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DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„Impact of sweet tastants on mechanisms regulating gastric acid secretion *in vitro*“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doktor der Naturwissenschaften (Dr. rer. nat.)

Wien, 2018 / Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 796 605 419
Dissertationsgebiet lt. Studienblatt / field of study as it appears on the student record sheet:	Chemie/ Chemistry
Betreut von / Supervisor:	Univ.- Prof. Mag. Dr. Veronika Somoza

Acknowledgement

The practical work was carried out at the Institute of Physiological Chemistry at the University of Vienna from 2015 to 2018 under the supervision of Prof. Dr. Veronika Somoza.

My special thanks go to my supervisor Prof. Dr. Veronika Somoza for the provision of the topic in carrying out the work as well the further career planning. Thank you very much for the dedicated cooperation, constructive discussions, and your confidence.

Furthermore, I would like to thank the company Symrise AG and its employees Dr. Jakob P. Ley and Dr. Joachim Hans for their excellent cooperation as well as encouraging technical discussions.

I would also like to thank the cooperation partner Dr. Maik Behrens at the German Institute for Nutrition (DIfE) for the outstanding contribution in Potsdam and the excellent knowledge exchange.

I am thankful to my dear colleagues Dr. Barbara Lieder and Dr. Ann-Kathrin Holik who provided crucial support along the way.

Besides being wonderful friends, I am also thankful to Dr. Jory Liétard for his great contributions during the preparation of this thesis and to Mathias Zaunschrim, M.Sc. for his support in translating Abstract into German.

I would also like to thank all of colleges who supported me during this work and assisted in achieving my goal.

Lastly, my deepest thanks are to my family for all their love and encouragement. And of course, Ana, my better half, whose constant love, support, guidance, and friendship made this achievement possible.

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I. Introduction

Sugar is a ubiquitous substance in the food we consume. However, too much sugar has adverse effects on our health according to multiple, though still controversial, investigations, suggesting that excessively consumed sugar may play a significant role in various progressive diseases like obesity and diabetes. Hence, sweet taste may play a critical role in the regulation of food preference and energy intake. One potential way to challenge the obesity problem is to decrease energy intake through high intensity non-caloric sweeteners (NCSs) since the satiety is influenced by the sweet taste signaling mechanisms identified in the gastrointestinal (GI) tract [1]. Therefore, NCSs, sugar substitutes providing a sweet taste without contributing to energy intake and without glycemic effects [1], have had an enormous impact in the market and continue to be the preferred choice for consumers wishing to restrict their caloric intake. Despite their popularity, there is increasing evidence that artificial NCSs cause health hazards. While several *in vivo* and *in vitro* studies have been conducted in order to unveil the effect of NCSs on the body, their impact on the mechanisms of gastric acid secretion (GAS) and serotonin (5-HT) secretion, key modulators of satiety [2,3], is poorly understood. Moreover, GAS is essential for the digestion and ingestion of proteins, absorption of iron and calcium, as well as for the prevention of bacterial overgrowth in the stomach. Control of GAS at the central, peripheral and intracellular levels is mediated by mechanisms involving several neurotransmitters, paracrine mediators, and hormones [4]. Furthermore, as a monoamine neurotransmitter, 5-HT is mostly located in the GI tract of animals and humans, and also predominantly regulates GI functions such as gastric acid secretion [5–9] as well as GI motility [10–12].

The goal of the present thesis was, therefore, to investigate and identify the impact of the selected sweet-tasting substances on the biological mechanisms regulating gastric acid and 5-HT secretion *in vitro*, as well as to elucidate the cellular mechanisms involved in GAS and 5-HT induced by NCSs. As a result, the knowledge and data gathered from this thesis can be used to further our understanding of GAS and 5-HT secretion in the stomach.

1.1 Regulation of GAS

The stomach is a unique organ that performs diverse functions in the GI tract and is composed of two main sections; the section of oxyntic gland, occupying the major part of the gastric mucosa, and the section of antral gland [13]. In addition, the stomach can anatomically be partitioned into the fundus, and the corpus that are representing the section of the oxyntic gland, and the antrum that corresponds to the section of antral gland. About two to three L of gastric juice is yielded by the gastric mucosa in the stomach. This juice primarily contains concentrated hydrochloric acid (HCl), destroying ingested pathogens and helping in the digestion of food nutrients. To do so, the stomach contents are acidified by specialized cells known as parietal cells and which are located in the gastric mucosa, in the section of oxyntic gland that pumps protons into the gastric lumen [14]. In addition to HCl, gastric juice also contains pepsinogens [15], the secretion of which is regulated during food digestion by various ways, such as paracrine, hormonal, neuronal pathways or *via* chemical and mechanical stimuli [14]. Pepsinogen, a proenzyme or "zymogen", is the inactive precursor of the active pepsin, a vital digestive enzyme that can solely be active in acidic environments, such as during secretion of HCl. Pepsin degrades the food proteins into peptides [14].

