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The steroid glycoside H.g.-12 from *Hoodia gordonii* activates the human bitter receptor TAS2R14 and induces CCK release from HuTu-80 cells

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Le Nevé B, Foltz M, Daniel H, Gouka R. The steroid glycoside H.g.-12 from *Hoodia gordonii* activates the human bitter receptor TAS2R14 and induces CCK release from HuTu-80 cells. *Am J Physiol Gastrointest Liver Physiol* 299: G1368–G1375, 2010. First published October 7, 2010; doi:10.1152/ajpgi.00135.2010.—Steroid glycosides extracted from the succulent plant *Hoodia gordonii* are suggested to have appetite-suppressant effects both in animals and humans. Yet, the mechanisms underlying the putative satiety action of *Hoodia* steroid glycosides are not fully understood. We found that H.g.-12, a steroid glycoside purified from *H. gordonii* extract, initiated cholecystokinin (CCK) secretion both ex vivo in rat intestine and in vitro in the human enteroendocrine (EC) cell line HuTu-80. CCK is known to exert central effects on appetite suppression via the vagus nerve which afferents terminate in the gut wall. Recent data show that G protein-coupled receptors signaling bitter taste (T2Rs) are expressed in both rodent and human gastrointestinal tract. It was further demonstrated that bitter sensing is functional in mouse STC-1 EC cells and leads to CCK secretion via increased intracellular Ca^{2+} concentrations. Based on the bitter taste of *H. gordonii* purified extracts, we assessed whether H.g.-12 could activate human bitter receptors. The steroid glycoside activated selectively TAS2R7 and TAS2R14, both heterologously expressed in HEK 293 cells. Removing an essential structural feature from the steroid glycoside inhibited H.g.-12-induced Ca^{2+} increase in TAS2R14-expressing HEK cells and abolished H.g.-12-induced CCK secretion from human EC cells. Similarly, a nonspecific bitter receptor antagonist abolished H.g.-12-induced CCK secretion in HuTu-80 cells. These results point to a potential route of action by which components of *Hoodia* might influence appetite control. Our data also provide additional evidence that bitter taste-sensing mechanisms are coupled to hormone release from EC cells in the intestine. Moreover, we identified a natural agonist of TAS2R7 and TAS2R14 for further studies on the role of bitter receptors in satiety control and food intake.

nutrient sensing; satiety; HuTu-80; cholecystokinin receptor 1

HOODIA GORDONII (MASSON) SWEET ex Decne is a perennial, succulent plant specie from the Apocynacaea (previously Asclepiadaceae) family that is indigenous to arid regions of South Africa, Botswana, and Namibia. Initial exploratory research with several species of *Hoodia* showed that *H. gordonii* extract contained a mixture of steroid glycosides (20, 29, 34) that decreased food intake and body weight in animals (34). The two major steroid glycosides H.g.-12 and H.g.-20 present in *H. gordonii* were purified and shown to exhibit food intake-suppressive effects in rats, strongly indicating that H.g.-12 and H.g.-20 are at least two of the pharmacologically active components in *H. gordonii* (34). However, the mechanisms of action by which the steroid glycosides might influence energy intake and appetite control are not fully understood. So far, only one study investigating potential mechanistic effects of one of the *H. gordonii* steroid glycosides has been published. Here it was hypothesized that H.g.-12 has a modulating effect on ATP production and its content in hypothalamic neurons after intracerebroventricular injection and after in vitro exposure of fetal hypothalamic neurons to the compound (24). However, it is not clear that the primary bioactivity of *Hoodia* components is exerted postabsorptively. Furthermore, bioavailability studies must demonstrate that, following ingestion, H.g.-12 has access to the brain-tested regions in vivo and that concentrations of the active form of the molecule are likely to be achieved at the site of action.

Based on the bitter taste of *H. gordonii* extracts, we hypothesized that the putative effects of *H. gordonii* could in part be mediated by luminal sensing of its steroid glycosides in the small intestine. Recent scientific evidence indeed suggests the existence of functional taste-sensing mechanisms in the intestine resembling those found in the neuroepithelium of the tongue (11). Various members of the T2R family, along with components of the taste signaling pathway such as α-gustducin, have been shown to be expressed in enteroendocrine (EC) cell lines and pancreas, as well as in the gastric and intestinal mucosa in rodents (8, 18, 21, 35, 36) and also in human colonic tissue (21, 30, 31). Data from these studies suggest that bitter taste receptors expressed in the small intestinal tract are involved in sensing of food components. In addition, some in vitro and in vivo animal studies showed that sweet and bitter compounds known to mediate gustatory signals in the oral cavity elicit gut hormone secretion from EC cells and lead to a neural activation in the brain (16, 17, 19).

The TAS2R family contains ~25 receptors as identified by genomic sequencing (1, 6, 9, 27). Agonists for almost all human TAS2Rs have been identified (2, 3, 26, 28, 32); all agonists identified so far have been bitter-tasting compounds (5, 26). Based on the presence of both bitter taste receptors and taste transduction proteins in EC cells, it has been postulated that TAS2Rs may be involved in regulation of food intake via initiation of secretion of satiety hormones such as glucagon like peptide-1 (GLP-1), peptide YY (PYY), and cholecystokinin (CCK). In vitro, bitter tastants like denatonium benzoate (DB) and phenylthiocarbamide (PTC) cause an increase in intracellular Ca^{2+} followed by CCK secretion as shown in the rodent EC cell line STC-1 (8). Ofloxacin, a bitter ligand for TAS2R9, elicits a GLP-1 secretion from human EC NCI-H716 cells (11). In rats, an intragastric infusion of TAS2R agonists was reported to excite neurons in the nucleus tractus solitarius (NTS), to condition flavor aversions and to delay gastric emptying (14, 16, 17). NTS activation is followed by reflex activation of vagal efferent neurons, which itself results in

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changes in gastrointestinal functions, including inhibition of gastrointestinal motility (15). However, to date, there is only one study reporting changes in gastrointestinal physiology in response to bitter compounds in rats (21), and, in humans, the only study available failed to show effects of an intragastric infusion of the bitter tastants quinine and naringin on gastric emptying (23). Direct effects of these bitter compounds on hormone secretion in EC models have not been shown yet.

