by caffeine and antagonized by HED, we also performed a CRISPR-Cas9 approach to knock out TAS2R43 in HGT-1 cells. In these TAS2R43-KO cells, the effect of caffeine on proton secretion was reduced in comparison with control cells. To further confirm our hypothesis that TAS2Rs are involved in mechanisms regulating GAS, TAS2R43 was transiently transfected into HEK-293T cells, which do not normally express any TAS2Rs. In this cell model, we demonstrated that HED antagonized caffeine-stimulated responses in TAS2R43-transfected cells. These results strongly indicate that TAS2R43 is involved in the proton secretory effect of caffeine. Nevertheless, involvement of other TAS2Rs or signaling pathways, such as adenosine receptors, or PDE inhibition cannot be excluded.

HED is also an agonist for TAS2R14, which is highly expressed in HGT-1 cells. The interaction of agonistic and antagonistic effects on TAS2Rs and the further activation of downstream signaling pathways seem highly complex, and not every TAS2R might be connected to the same downstream signaling cascade. One downstream signal for the induction of proton secretion is cAMP. Here, we show that caffeine increased cAMP levels in HGT-1 cells, an effect that was inhibited by HED. HED itself reduced the cAMP level in HGT-1 cells, but did not affect proton secretion in HGT-1 cells. Therefore, it remains unclear whether or which signaling pathways are affected by HED. Caffeine signaling via cAMP is supported by the fact that the adenylyl cyclase inhibitor NKY80 reduced the caffeine-evoked stimulation of proton secretion. Caffeine-induced activation of PLCβ2 and IP3 signaling can be excluded by the failure of the specific inhibitors U73122 (PLCβ2) and neomycin (IP3) to reduce proton secretion. Experiments in which caffeine and HED were tested in combination with the inhibitors showed no difference in proton secretion in comparison with caffeine and HED tested alone. That leaves the question of how caffeine can stimulate GAS via TAS2Rs. So far, only for sweet and glutamate taste receptors (TAS1R1/3) has an increase of cAMP levels via activation of adenylyl cyclase been demonstrated (39). The signaling cascade of bitter taste receptors has been proposed to reduce cAMP levels by activation of PDE via α-gustducin or transducin (23, 26, 40).

Based on our results, we hypothesize that bitter perception of caffeine in the mouth generates a signal of aversion, which leads, via vagal withdrawal, to inhibition of GAS (41). However, when bitter compounds reach the stomach, increased GAS could aid degradation or removal of the potential toxins. This hypothesis is
supported by previous studies demonstrating that extraoral TAS2Rs are probably involved in defense mechanisms in other parts of the gastrointestinal tract and form a "chemofensor complex" (18, 19, 42). The differential effect of site-specific TAS2R activation on GAS we demonstrated has not been reported so far to our knowledge and warrants further investigations. The expression of TAS2Rs in murine goblet cells (18), a cell type that secretes mucus to protect the epithelium, and the fact that bitter substances increase anion transport and fluid secretion in human and rat colon tissue (42), indicate defense-related functions of bitter taste receptors. Furthermore, in intestinal cells, Jeon et al. (43) identified a TAS2R38-dependent activation of the ATP-binding cassette B1 (ABCBI) via phenylthiocarbamide (PTC). As ABCBI is an efflux transporter located on the apical membrane of intestinal epithelial cells to limit absorption of toxic substrates contained in food, TAS2R signaling has been assumed to limit the absorption of potentially hazardous bitter-tasting substances in the intestine (43).

Our results clearly demonstrate that the route of application of caffeine determines its effects on GAS, and suggest that other bitter tastants and bitter-masking compounds are also potentially useful therapeutics to regulate gastric pH. Finally, our results support the pleiotropic functions of taste receptors far beyond their role in taste.

Materials and Methods
Chemicals. The sodium salt of HED (3'-methoxy-4,5,7-trihydroxyflavonane) and ED was provided by Symrise. All other chemicals were obtained from Sigma-Aldrich unless stated otherwise.