In addition to the parietal cells, various enteroendocrine cells such as ghrelin-secreting cells, somatostatin (STT)-secreting D cells, histamine-secreting enterochromaffin-like (ECL) cells, as well as leptin-secreting chief cells and mucus-producing neck cells are also located in the oxyntic glands of the stomach [16]. Since concentrated HCl is a harmful constituent, the gastric mucosa takes broad actions to protect itself from tissue damage by either secreting mucus from mucus-producing cells or firmly modulating the acid secretion. Specific endocrine cells located in the gastric mucosa are involved in the regulation of GAS. A failure of either defense mechanism can result in critical tissue injury causing, in the long run, gastric ulcers.

The antral gland section contains G-cells that are responsible for producing, storing and releasing the major gastric hormone gastrin [16], and are in addition also responsible for secreting histamine from the ECL cells [16]. Gastrin jointly works with histamine and

acetylcholine (ACh) in the activation of the parietal cells. Gastrin stimulates the release of histamine from the ECL cells, which then acts on the parietal cells *via* the cell membrane histamine-H2 receptor, positively coupling to adenylate cyclase and, consequently, to the formation of adenosine 3,5-cyclic monophosphate (cAMP). cAMP is the key component of the secondary messenger signaling cascade, performing a crucial role in the action of GAS [13]. Additionally, acid secretion is inhibited by STT *via* either paracrine pathway by functioning directly on the parietal cells and or indirectly by inhibiting the secretion of histamine from ECL cells [4].

GAS, in relation to the consumption of food, is divided into three phases: the cephalic, gastric and intestinal phases of digestion [17], their designation being related to the regulatory site: brain, stomach or duodenum, respectively. These phases can happen at the same time [15]. The cephalic phase can be provoked by imagining, smelling and tasting food and is controlled by the vagus nerve in the central nervous system (CNS). Studies suggest that the vagus nerve may parasympathetically activate GAS [15, 18]. Importantly, GAS can be influenced by emotions; stress pain, anger or sorrow, and hypoglycemic circumstances can affect GAS [15].

1.1.1 Parietal cells

The gastric parietal cells are in charge of the acidification of the stomach through the secretion of concentrated HCl. The apical extrusion of the ions mediates GAS. Firstly, the protons (H^+) are pumped into the gastric lumen by the gastric H^+-K^+-ATPase, so called proton pumps, in order to acidify the gastric lumen to a pH level of 1. Then, apical selective chloride channels release chloride (Cl^-) to guarantee the formation of H^+Cl^-. Finally, potassium (K^+) apically departs the parietal cells in a recycling manner, in turn increasing the reciprocal transportation of H^+ through H^+-K^+-ATPase. The exchange of one intracellular H^+ ion with one extracellular K^+ ion occurs at an energy cost provided by adenosine triphosphate (ATP), which is supplied by the mitochondria occupying approximately 40 % of the cell capacity. It has been showed that interruption of these ion transport mechanisms reduces the parietal cell's ability to secrete gastric acid [19]. During the resting stage, H^+-K^+-ATPase is found in

tubulovesicles within the parietal cell, and its stimulation causes the transformation of the cell membrane [20].

In order to stimulate the parietal cell, binding of either histamine or acetylcholine to the corresponding receptor is needed (Figure 1). The activation of these receptors induces two separate routes.

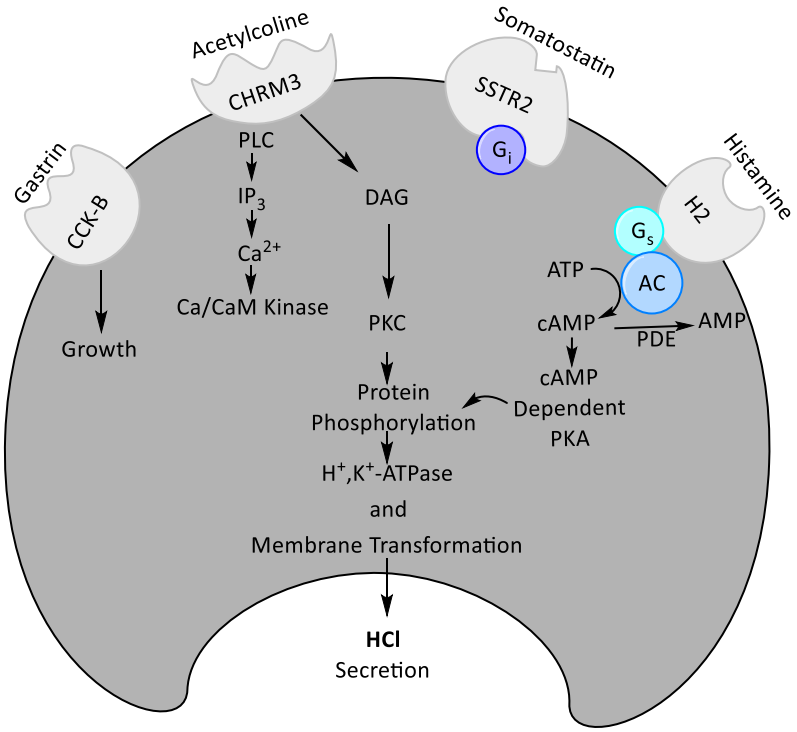

Figure 1. A parietal cell model demonstrating signaling pathways leading to membrane transformation and the secretion of HCl (adapted from Forte 2010 [21])