Here, we demonstrate that the *H. gordonii* steroid glycoside H.g.-12 elicits CCK secretion both ex vivo in rat intestine and in vitro in cultured human EC cells. We also investigated whether this hormone secretion could be mediated via intestinal bitter receptors and demonstrated that H.g.-12 selectively activates TAS2R7 and TAS2R14 out of 25 TAS2Rs. Finally, bitter receptor activation and subsequent satiety hormone secretion in vitro were linked to essential structural features of H.g.-12.

MATERIALS AND METHODS

Materials. HuTu-80 cells (HTB40; ATCC) were obtained from the American Type Culture Collection (WZ). HEK 293 cells were obtained from Invitrogen (Breda, The Netherlands). A Chinese hamster ovary cell line (CHO) functionally expressing rat CCK receptor 1 (CHO-CCK_1R) was kindly provided by Dr. R. Smeets (University of Nijmegen, The Netherlands). Other reagents used in this study were purchased from Sigma Aldrich unless indicated differently. The *H. gordonii* steroid glycoside H.g.-12 was purified from a *H. gordonii* extract and had a chemical purity of 92% (Russell PJ and Swindells C, unpublished observations). Removal of the tigloyl group from the steroid core by alkaline cleavage was used to generate detiglated H.g.-12; acid cleavage was used to generate the aglycon of H.g.-12, and combination of both processes led to the detiglated-aglycon of H.g.-12. Chemical structures are presented in Fig. 1.

A synthetic compound library containing 10,000 different compounds was obtained from Specs. The compounds were dissolved in DMSO at a 20 mM stock concentration.

Cell culture conditions. HuTu-80 human duodenal cells were grown in minimum essential Eagle's medium containing 10% FBS, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 100 μg/ml L-glutamine in plastic flasks or Poly-Lysin-coated 24-wells plates. Cells were used until passage 32.

HEK 293 cells (human embryonic kidney cells) expressing each of the 25 bitter receptors were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and L-glutamine and supplemented with 10% tetracycline-free FBS (Cambrex), blasticidin (5 μg/ml), geneticin (400 μg/ml), and hygromycin (100 μg/ml) in plastic flasks or Poly-Lysin-coated 96-wells plates. Cells were used until passage 25.

CHO-CCK_1R cells were grown in DMEM and Ham's F-12 medium 1:1 with 15 mM HEPES and L-glutamine supplemented with 10% FBS (GIBCO, Paisley, UK), 500 μg/ml penicillin, 500 U/ml streptomycin (Cambrex, Baltimore, MD), and 500 μg/ml geneticin (GIBCO). As negative control cells, native CHO-K1 cells (CCL61; ATCC) were used and grown in the same medium without geneticin. Cells were used until passage 25. All cells were grown and maintained at 37°C/5% CO_2.

Cloning and expression of human TAS2 receptors in HEK 293 cells. For functional expression of the human bitter taste receptors, HEK 293 cells (Invitrogen) stably expressing the chimeric G protein α-subunit Gα16-gust44 (cloned into pcDNA4; Invitrogen, San Diego, CA) and one of each 25 bitter receptor genes (cloned into pcDNA5/FRT; Invitrogen) were used. To improve receptor membrane targeting, each bitter receptor gene contained the first 45 amino acids of rat somatostatin receptor type 3 at its amino terminus. Cells were maintained in DMEM and 10% tetracycline-free FBS (Cambrex) supplemented with blasticidin (5 μg/ml), geneticin (400 μg/ml), and hygromycin (100 μg/ml).

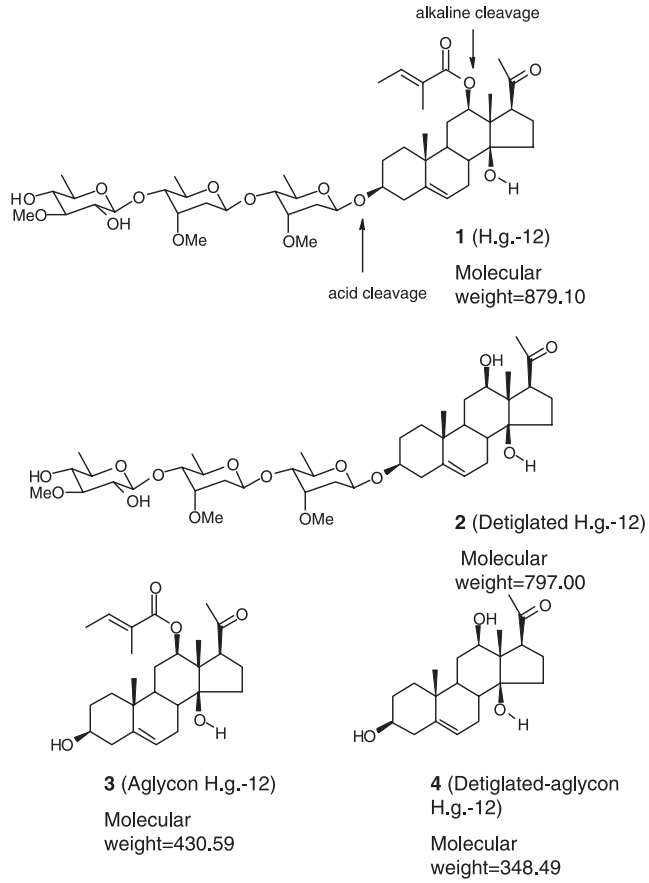

Fig. 1. Chemical structures of H.g.-12, a steroid glycoside purified from *Hoodia gordonii* extract, and its aglycon, detiglated, and detiglated-aglycon forms. Removal of the tigloyl group from the steroid core by alkaline cleavage was used to generate detiglated H.g.-12; acid cleavage was used to generate the aglycon of H.g.-12; and a combination of both processes led to the detiglated-aglycon of H.g.-12.