Identification of the Influence of Bitter Taste on GAS in Vivo. The human intervention study was designed as a single-blinded, randomized, controlled, longitudinal trial and was performed in accordance with good clinical practice guidelines and the Declaration of Helsinki. The experimental protocol was reviewed by the ethics committee of the city of Vienna (registration no. EK 13–180–VK_N2), and the study has been registered at ClinicalTrials.gov (ID code NCT02372188). The subjects provided written informed consent after they had been given a detailed oral and written description of the study. The 13 healthy test subjects had no gastrointestinal complaints, were nonsmokers, did not take antibiotics for 2 mo before the test, and were between 21 and 32 y of age, with a body mass index between 19 and 25 kg/m². Helicobacter pylori infection was excluded by an immunochromatographic rapid capillary blood test (Diagnostik Nord). Average habitual caffeine consumption was 125 mg/d and determined by a food frequency questionnaire of caffeine-containing food and beverages. The Heidelberg capsule measurements were carried out at the Department of Nutritional and Physiological Psychology.
Human stomach was α-value. Total RNA was extracted from HGT-1 cells and two human × could be verified, The human gastric tumor cell line HGT-1 was obtained 45
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of the tested substances and treatment reagents was excluded by MTT test
feine, from 25 to 225 mg/L. Furthermore, the subjects had to rank the bitterness
in baseline tension. The number of patients is given as an
detected changes in muscle changes. Data are presented as percent changes
vaseline (calibrated at 2 g; AD Instruments) and the software AcqKnowledge (Biopac)
HGT-1 Cell Culture. The human gastric tumor cell line HGT-1 was obtained from C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France) and cultured under standard conditions as described previously (8). Cytotoxicity of the tested substances and treatment reagents was excluded by MTT test as described before (8), and cell viability was determined by trypan blue staining. Tested cells had at least 90% cell viability.

Immunochemical Staining of Gastric Tissues. Histological specimens were obtained from two patients from the Pathologisch-Bakteriologisches Institut, Donaupital, Vienna, Austria. The gastric fundus was derived from a sleeve gastrectomy of a 42-y-old adipose but otherwise healthy patient. The gastric antrum was derived from a 71-y-old patient undergoing distal partial gastrectomy for a benign gastrointestinal stroma tumor. Immunohistochemistry was performed on 5-μm-thick formalin-fixed, paraffin-embedded whole tissue sections. Slides were processed in the fully automated staining instrument Benchmark ULTRA by using an ultraView Universal DAB Detection Kit (Ventana Medical Systems). The following primary antibodies were applied: TAS2R10 (OSR00158W; Thermo Scientific), 1:750 for 28 min at 37 °C after heat-mediated antigen retrieval using EDTA buffer, pH 8.0, at 95 °C for 36 min (CC1 buffer; Ventana Medical Systems) and GNAAT2 (transducin α-2 chain; AP11077c; Abgent), 1:50 for 28 min at 37 °C after heat-mediated antigen retrieval using EDTA buffer, pH 8.0, at 95 °C for 64 min (CC1 buffer; Ventana Medical Systems) and amplification at 95 °C (Amplification Kit; Ventana Medical Systems). All counterstaining was performed with hematoxylin. Blocking experiments to control for unspecific staining were performed by using the TAS2R10 control peptide (GST00040P; Thermo Scientific) and GNAAT2 antibody blocking peptides (BP11077c; Abgent). For the TAS2R10 taste receptor, the blocking experiment consisted of the control peptide, 1:200, incubated together with TAS2R10 antibody, 1:750, for 120 min at 4 °C, and, thereafter, incubation of the slide at 37 °C for 28 min. The GNAAT2 antibody blocking peptide, 1:10, was incubated together with GNAAT2 antibody, 1:50, for 120 min at 4 °C, and, thereafter, incubation of the slide for 28 min at 37 °C. All other steps were performed similarly to the staining procedure as described earlier.