Figure 1 demonstrates the signaling pathway in parietal cells. Binding of histamine to the cell membrane histamine-H2 receptor induces the activation of adenylate cyclase (AC) that is converted into cAMP which, further, stimulates cAMP-dependent protein kinase (PKA). The cAMP is degraded to AMP by phosphodiesterase (PDE). The activation of cell membrane cholinergic-M3 receptor by acetylcholine leads to the stimulation of phospholipase C (PLC). The membrane-bound of the phosphatidylinositol bisphosphate (PIP_2) is cleaved by PLC to produce inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of Ca^{2+} from the membrane storage. The activation of the H^+-K^+-ATPase into the apical membrane is induced after protein phosphorylation by PKA, PKC, and calmodulin (CaM) kinases. In the

event of binding the somatostatin to the SSTR2 receptor causes direct inhibition of HCl secretion in the parietal cell [21]. In addition to major secretagogues histamine and acetylcholine, gastrin is also a potential pro-secretory transmitter. The promotion of the acid output is indirectly mediated by gastrin, which induces gastrin/CCK-B receptors to stimulate histamine release that directly acts on the parietal cells in a paracrine manner. It is important to note that, by activating protein tyrosine kinases, gastrin/CCK-B receptors play a direct and major role in the regulation of cell maintenance and growth (Figure 1) [21].

A cell system, the human gastric adenocarcinoma cell line (HGT-1) is a well-established *in vitro* parietal cell model to investigate the regulatory mechanisms involved in GAS, as all functional and regulatory proteins related to GAS are expressed by HGT-1 cells. Therefore, HGT-1 cells can be considered characteristic gastric parietal cells [22–24]. Although HGT-1 cells have been widely utilized for the *in vitro* investigations of flavoring food compounds (in particular bitter-tasting substances) in the regulatory mechanisms of GAS [25–29], the impact of NCSs, sweet-tasting compounds, on mechanisms regulating GAS have not been studied until now. For those reasons, evaluating the impact of the NCSs on the regulatory mechanisms related to GAS is an attractive prospect.

1.2 Localization and functional role of sweet taste receptor (T1R2/T1R3)

As explained previously, the taste of food influences the preliminary, cephalic phase of GAS which is regulated by the central nervous system [15]. The detection of essential food nutrients, stimulation of the digestive system, and protection against unsafe elements in food are the major functions of the gustatory system [30]. In the oral cavity, the recognition of taste takes place in the taste buds, which are able to identify sweet, umami, salty, bitter, and sour flavors *via* taste receptors that belong to the G-protein-coupled receptor family (GPCR). In the taste buds, four types of taste receptor cells have been identified and divided into four types called; Type I, II, III and IV. The two main categories are Type II and III. Sweet, bitter, and umami tastants can stimulate Type II cells, known as sensory cells, which express GPCRs [31]. Bitter tastants are detected by bitter taste receptors family which is encoded by the taste receptor type 2 (T2R) genes [32]. The taste receptor type 1 (T1R) family consists of three sub-units; T1R1, T1R2, and T1R3. The heterodimer T1R1/T1R3 receptor responds to

umami substances [33]. The sweet taste receptor is a heterodimeric structure consisting of T1R2 and T1R3. The most fascinating feature of the T1R2/T1R3 is that it can be activated by diverse sweet-tasting compounds of various chemical structures including natural sugars, intensively sweet proteins, D-amino acids, and artificial NCSs [33–35]. Moreover, lactisole, a well-known selective sweet taste inhibitor acting on T1R3, inhibits sweet taste in humans [36]. Study conducted on knockout mice have showed that most of the sweet tasting mechanism is regulated by the heterodimeric complex T1R2/T1R3 [37]. However, some activity was detected in response to sugars in T1R3 knockout mice [38, 39]. Therefore, it seems probable that there are other mechanisms for the detection of sweet-tasting substances and that a different sweet-sensing action is mediated by different unidentified GPCRs.