Activation measurement of TAS2R via monitoring of intracellular Ca^{2+} concentrations using a fluorescent plate reader. Activation of human TAS2 receptors was measured by monitoring variations in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) using the FlexStation II 384 (Molecular Devices). TAS2R-expressing cells and nontransfected cells were seeded in supplemented DMEM into Poly-Lysin-coated 96-well plates (black wall, clear bottom; Greiner) at a density of 1×10^5 cells/ml, 100 μl/well and cultured overnight. The following day, transcription of the receptors was induced by adding 0.25 μg/ml doxycyclin. Cells were induced for 24 h and then loaded with the Ca^{2+}-sensitive fluorescent dye fluo 4-AM (Molecular Probes), following suppliers guidelines. Dye loading medium composition was the following: 0.01% Pluronic F-127 (Molecular Probes), 0.5 mM Probenecide (Sigma), and 2.5 μM fluo 4-AM in Tyrode's buffer, pH 7.4 (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, and 20 mM HEPES). Compounds to test were prepared in parallel in Tyrode's buffer in a 96-well plate (V96 Microwell; Nunc) and automatically pipetted onto the cells during the assay. Measurements were carried out for 90 s with an interval time of ~1.6 s, giving 55 data points per measurement. To obtain a baseline, fluorescence signals (excitation 485 nm/emission 520 nm) were measured for 20 s before the addition of compounds on the cells (80 μl, injection speed of 100 μl/s). After agonist addition, the fluorescence

signals were measured for an additional 70 s at 37°C. The fluorescence values (ΔF) were calculated by subtracting the maximum fluorescence from the average fluorescence of the first 10 time points (baseline). Noninduced cells were measured in parallel as a negative control.

TAS2R antagonists screening from chemical libraries. A commercially available library (Specs) consisting of 10,000 chemical compounds was screened for TAS2R antagonists. Around 3,000 compounds (final concentration 100 μM) were coincubated with 0.25 mM of activating ligand on TAS2R-expressing HEK cells for identification of receptor blockers.

CCK secretion studies in HuTu-80 cells. For each secretion study, HuTu-80 cells were seeded at 2×10^5 cells/ml, 0.5 ml/well in a Poly-Lysin-coated 24-well plate 48 h before the experiment. HBSS buffer (GIBCO) supplemented with 10 mM HEPES (Sigma) was used for the secretion studies. Test compounds were prepared in HBSS buffer and incubated on the cells (0.5 ml/well) for 2 h at 37°C in a humidified incubator at 5% CO_2. Medium (0.5 ml) was collected and centrifuged at 4°C for 5 min at 1,000 *g* to remove cell debris, and the supernatants were stored at −20°C until further analysis. A 100 mM stock solution for H.g.-12 was prepared in DMSO; the final concentration of DMSO never exceeded 0.5% on the cells.

Determination of CCK concentrations using a CCK receptor-1 activation assay by monitoring $[Ca^{2+}]_i$. Samples derived from CCK secretion studies in HuTu-80 cells and rat gut rings were tested for CCK in a CCK receptor 1 (CCK_1R) activation assay as described previously (12). This method has been shown to display higher sensitivity and lower variability than an ELISA (12). In brief, CHO-K1 and CHO-CCK_1R cells (100 μl/well) were seeded in a Poly-Lysin-coated 96-well microtiter plate (black wall, clear bottom; Greiner) at a density of 3×10^5 and 4×10^5 cells/ml, respectively, and cultured overnight. Cells were loaded with fluo 4-AM as described above. Supernatants from the secretion studies were thawed from −20°C and prepared in parallel in a 96-well plate (V96 Microwell; Nunc). The Flexstation measurement settings were identical to those used for the HEK cells expressing human bitter taste receptors. CCK_1R activation was measured by monitoring fluorescence signals in CCK_1R-expressing CHO cells after the addition of the supernatants derived from CCK-release experiments as described above. Each experiment was conducted in parallel using wild-type CHO cells to assess any nonspecific signal. Calibration was performed using sulfated CCK-8 as a standard.

Animals and tissue preparation. The use of animals was in accordance and approved by the Animal Care and Use of Laboratory Animals committees at the Technical University of Munich. Male Sprague Dawley rats (Charles River Laboratories) weighing 240–260 g ($n = 6$) were used for this study. Rats were maintained on a regular laboratory chow and were fasted overnight previous to the experiment. Water was supplied ad libitum before all experimental procedures. Rats were anesthetized with ether (Sigma) and killed by cervical dislocation. Median laparotomy was performed, and the entire small intestine was carefully removed by cutting along the mesenteric border. Duodenum was defined as a 10-cm section downstream of the pyloric sphincter and ileum as a 10-cm section upstream of the cecum. The tissue in between was defined as jejunum.

Hormone secretion studies using rat intestinal rings. The protocol used by Jang et al. (19) was adapted to everted rat intestine. All steps before incubation were performed on ice. Briefly, luminal contents were gently removed using a fresh-made Krebs buffer saturated with 95% O_2-5% CO_2 (pH 6.5, all in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 20 MES). Duodenum and ileum segments were everted using a customized metallic rod. Rings of 0.5 cm were prepared and briefly incubated in Krebs supplemented with 0.5 mM dithiothreitol to prevent excessive mucus production. The gut rings were then randomly transferred in 200 μl of effectors (triplicates) prepared in Krebs buffer containing dipeptidyl peptidase-IV inhibitor (20 μl/ml; Millipore) in a 96-well plate. A stock solution of H.g.-12 was prepared in DMSO (100 mM) and freshly diluted in Krebs buffer on the day of the experiment. The final concentration of DMSO during incubation never exceeded 0.5%. The gut rings were incubated for 30 min at 37°C in a shaking incubator (60–100 rpm), after which the total content of each well was centrifuged at 4°C for 15 min at 5,000 *g*. CCK-8S levels were measured in the supernatants using the CCK_1R activation assay. PYY (total) and GLP-1 (active) levels were measured using a Milliplex MAP rat-gut-hormone kit (RGT-88K; Millipore, Billerica, MA) read on the Luminex 100 IS system (Luminex, Austin, TX).