Immunocytochemical Staining of HGT-1 Cells and HEK-293-TorGlu444 Cells. Cells were transiently transfected with microplasmids containing TAS2R45 (60 μg DNA) were prepared as described previously (19), and HGT-1 cells were seeded on coverslips 24 h before the staining procedure. Cells were fixed and stained as described previously (18) by using anti-HSV (1:15,000; Novagen), anti-TAS2R10, and anti-GNAAT2 antibodies (Immunohistochemical Staining of Gastric Tissues) for 1 h at room temperature. Specificity of labeling was ensured as described in Immunohistochemical Staining of Gastric Tissues, Sensory Study. Intracellular pH, as indicator for proton secretion in HGT-1 cells, was measured using the pH-sensitive fluorescent dye 1,5-carboxy-sesaminaphto-rhodaflox (SNARF-1-AM; Life Technologies) as described before (8, 29). The intracellular proton index (IPX) in the cells was calculated by log₂ transformation of the ratio between treated and untreated (i.e., control) cells. The lower the IPX, the fewer protons are in the cell, indicating a higher secretory activity in HGT-1 cells.
mRNA Expression of Bitter Taste Receptors in HGT-1 Cells and Human Biopsies Using RT-qPCR. Total RNA was extracted from HGT-1 cells and two human biopsy specimens by using thepeqGold Total RNA Kit (Peqlab). Quality and quantity were checked spectrophotometrically. Reverse transcription was carried out with 2 μg RNA and the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed with an Applied Biosystems StepOnePlus Real Time PCR system and Fast SYBR Green Master Mix (Thermo Fisher Scientific). Primers were designed using the National Center for Biotechnology Information (NCBI) primer design tool (using Primer 3 and BLAST; Table S2). Cycling conditions were 20 s 95 °C (activation), 3 s 95 °C (denaturation), 30 s 60 °C (annealing), and 15 s 67 °C (elongation with fluorescence measurement). The PCR products were verified by melting curve analysis, agarose gel electrophoresis, and sequence analysis (Eurofins Genomics). Sequences were checked by using the NCBI BLASTn tool. Primers showing no product in HGT-1 in at least one of the three replicates (TAS2R8 8, 9, 45, and 60) were tested with cDNA derived from a human tongue biopsy provided by J.-D. Rague (Charité, Berlin, Germany). Whereas primers for TAS2R8 8, 9, and 60 could be verified, TAS2R45 was not detected. For TAS2R46, high-frequency copy-number variants are known, and some people do not possess the tested variant of the mRNA for this gene (44). TAS2R46 could not be detected in the second human biopsy sample. The open-source software LinRegPCR was used for quantitative PCR

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data analysis. This software enables the calculation of the starting concentration \(N_0\) of each sample, expressed in arbitrary fluorescence units. The calculated starting concentrations of the TAS2R2 genes were compared with the starting concentrations of the acetylcholine receptor (CHRHM3), with previously described primers (8), which is typically expressed in parietal cells on a functional level.

**Generation of TAS2R43 Homozygous KO HGT-1 Cell Line Using CRISPR-Cas9.** A total of 40,000 cells were seeded in a 24-well plate. After approximately 24 h, cells were transfected with 495 ng GeneArt CRISPR Nuclease (CD4 Vector; A21175; Invitrogen) containing the gRNA targeting TAS2R43 gene TTTTTTGCAATAAGCTGCCTG (5′–3′) and, as a control, a scrambled gRNA GTTGCAGGTGGTGCCTGTG (5′–3′) with no target by using the transfection reagent Viromer RED (Lipocalyx) according to the manufacturer’s protocol. Transfection efficiency was approximately 25%, verified with a CD4 monoclonal antibody (07-0403; Invitrogen)/Thermo Fisher Scientific) by using a Guava soft-flow cytometer (Millipore) on the basis that only positively transfected cells express a CD4 protein. Cells were transfected in a six-well plate to increase cell number. After 3 d, CD4-positive cells (i.e., positively transfected cells) were enriched by using the Dynabeads CD4 Positive Isolation Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Cells were analyzed with a Genomic Cleavage detection kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. For genomic cleavage detection, the following primers were used: forward primer AGACTGTCAAGGAGCGGAAAGC (5′–3′) and reverse primer GATGTTGTTGAGGCTCTTG (5′–3′). The following temperature protocol was used: 95°C for 10 min, 40 cycles of 95°C/30 s, 52°C/30 s, and 72°C for 2 min. A genomic cleavage of approximately 22% was detected. Single cells were isolated by serial dilution of positively transfected cells into two 96-well plates and observed for colony forming for 2 wk. Total cells of 39 wells in which clearly only one colony formed were harvested by trypsin/EDTA and first transferred to a 48-well plate, and, after confluence, to a 12-well plate to increase cell number. From each clone, half of the cells were frozen and the other half were used to extract DNA with a PureLink Genomic DNA Mini Kit (Life Technologies) according to the manufacturer’s protocol. Before Sanger sequencing by Eurofins Genomics, the DNA extracts were purified with AMPure/AmpTaqGold 360 Mastermix (Thermo Fisher Scientific), and PCR was carried out as described earlier for the genomic cleavage detection. Of 20 clones, 15 showed no deletion, four a heterozygous deletion, and one a homozygous deletion. Deletion on an mRNA level was also analyzed by means of Sanger sequencing following total RNA isolation of the WT, cells transfected with the scrambled gRNA, and the TAS2R43 KO cells as described earlier (Fig. S7).