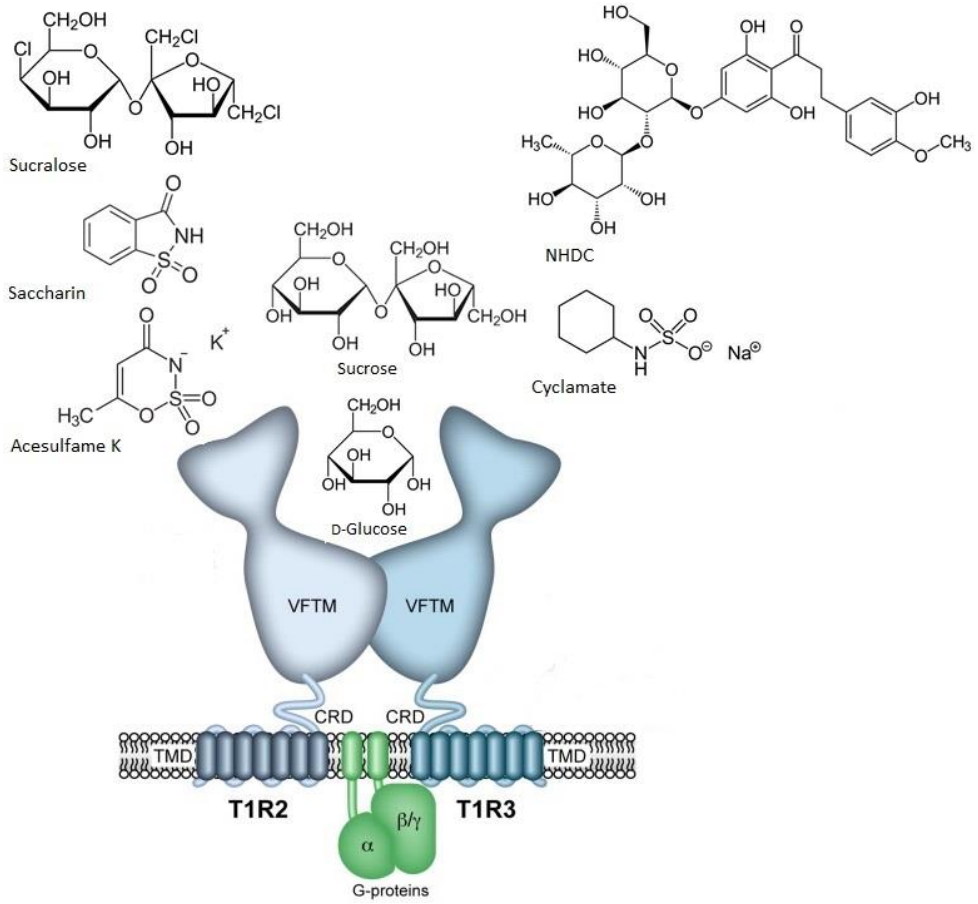

Figure 2. Structure of the heterodimeric sweet taste receptor and its agonists.

Figure 2 illustrates sweet taste receptor and the binding site of its agonists. The sweet taste receptor consists of two subunits, namely T1R2 and T1R3. The identified binding site for sucralose, saccharin and acesulfame K on the sweet taste receptor is T1R2 [40]. T1R3 is the responsive receptor subunit of NHDC and cyclamate [41]. The natural sugars sucrose and glucose are assumed to bind to the both subunits, T1R2 and T1R3 with distinct binding affinities [42].

In addition, it has been suggested that the sweet taste receptor could also be T1R3 homodimers instead of the well-known heterodimer T1R2/T1R3 in various cell lines, as in β-cells [43], adipocytes [44], HuTu-80 cells [45], and ghrelinoma cell line MGN3-1 which is of gastric origin [46]. T1Rs have been detected in tissues other than the gustatory system including the small intestine [47]. Also, T1R3 and α-gustducin are expressed in various region of stomach and intestine of mice [48]. Expression of the T1R subtype T1R3, which is crucial for the recognition of sugars and amino acids in the gustatory system, has been discovered in ghrelin-producing cells of murine stomach and brush cells [49].

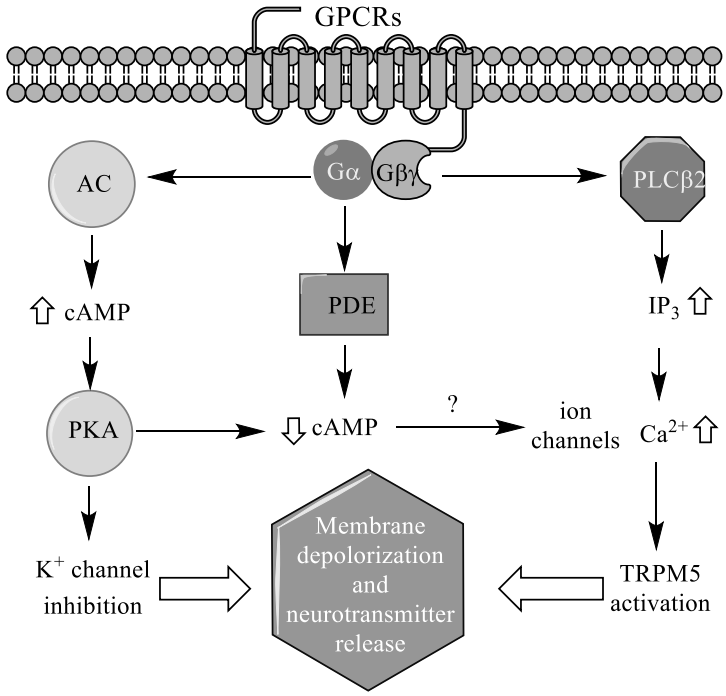

Figure 3. Graphic illustration of taste signaling downstream for G protein-coupled taste receptors (adapted from Roper 2007 [31]).

It has been suggested that activation of T1R and T2R receptors by sweet, umami, and bitter ligands leads to dissociation of G proteins and stimulation of two major downstream signaling cascades. First taste signaling pathway occurs via cAMP-dependent manner. Second one relies on phospholipase C-ß2 (PLC-ß2) [31]. When activated by an agonist, taste receptor conveys a signal to a specific trimeric G protein, gustducin, and following activation of phospholipase C-ß2 (PLCß2) leads to the release of two messengers, and inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates the release of Ca^{2+} from the endoplasmic reticulum, therefore elevating cytosolic calcium concentration, which induces a non-selective cation channel (TRPM) that is accompanied by membrane depolarization, allowing ATP release, and ultimately, neurotransmitter secretion (Figure 3) [31].