PCR of TAS2R7 and TAS2R14 in HuTu-80 ECs and human duodenum and jejunum. PCR (Lightcycler; Roche Applied Science, Mannheim, Germany) were performed using HuTu-80 cells and human adult normal RNA for duodenum (R1234101-50) and jejunum (R1234230-50) purchased from BioChain from four different male donors. Total RNA from HuTu-80 cells was isolated using the RNeasy mini kit (Qiagen), and cDNAs were synthesized by a ThermoScript RT-PCR system (Invitrogen). Specific PCR primers for TAS2Rs and GAPDH (to control the absence of genomic contamination in the probes) were designed using the Lightcycler Probe design software version 1.0 (Roche Applied Science) and synthetized by Eurofins (Eurofins MWG Operon, Ebersberg, Germany). Primer sequences were as follows: human (h) *TAS2R7* (amplicon size = 215 bp), forward primer 5′-ATTGTTCTTAGCAGTTGGAG-3′, reverse primer 5′-CTTTACCAGTGGCATAGAC-3′; *hTAS2R14* (amplicon size = 344 bp), forward primer 5′-AAGACTTGCAGTTCTGATT-3′, reverse primer 5′-GAGTGACATGAAGGATAAGC-3′; *hGAPDH* (amplicon size = 215 bp), forward primer 5′-CATCGCTCAGACACCA-3′, reverse primer 5′-AGCTTCCCGTTCTCAG-3′. The sequence specificities of the primers were verified with NCI BLAST. In addition, RT-PCR on cDNAs from TAS2R7- and TAS2R14-expressing HEK cells were performed to test primer reliability. PCR products were separated on agarose gel and stained with ethidium bromide. Gel images were recorded by a digital camera.

Data processing and statistical analysis. Data in Figs. 1–7 are presented as means ± SE. GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences in the potency of the agonists/effectors toward receptor activation/hormone secretion were analyzed using one-way ANOVA followed by the appropriate multiple-comparison test. Differences were considered to be significant at $P < 0.05$. Receptor activation data (TAS2Rs, CCK_1R) are expressed in ΔF values. All in vitro experiments were performed at least in duplicate. Hormone secretion data from the rat gut rings study are expressed in fold changes of the control (Krebs buffer).

RESULTS

H.g.-12 elicits a significant CCK-8S secretion in a rat ex vivo model. A study with rat gut rings was conducted to assess whether H.g.-12 could elicit gut satiety hormone secretion. When duodenal tissue was exposed to 0.5 mM of H.g.-12, a significant 1.4 ± 0.2-fold increase in CCK-8S secretion was observed ($P < 0.001$, Fig. 2). Here as a positive control, a protein hydrolyzate (4%) was shown to significantly increase CCK secretion around 1.7 ± 0.2-fold ($P < 0.001$). In ileal tissue specimens, H.g.-12 tended to elicit a 1.6 ± 0.8-fold GLP-1 secretion ($P > 0.05$, data not shown) and a 1.5 ± 0.4-fold increased PYY output ($P > 0.05$, data not shown).

H.g.-12 induces CCK release from the human EC cell line HuTu-80. To investigate whether the steroid glycoside H.g.-12 could elicit hormone secretion from human EC cells, HuTu-80 cells were exposed to increasing concentrations of H.g.-12 for 2 h. H.g.-12-mediated CCK secretion in the medium was demonstrated by increased CCK_1R activation in a receptor cell

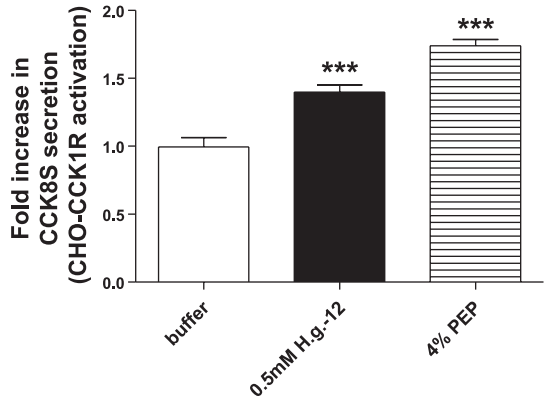

Fig. 2. H.g.-12 induced cholecystokinin (CCK) secretion in the rat intestine ex vivo model. Duodenum sections (0.5 cm) prepared from everted rat intestine were incubated with 0.5 mM H.g.-12 and 4% peptone mix (positive control, protein hydrolyzate) for 30 min at 37°C. After centrifugation (4°C) of the samples, CCK-8S levels were measured in the supernatants using the CCK receptor 1 (CCK_1R) activation assay. The data are averages of triplicate determinations from $n = 6$ animals and are expressed as control fold (Krebs buffer); $***P < 0.001$ (one-way ANOVA + Bonferroni's multiple-comparison test). CHO-CCK_1R, Chinese hamster ovary cell line functionally expressing rat CCK receptor 1.

line. A significant mean threefold increase ($P < 0.001$) in CCK_1R activation compared with the control cells was observed (Fig. 3*A*). Whereas H.g.-12 exhibited potent effects on CCK secretion, removal of the tigloyl group in the detiglated and detiglated-aglycon forms completely abolished the CCK secretion from HuTu-80 cells (Fig. 3*B*), and the aglycon form was not able to significantly induce hormone secretion from the cells. H.g.-12 effects on CCK secretion in HuTu cells did not exhibit a clear dose dependency. Concentrations of 30 μM and below failed to elicit a significant CCK output, whereas concentrations ranging from 60 to 500 μM displayed similar effects on CCK_1R activation. To determine whether the effect of H.g.-12 on CCK secretion in HuTu-80 cells is specific or a more generic effect, we assessed whether bitter tastants like DB, PTC, or quinine could as well elicit CCK secretion with H.g.-12 as a positive control. Whereas 0.25 mM H.g.-12 in a robust manner increased CCK release threefold, DB, PTC, and quinine at concentrations up to 10 mM failed to cause a stimulation of CCK release in HuTu-80 cells (data not shown), thus demonstrating the selectivity of H.g.-12.