**Exclusion of Off-Target Effects Using Whole-Genome Sequencing.** The quality-checked DNA was fragmented with a Covaris ultrasonicator. The resulting DNA fragments were purified, end-blunted, A-tailed, and adaptor-ligated. Checked DNA was fragmented with a Covaris ultrasonicator. The resulting DNA fragments were purified, end-blunted, A-tailed, and adaptor-ligated. The concentration of the libraries was quantified by Bioanalyzer and real-time PCR. Each library was sequenced with Illumina manufacturer’s protocol. Cells transfected with 495 ng GeneArt CRISPR Nuclease (CD4 Vector; A21175; Invitrogen) containing the gRNA targeting TAS2R43 gene TTTTTTGCAATAAGCTGCCTG (5′–3′) and, as a control, a scrambled gRNA GTTGCAGGTGGTGCCTGTG (5′–3′) with no target by using the transfection reagent Viromer RED (Lipocalyx) according to the manufacturer’s protocol. Transfection efficiency was approximately 25%, verified with a CD4 monoclonal antibody (07-0403; Invitrogen)/Thermo Fisher Scientific) by using a Guava soft-flow cytometer (Millipore) on the basis that only positively transfected cells express a CD4 protein. Cells were transfected in a six-well plate to increase cell number. After 3 d, CD4-positive cells (i.e., positively transfected cells) were enriched by using the Dynabeads CD4 Positive Isolation Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Cells were analyzed with a Genomic Cleavage detection kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. For genomic cleavage detection, the following primers were used: forward primer AGACTGTCAAGGAGCGGAAAGC (5′–3′) and reverse primer GATGTTGTTGAGGCTCTTG (5′–3′). The following temperature protocol was used: 95°C for 10 min, 40 cycles of 95°C/30 s, 52°C/30 s, and 72°C for 2 min. A genomic cleavage of approximately 22% was detected. Single cells were isolated by serial dilution of positively transfected cells into two 96-well plates and observed for colony forming for 2 wk. Total cells of 39 wells in which clearly only one colony formed were harvested by trypsin/EDTA and first transferred to a 48-well plate, and, after confluence, to a 12-well plate to increase cell number. From each clone, half of the cells were frozen and the other half were used to extract DNA with a PureLink Genomic DNA Mini Kit (Life Technologies) according to the manufacturer’s protocol. Before Sanger sequencing by Eurofins Genomics, the DNA extracts were purified with AMPure/AmpTaqGold 360 Mastermix (Thermo Fisher Scientific), and PCR was carried out as described earlier for the genomic cleavage detection. Of 20 clones, 15 showed no deletion, four a heterozygous deletion, and one a homozygous deletion. Deletion on an mRNA level was also analyzed by means of Sanger sequencing following total RNA isolation of the WT, cells transfected with the scrambled gRNA, and the TAS2R43 KO cells as described earlier (Fig. S7).

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