The signal transduction pathways initiated by the T1R2/T1R3 upon binding to an agonist have been thoroughly investigated, but is not entirely understood. Mice lacking either T1R2 or T1R3 show significantly reduced but not eliminated sweet perception in response to sweet substances [37–39]. Gustducin or TRPM5 knockout mice are not entirely unresponsive to sweet tastants [50–52]. Conversely, one study showed that TRPM5 knockout mice do not perceive the taste of sucrose from water over a wide concentration range [53]. In addition, a total loss of sweet response was seen in PLCß2 knockout mice [53]. These observations also suggest that other undefined sweet taste receptors and signal transduction mechanisms may participate in the detection of sweet stimuli. Moreover, activation of the T1R2/T1R3 by sweet tastants leads to GLP-1, GIP, PYY, CCK, as well as insulin secretion [54–58]. It was found in addition that lactisole, a T1R3 inhibitor [36], decreased GLP-1 secretion induced by acesulfame K, sucralose, and saccharin in T1R2-lacking Hutu-80 cells [45]. These results suggest that T1R2/T1R3 receptor has a regulatory role in the GI tract beyond taste perception, and is functional upon activation by its agonist.
In summary, while the role of T1R2/T1R3 has widely been examined in the GI tract its role in parietal cells *in vitro* has not been elucidated. Therefore, the function of the T1R2/T1R3 in mechanisms regulating GAS, and 5-HT secretion needed to be clarified.

1.2.1 Glucose mechanism and its mediation by T1R2/T1R3

Glucose is the essential energy source for all mammalian cells as well as a vital substrate for the synthesis of protein and lipids. In mammals, most of the cells do uptake glucose by a passive or carriage process. Glucose is driven across the plasma membrane, and blood glucose levels remain relatively constant *via* homeostatic mechanisms [59]. Thirteen members of glucose transporters (GLUTs) have been identified, namely GLUTs [60]. In addition, six members of the sodium-dependent glucose transports, namely SGLTs, have been described so far but only SGLT1 and SGLT2 have been identified [61]. Glucose transport can be inhibited by GLUT transporter inhibitors cytochalasin-B or phloretin [62]. Several studies have suggested that T1R2/T1R3 play a role in the secretion of enteroendocrine hormone by regulating glucose absorption via GLUT transporters [54–56]. It has been demonstrated that glucose can promote its own metabolism by acting on T1R2/T1R3, leading to an increase in intracellular ATP. In accordance with this finding, the glucose-induced increase in the intracellular ATP was reduced when T1R3 was knocked down. It was also hypothesized that glucose is not able to fully function in the absence of T1R3 [63]. Overall, these results suggest that glucose mechanism is controlled by T1R2/T1R3, and especially by the T1R3 receptor.

The ATP-gated potassium sensors (K_{ATP}) are important for glucose sensing in mouse T1R3-expressing taste cells [64]. Studies have reported the co-expression of SUR1 (the subunit of the ATP-gated potassium sensors (K_{ATP}) sulfonylurea receptor subtype 1, SUR1) and GLUTs along with T1R3, which are crucial components in mechanisms regulating glucose homeostasis [64]. They were also found to be essential for insulin secretion induced by glucose as well as critical for glucose-stimulated insulin secretion, and are involved in glucose uptake. Importantly, impaired K_{ATP} channel genes lead to negative physiological effects, contributing factor to hyperinsulinemia and diabetes [65]. It has been hypothesized that the concomitant injection of a mixture of a non-caloric sweetener, leading to a secondary messenger signaling cascade *via* T1R2/T1R3, and a caloric sweetener such as glucose, which is taken up, metabolized and is promoting the closure of K_{ATP} channels, would have a synergized effect enhancing the sweet taste perception more than either

sweetener taken alone. As GLUTs are co-expressed along with K_{ATP} channels in mouse T1R3-expressing taste cells, sugar uptake by a transporter might lead to an elevation of ATP levels, provoke K_{ATP}, a depolarization of the cells, and a regulation of hormone or neurotransmitter release from the cells, as is the case in insulin-releasing β-cells [63]. In line with rationale, it has been shown that both insulin and glucagon secretion is mediated by K_{ATP} channels [65]. Additionally, data from a study performed in MGN-1 cells suggest that K_{ATP} channels play a role in ghrelin secretion induced by 25 mM D-glucose [66]. Ghrelin secretion is stimulated in presence of 1, 5 or 10 mM glucose in the primary cultures of gastric mucosal cells [67]. Although the role and function of K_{ATP} channels along with GLUTs have been investigated in the GI tract, how and if K_{ATP} channels as well as GLUTs are involved in the mechanisms modulating GAS in presence of NCSs remains elusive. Relevant studies, thus, needed to be carried out.

1.3 5-HT and its key functions in the GI tract

A monoamine neurotransmitter, serotonin (5-HT) is synthesized from the essential amino acid L-tryptophan, which is firstly catalyzed by the rate-limiting enzyme tryptophan hydroxylase (TPH) into L-hydroxytryptophan, then transformed into 5-hydroxytryptamine (5-HT) by the aromatic amino acid decarboxylase (AADC). 5-HT is not able to cross the blood-brain-barrier and is stored in the body of humans and animals. Approximately 80% to 90% of the entire amount of 5-HT in the human body is stored throughout the GI tract, in the granules of enterochromaffin (EC) cells and enteric neurons [68].