H.g.-12 selectively activates the human bitter receptors TAS2R7 and TAS2R14. Purified *H. gordonii* extracts taste bitter. To identify whether the *H. gordonii* steroid glycoside H.g.-12 selectively activates one or more human TAS2 bitter receptors, a screening of 25 human bitter receptors each expressed heterologously in HEK 293 cells was conducted (Fig. 4). Monitoring $[Ca^{2+}]_i$ revealed that 0.25 mM H.g.-12 specifically activated hTAS2R7 (*wells E4* and *E10*) and hTAS2R14 (*wells B5* and *B11*), whereas receptors TAS2R38 (*well D11*) and TAS2R43 (*well A6*) displayed a nonspecific response in induced cells that also failed to show a dose dependency. In contrast, the increase in $[Ca^{2+}]_i$ for cells expressing TAS2R7 and TAS2R14 was dose dependent for both H.g.-12 and its aglycon at concentrations ranging from 30 to 250 μM (Fig. 5, *A* and *B*). The absence of a plateau at high compound concentration, which would allow calculation of an EC_{50} value, is due to the poor solubility of H.g.-12 and its aglycon in buffer at concentrations >250 μM.

Structural determinants of H.g.-12-induced activation of TAS2R7 and TAS2R14 and CCK secretion from EC cells. The common structural elements in all *H. gordonii* steroid glycosides are the steroid core, the tigloyl group, and the chain of (deoxy and/or methoxy) sugars (20). To understand which structures of H.g.-12 are essential for TAS2R activation, we tested H.g.-12, its aglycon, the detiglated compound, and the detiglated aglycon on both TAS2R7 and TAS2R14 (Fig. 5, *C* and *D*). Whereas the removal of the tigloyl group abolished the activation of both TAS2R7 and TAS2R14, the removal of the sugar residues did not significantly alter TAS2R7 activation. The aglycon even appeared to be a better agonist for TAS2R14 when compared with H.g.-12 ($P < 0.001$, Fig. 5*D*). Similarly to detiglated H.g.-12, the detiglated aglycon did not exhibit any effect on $[Ca^{2+}]_i$ in TAS2R7- and TAS2R14-expressing HEK cells (data not shown).

To further investigate the role of the tigloyl group on TAS2R7 and TAS2R14 activation, we tested a number of structural unrelated compounds containing a tygloyl function such as geranyl-tiglate, citronellyl-tiglate, and phenethyl-tiglate. None of the compounds activated either TAS2R7 or TAS2R14 (data not shown).

A bitter receptor inhibitor was identified by screening of a chemical library with 10,000 compounds against the receptors TAS2R14, TAS2R16, and TAS2R39. Coincubation with the lead candidate 03A3 (100 μM) in the presence of the activating ligand (0.25 mM) caused a significant inhibition of $[Ca^{2+}]_i$ in TAS2R-expressing cells, up to 35% in the case of H.g.-12-induced TAS2R14 activation (Fig. 6*A*). Similarly, coincubation of HuTu-80 cells with 03A3 (100 μM) and H.g.-12 (0.25 mM) abolished CCK secretion from EC

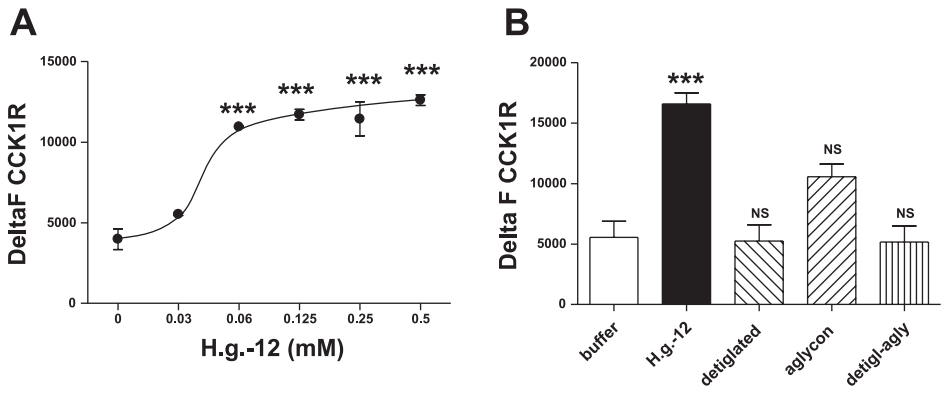

Fig. 3. H.g.-12 structure-activity relationship on CCK secretion from HuTu-80 cells. Indirect determination of CCK-8S levels in HuTu-80 supernatants using the CCK_1R activation assay. *A*: cells were incubated for 2 h with increasing concentrations of H.g.-12 (0.03–0.5 mM). The data are averages of triplicate determinations from 3 independent experiments; $***P < 0.001$ (one-way ANOVA + Bonferroni's multiple-comparison test). *B*: cells were incubated for 2 h with 0.25 mM of H.g.-12, detiglated, aglycon, or detiglated-aglycon. The data are averages of triplicate determinations from 3 independent experiments; $***P < 0.001$ (one-way ANOVA + Bonferroni's multiple-comparison test). All data are reported in changes of fluo 4-AM fluorescence (ΔF). NS, not significant.