Upon vagal stimulation, 5-HT is released into the circulation and the gastric lumen [69]. Another stimulus for 5-HT secretion is intraluminal acidification [70]. 5-HT has an inhibitory effect on GAS [5–9] and regulates gastric motility [10–12]. Gastric emptying in the duodenum is inhibited upon food intake through a feedback mechanism involving 5-HT secretion from neuroendocrine cells that act on the $5\text{-}HT_3$ receptor in the regulation of gastric motility [11]. Furthermore, it has been shown that the activation of the $5\text{-}HT_3$ receptor by its agonist resulted in a delayed gastric acid emptying that elicited a notable augment in cross-sectional region of proximal stomach in human [10].

There is evidence of 5-HT presence in the stomach. Data from a study conducted in male Sprague-Dawley rat's isolated stomach indicate detectable 5-HT levels in various sections of the stomach, and the authors suggest that GAS is modulated by serotonin via 5-HT_3 receptors [9]. Studies on human tissues have also been reported. A study conducted in the stomachs of healthy human donor shows distinct patterns of 5-HT-positive cells in the antrum and proximal stomach [71]. In addition, the occurrence of 5-HT-positive cells in embryonic, fetal and infantile stomach tissue was demonstrated [72]. Importantly, abnormalities in somatostatin, gastrin and 5-HT endocrine cells have been detected in the antrum of the patients diagnosed with irritable bowel syndrome [73].

1.3.1 Mechanisms of 5-HT secretion induced by tastants

NCSs are used in food and beverage products, yet little is known about their impacts on the GI tract, and if they participate in the regulation of the GI symptoms, especially 5-HT secretion. Data from examinations carried out on human EC cells surgically resected from human small intestinal mucosa and neoplastic EC cells (KRJ-I) suggest that the stimulation of normal and neoplastic EC cells with bitter tastant caffeine, as well as with the sweet artificial non-caloric tastant sucralose at various concentrations, activated the release of 5-HT, although neoplastic EC cells respond with dissimilar secretory profiles compared to the normal, non-neoplastic, EC cells [74].

Likewise, D-glucose stimulated the release of 5-HT in a concentration-dependent manner, and this release was significantly diminished by phloridzin in human BON cells derived from EC cells, which do express glucose transporters GLUT1 / GLUT3 [75].

Luminal sweet tastant-stimulation of GPCRs leads to the induction of cAMP/ERK/Ca^{2+} signaling via activation of adenylate cyclase (AC), diacylglycerol (DAG), and inositol trisphosphate (IP_3) through coupling Gαs in EC cells. Transportation of glucose into the cell membrane via glucose transporters (GLUTs) activates ERK phosphorylation (pERK). Gene transcription is induced by augmented cAMP through rate-limiting 5-HT synthesis enzyme tryptophan hydroxylase (TPH), which synthesizes 5-HT, the secretion of which is positively modulated via cAMP-activated PKA and ERK (both participating in the maturation and

translocation of vesical) and *via* augmented intracellular Ca^{2+} (vesicle-membrane docking and exocytosis of 5-HT) (Figure 4) [74].

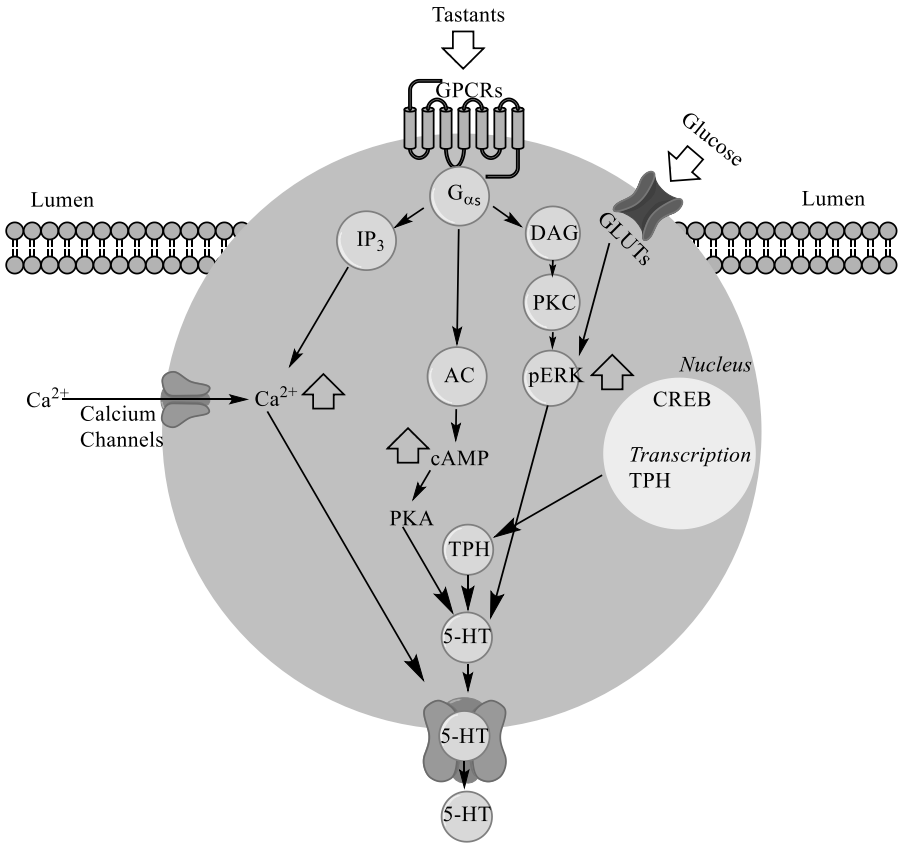

Figure 4. An EC cell model illustrating GPCR-mediated 5-HT secretion induced by tastants (adapted from Kidd et al., 2008 [74]).

In summary, there is only fragmentary evidence in the scientific literature of the functional role of sweet tastants, and especially of NCSs other than sucralose, on the release of 5-HT. In addition, the impact of the NCSs on 5-HT release in parietal cells has not been studied so far. Thus, it seems particularly attractive to shed some light into the NCSs involved in the regulation of 5-HT secretion in parietal cells.

II. Objectives

The impact of bitter-tasting substances on the mechanisms of GAS has been comprehensively examined in the research group of V. Somoza [25–28]. Sweet-tasting substances have, on the other hand, received less attention and their effects on the mechanisms regulating the release of GAS and 5-HT, key modulators of satiety [2, 3], have not yet been studied. In addition, the effect of a stimulation of sweet taste receptors by sweet tastants on the secretion of 5-HT and on the mechanisms modulating GAS has also not been investigated so far. The work presented in the current thesis aimed at investigating the impact of NCSs on 5-HT release and on the mechanisms regulating GAS. An approach of *in vitro* analyses performed in well-established parietal cells in culture (HGT-1 cells) in order to elucidate the underlying mechanisms of action. First, the effect of selected sweet-tasting substances, namely cyclamate, acesulfame K as NCSs and D-threonine as a sweet-tasting amino acid, on proton secretion was examined and the results revealed that cyclamate, acesulfame K, and D-threonine are potent regulators of proton secretion, but their action on proton secretion differed from each other. While cyclamate promoted proton secretion, acesulfame K, and D-threonine had an adverse effect and resulted in inhibition of proton secretion. Detailed examination of the human sweet taste receptors T1R2/T1R3 in HGT-1 cells revealed that the T1R3 site is the major unit of the T1R2/T1R3 couple in HGT-1 cells. Mechanistic studies using siRNA knockdown tests then showed an association between T1R3 receptor activation and proton secretion in HGT-1 cells. Next, an investigation of the 5-HT secretory potentials of HGT-1 cells was conducted, and the results indicate that HGT-1 cells are able to synthesize and release 5-HT. Subsequently, the effect of NCSs cyclamate, acesulfame K, saccharin, sucralose, and NHDC on 5-HT secretion was examined and the results revealed that the selected NCSs are potent stimulators of 5-HT secretion. Subsequent mechanistic studies using siRNA knockdown tests showed that the activation of the T1R3 receptor is involved in 5-HT secretion in HGT-1 cells. Taken together, our studies and the collected data corroborate the hypothesis that NCSs are the proton and 5-HT secretory modulators in the HGT-1 cell line, which is a promising multi-functional cell system for the study of the impact of tastants on the mechanisms of GAS *in vitro*.

III. Results

(1) "Human Sweet Receptor T1R3 is Functional in Human Gastric Parietal Tumor Cells (HGT-1) and Modulates Cyclamate and Acesulfame K-Induced Mechanisms of Gastric Acid Secretion"

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Published in Journal of Agricultural Food Chemistry, 2018, 66, 4842−4852.

This study investigated the effect of cyclamate, acesulfame K and D-threonine on proton secretion from HGT-1 cells. Proton secretion was regulated by cyclamate, acesulfame K and D-threonine, effects of whose were reduced by T1R3 receptor inhibitor lactisole. In addition, the impact of cyclamate and acesulfame K on proton release was decreased in *TAS1R3*-knocked down cells.

I participated in the experimental plan and analyzed the effect of the sweet-tasting substances on proton secretion, cAMP and cell viability in HGT-1 cells. Additionally, I did the immunocytostaining analysis under the supervision of Dr. Maik Behrens in HGT-1 cells. Also, I performed the siRNA knockdown and qPCR experiments of genes relevant for proton secretion. Furthermore, I did the statistical analysis and prepared the manuscript of draft.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 4842−4852

pubs.acs.org/JAFC

Human Sweet Receptor T1R3 is Functional in Human Gastric Parietal Tumor Cells (HGT-1) and Modulates Cyclamate and Acesulfame K-Induced Mechanisms of Gastric Acid Secretion