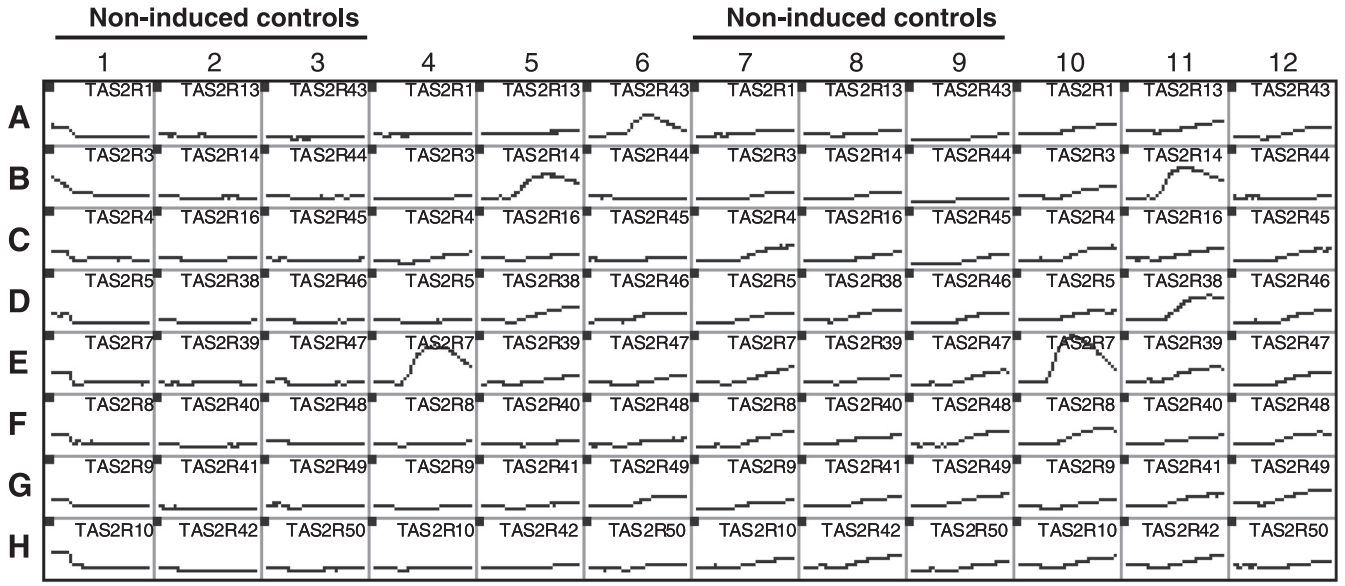

Fig. 4. Screening of human (h) TAS2R transfected HEK 293T cells for activation by H.g.-12. Activation of hTAS2Rs by H.g.-12 (0.25 mM) was measured over 90 s in TAS2R-expressing HEK cells by monitoring of variations in fluo 4-AM fluorescence (ΔF) induced by changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Non-doxycyclin-induced HEK cells were used as controls. Receptor identity is shown for each well of the 96-well plate. *Columns 1–3* and *7–9* are control cells, and *columns 4–6* and *10–12* are induced cells (all duplicates).

cells (Fig. 6*B*). However, the compound 03A3 also inhibited other TAS2Rs, suggesting that it is not specific for TAS2R14.

TAS2R14 but not TAS2R7 is expressed in HuTu-80 cells and human proximal intestine. PCR experiments were conducted in the EC cell model and in human proximal small intestine to establish the expression pattern of the two TAS2Rs activated by H.g.-12. The presence of the TAS2R14 transcript was found in the HuTu-80 EC cell line of duodenal origin and in human normal duodenum and jejunum (Fig. 7). The presence of a TAS2R7 transcript could be detected neither in HuTu-80 cells nor in human proximal intestinal tissues, whereas, in the positive control, TAS2R7-expressing HEK cells, the transcript was detected (data not shown).

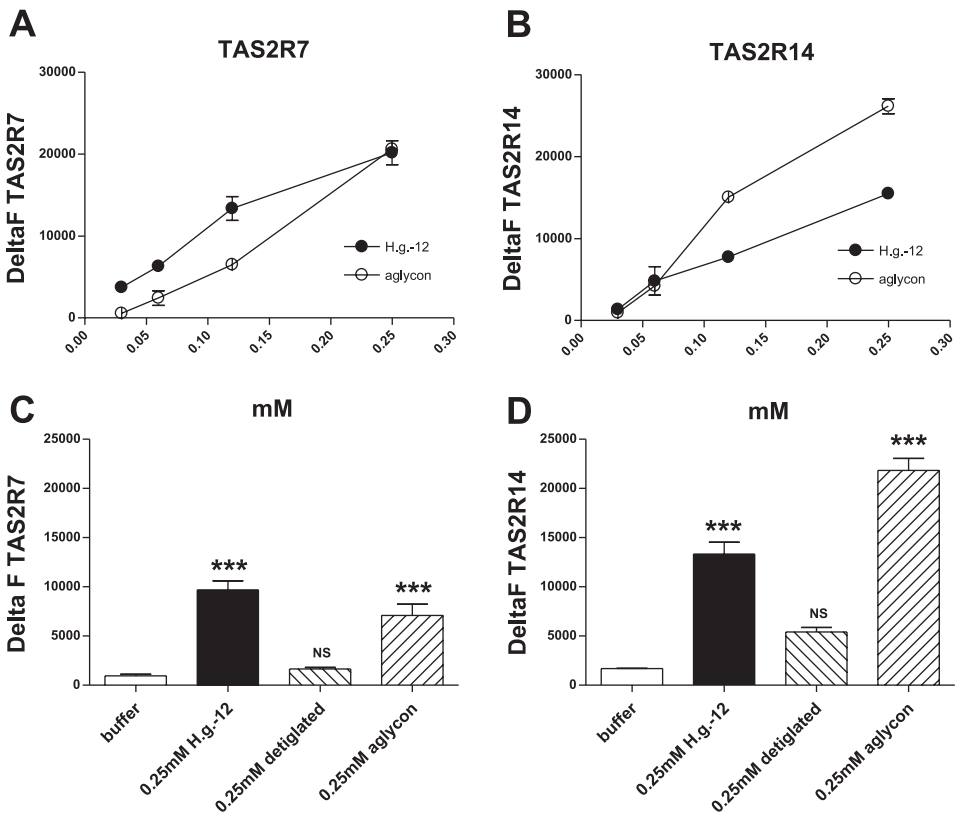

Fig. 5. H.g.-12 structure-activity relationship on TAS2R7 and TAS2R14 activation. *A* and *B*: changes in $[Ca^{2+}]_i$ in TAS2R7- and TAS2R14-expressing HEK cells activated by H.g.-12 and its aglycon (0.03–0.25 mM). Data are averages of duplicate determinations from a representative experiment. *C* and *D*: $[Ca^{2+}]_i$ answer in TAS2R7- and TAS2R14-expressing HEK cells to either 0.25 mM of H.g.-12, detiglated or aglycon. The data are averages of triplicate determinations from 3 independent experiments; $***P < 0.001$ (one-way ANOVA + Bonferroni's multiple-comparison test). All data are reported in changes of fluo 4-AM fluorescence (ΔF).

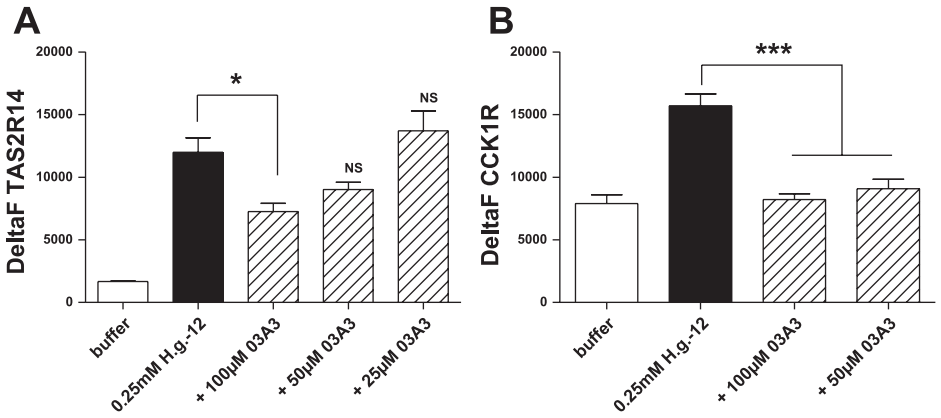

Fig. 6. Inhibition of H.g.-12-induced activation of TAS2R14 and CCK secretion in HuTu-80 cells by compound 03A3. *A*: $[Ca^{2+}]_i$ answer in TAS2R14-expressing HEK cells to 0.25 mM H.g.-12 alone (positive control) or coincubated with compound 03A3 (25, 50, or 100 μM). $[Ca^{2+}]_i$ were determined using fluo 4-AM and are reported in ΔF. The data are averages of triplicate determinations from 2 independent experiments; $*P < 0.05$ (one-way ANOVA + Dunnett's multiple-comparison test). *B*: indirect determination of CCK-8S levels in HuTu-80 supernatants using the CCK_1R activation assay. Cells were incubated for 2 h with 0.25 mM H.g.-12 alone (positive control) or coincubated with compound 03A3 (50–100 μM). The data are averages of triplicate determinations from 2 independent experiments; $***P < 0.001$ (one-way ANOVA + Bonferroni's multiple-comparison test).

DISCUSSION

In this paper, we report for the first time that the steroid glycoside H.g.-12 isolated from *H. gordonii* can elicit a CCK release from rat ex vivo tissue preparations and from a human EC cell line. In addition, we have established that H.g.-12 is a natural and specific agonist for the human bitter receptors TAS2R7 and TAS2R14 but not for any of the other human bitter receptors. We demonstrated that TAS2R14 but not TAS2R7 is expressed in the human EC cell line HuTu-80 as well as in human proximal intestinal tissues. The tigloyl group in the steroid molecule was found in different experimental models to be an essential structural feature needed for both bitter receptor activation and hormone secretion. Thus, by demonstrating specificity of solely TAS2R7 and TAS2R14 activation and employing derivatives of H.g.-12 in both TAS2R14 overexpressing cells and in human EC cells, we have supportive but not conclusive evidence that CCK secretion from HuTu-80 cells is mediated via TAS2R14. Our experiments emphasize the notion that the steroid glycoside H.g.-12 could, via bitter receptor activation in endocrine cells, cause CCK-8 release. CCK-8 has central effects on appetite suppression (7, 33); thus, bitter receptor activation by *H. gordonii* steroid glycosides in the intestine might be a part of the mechanistic route of its effects in suppressing food intake.

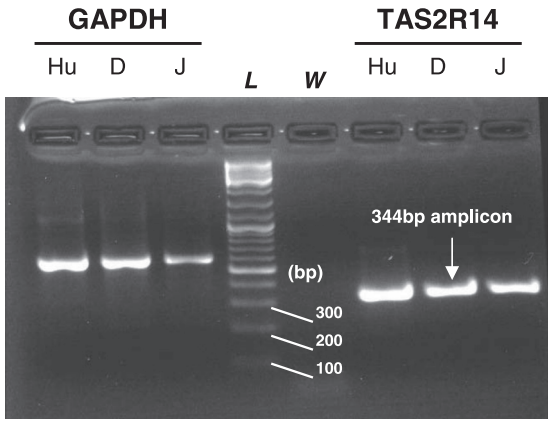

Fig. 7. TAS2R14 is expressed in HuTu-80 human enteroendocrine cells and human proximal intestine. Expression of human taste receptor TAS2R14 in HuTu-80 enteroendocrine cells (Hu) and human normal duodenum (D) and jejunum (J). PCR was performed using the specific primers listed in MATERIALS AND METHODS to detect the expression of TAS2R14 (predicted amplicon size 344 bp). PCR for GAPDH was performed to verify the absence of genomic contamination in the probes. L, DNA sizing ladder; W, PCR performed without DNA as a negative control.

Rat tissues exposed ex vivo to H.g.-12 responded with a significantly increased CCK secretion. This tissue model takes into account the morphology in which EC cells are surrounded by enterocytes and an intact enteric nervous system. Although the finding that H.g.-12 induced CCK secretion in this system is considered to be physiological, future studies are needed to confirm that H.g.-12 and other *Hoodia* components increase CCK in vivo to plasma concentrations, which could explain the effects of *Hoodia* extracts on food intake. Furthermore, it remains speculative whether the effect of H.g.-12 on CCK secretion in rat tissues is mediated by bitter receptor activation. It has been shown that rT2R6, the rat ortholog gene to TAS2R7 (70% of sequence identity), is expressed in gastric and duodenal mucosa (35, 36), but, for TAS2R14, no close ortholog has yet been found in rats. Thus, whether CCK secretion in rat duodenum depends on the TAS2R7 ortholog and whether this receptor is activated in a similar manner as the human receptor is currently not known. However, we expect some differences between human and rodent bitter receptor activation by *H. gordonii* steroid glycosides based on the observations that, in rodents, only *H. gordonii* steroid glycosides with at least two sugar moieties have an appetite-suppressant effect (34). In our cell systems, the aglycon possessed similar activities with respect to TAS2R7 and TAS2R14 activation.

By employing the human EC cell line HuTu-80, we demonstrated that H.g.-12 can increase CCK secretion by two to three times, although with an unusual dose dependency. It is likely that, in this endocrine cell line that possesses a wide range of different receptors and signaling pathways, H.g.-12 effects at low concentrations are masked and a threshold concentration is needed for final stimulus-secretion coupling. However, H.g.-12-induced stimulation in CCK secretion from HuTu-80 cells was specific, since other bitter-tasting com-

pounds such as DB, PTC, and quinine, even at concentrations as high as 10 mM, did not induce CCK secretion. Although PTC was shown previously to increase $[Ca^{2+}]_i$ in HuTu-80 cells (31), it failed to cause a CCK output in our experiments. In this respect, the capability of H.g.-12 to induce a CCK release from HuTu-80 cells is an original feature among the various bitter tastants.

The structure-specificity in the ligands that cause CCK release was demonstrated by the removal of the tigloyl group (detiglated and detiglated-aglycon forms) that abolished the capability for stimulation of CCK secretion. Removal of the sugar moieties from H.g.-12 (aglycon) decreased its stimulation of CCK secretion from HuTu cells in contrast to its increased capability to activate TAS2R14 in HEK cells. We assume that this is related to a lower receptor density on HuTu cells and a much higher hydrophobicity of the aglycon with increased diffusion and removal by the cells over the time of incubation. This problem could not be overcome by increasing concentrations as those used are already at the limits of solubility.

The H.g.-12 steroid glycoside from *H. gordonii* extract is identified as a new agonist for both TAS2R7 and TAS2R14. TAS2R7 displays a quite broad agonist spectrum with currently nine ligands known (28, 32) that are all structurally unrelated to H.g.-12. As for TAS2R14, 33 compounds have been identified as agonists, yet without any obvious common structural motif in the ligands (3, 28). This makes TAS2R14 one of the broadest human bitter receptors in terms of agonist spectrum along with TAS2R10 and TAS2R46, of which all three tend to display a slight preference for natural compounds (28).

We have observed that removal of the tigloyl group from H.g.-12 abolishes its interaction with both tested TAS2 receptors, thus mirroring the situation in HuTu-80 cells where removal of the tigloyl group abolished CCK secretion. Although the compound 03A3 was shown to inhibit TAS2R14 and also to markedly reduce hormone secretion in response to H.g.-12 activation, it also interacted with some other bitter receptors.

Tiglic acid alone and three structurally unrelated compounds containing a tigloyl group failed to activate TAS2R7 or TAS2R14. This demonstrates that the tigloyl function as such is not the sole structural feature needed for TAS2R activation. Quantitative structure-activity relationship studies are currently not possible because of a very limited availability of H.g.-12-related structures.

We observed that removal of the glycoside groups (aglycon) in H.g.-12 increased the selectivity toward receptor TAS2R14. With the use of a simulated gastric and small intestinal digestion model, it was recently suggested that H.g.-12 is partly deglycosylated during gastrointestinal digestion (25). In the same study, the aglycon molecule was shown to cross both the intestinal and blood-brain barriers by passive diffusion. However, further studies would have to demonstrate that the aglycon is not extensively metabolized during intestinal absorption and/or first liver passage before postabsorptive systemic effects are proposed. This is, however, conceptually different from the preabsorptive effects that H.g.-12 can have by interacting with luminal-facing taste receptors in the gastrointestinal tract.

We identified the transcript for TAS2R14 in HuTu-80 cells as well as in human duodenal and jejunal tissue samples. In contrast, the TAS2R7 transcript was not detectable with the probes employed in the cell and tissue samples. Our data confirm recent findings on the presence of TAS2R14 but not TAS2R7 in HuTu-80 cells together with 13 other TAS2Rs (31). Although TAS2R7 and TAS2R14 are both found in the neuroepithelium of the tongue (4), they could not be detected in human colon (31), and TAS2R7 could also not be identified in human ileal NCI-H716 cells or human cecum samples (11). Concerning the proximal intestine, this is, to our knowledge, the first report demonstrating the expression of TAS2R14 in human duodenum and jejunum. However, detailed cellular and subcellular distribution of TAS2R14 proteins in intestinal tissues, combined with colocalization data for gut hormones, is needed before more profound suggestions for the physiological relevance of TAS2R14 activation can be made.

In conclusion, we provide supportive evidence for a mechanism through which the proposed appetite-suppressant properties of *H. gordonii* extract may partly originate from its main steroid glycoside H.g.-12. We have shown that H.g.-12 elicits in vitro CCK release from EC cultured cells, most probably through the activation of one subtype of human bitter receptors. The same mechanism could possibly be involved in CCK secretion from rat duodenal tissues ex vivo. CCK is known to induce satiety in rodents (13, 33) and in humans (10, 22, 23). In addition, T2R agonists administered in the stomach of rats by oral gavage were found to increase c-Fos gene expression and c-Fos positive neuron numbers in the NTS, and CCK_1 receptors located on vagal afferent terminals in the gut wall are thought to form this gut-brain axis (16, 17). The vagal afferent terminals found in the lamina propria are in close vicinity to the EC cells, suggesting that the transformation of the chemosensor input signals into neuronal signals could take place at this location. Combined with a possible direct interaction of CCK secreted from the intestine with the CCK_2 receptor in the brain, the inhibition of food intake associated with *H. gordonii* consumption in rats and putatively in humans could result, in part, from this intestine-to-brain communication route.

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DISCLOSURES

M. Foltz and R. Gouka are employed by Unilever. Unilever had licensed from Phytopharm plc the rights to research *Hoodia gordonii* for commercial applications. Unilever has no longer commercial interest in *Hoodia gordonii*.

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