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Supporting Information

ABSTRACT: The noncaloric sweeteners (NCSs) cyclamate (Cycl) and acesulfame K (AceK) are widely added to foods and beverages. Little is known about their impact on gastric acid secretion (GAS), which is stimulated by dietary protein and bitter-tasting compounds. Since Cycl and AceK have a bitter off taste in addition to their sweet taste, we hypothesized they modulate mechanisms of GAS in human gastric parietal cells (HGT-1). HGT-1 cells were exposed to sweet tastants (50 mM of glucose, D-threonine, Cycl, or AceK) and analyzed for their intracellular pH index (IPX), as an indicator of proton secretion by means of a pH-sensitive dye, and for mRNA levels of GAS-associated genes by RT-qPCR. Since the NCSs act via the sweet taste-sensing receptor T1R2/T1R3, mRNA expression of the corresponding genes was analyzed in addition to immunocytochemical localization of the T1R2 and T1R3 receptor proteins. Exposure of HGT-1 cells to AceK or D-threonine increased the IPX to 0.60 ± 0.05 and 0.80 ± 0.04 ($P \leq 0.05$), respectively, thereby indicating a reduced secretion of protons, whereas Cycl demonstrated the opposite effect with IPX values of −0.69 ± 0.08 ($P \leq 0.05$) compared to controls (IPX = 0). Cotreatment with the T1R3-inhibitor lactisole as well as a *TAS1R3* siRNA knock-down approach reduced the impact of Cycl, AceK, and D-thr on proton release ($P \leq 0.05$), whereas cotreatment with 10 mM glucose enhanced the NCS-induced effect ($P \leq 0.05$). Overall, we demonstrated Cycl and AceK as modulators of proton secretion in HGT-1 cells and identified T1R3 as a key element in this response.

KEYWORDS: *cyclamate, acesulfame K, gastric acid secretion, sweet taste receptor, K_{ATP}-channels, HGT-1 cells*

INTRODUCTION

Noncaloric sweeteners (NCSs) are widely used as sugar substitutes to increase the sweetness of foods and beverages without contributing to their caloric load. NCSs are known agonists of the human sweet taste receptor (T1R2/T1R3), which consists of the subunits T1R2 and T1R3 that respond to a variety of compounds, including natural sugars, sweet-tasting D-amino acids, and artificial sweeteners, such as cyclamate (Cycl) and acesulfame K (AceK).[1,2] Cycl and AceK are classified as high-intensity sweeteners since their sweet taste is 30 and 200 times more intense than that of sucrose, respectively, whereas glucose evokes a 30% less intense sweet sensation compared to sucrose (see review by Edwards et al., 2016).[3] Lactisole, a sweet taste receptor antagonist, inhibits sweet taste by acting on T1R3.[4] Previous studies have been aimed at identifying the yet not fully elucidated binding sites for sweet tastants on the sweet taste receptor. On one hand, it has been reported that Cycl and AceK, e.g., act on the heteromeric T1R2/T1R3.[2,5] On the other hand, it has been hypothesized that the homodimeric T1R3 receptor may respond to sweet-tasting compounds in various cell lines[6−8] and in humans.[9]

Apart from their molecular mechanism of action, the health effects of NCSs beyond their impact on sweet taste perception are also still controversial.[10] One concern is that regular intake of NCSs may stimulate food intake through their impact on the release of orexigenic and anorexigenic peptides in the gastrointestinal tract,[11] thereby contributing to the global increase of obesity and associated degenerative diseases.[10]

From a physiological perspective, the gastrointestinal tract and the central nervous system play a pivotal role in the control of energy homeostasis, not only by secreting hormones regulating the complex cascade of hunger and satiety[12] but also by regulating gastric motility and gastric acid secretion (GAS).[13] Hypersecretion of gastric acid may cause gastric discomfort accompanied by symptoms like epigastric pain, reflux, or heartburn[16] and may result in accelerated gastric emptying which counteracts satiation due to reduced gastric distention.[17] Satiating foods,

Received: February 4, 2018
Revised: April 15, 2018
Accepted: April 17, 2018
Published: April 17, 2018

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

in particular dietary proteins and amino acids,[14] but also endogenous satiating compounds, such as the sugar acid 2-buten-4-olide,[15] have been shown to suppress GAS via reduction of activity of the vagus nerve and gastric-related hypothalamic neurons. However, the effects of NCSs on mechanisms regulating gastric acid release still have to be elucidated.

One cell model to study the mechanisms of GAS *in vitro* is the HGT-1 cell line, established from the primary tumor of a 60-year-old patient[18] and known to express the principal transport proteins facilitating GAS.[19] Recently, HGT-1 cells have been verified as a suitable model for the identification of bitter and bitter-modulating compounds,[20] showing a high correlation between the proton secretory potential of a bitter compound and its sensory bitter perception,[20] whereas the associations between sweet-tasting substances and proton release have not been studied. Since some NCSs show a bitter off taste, we hypothesized that NCSs modulate mechanisms of GAS in HGT-1 cells and aimed at elucidating whether the sweet taste sensing receptor proteins (T1R2/T1R3) are involved in this response. Following this hypothesis, we investigated the proton secretory potentials of NCS Cycl, AceK, and the sweet-tasting amino acid D-thr in HGT-1 cells and studied the impact of the T1R3 antagonist lactisole and a *TAS1R3*-siRNA knock down. Moreover, involvement of cAMP and glucose in the regulation of NCS-stimulated proton secretion as well as the regulation of gastric acid associated genes, the H^+/K^+-ATPase alpha-subunit (*ATP4A*), the histamine H2 receptor (*HRH2*), the somatostatin receptor (*SSTR2*), the acetylcholine receptor M3 (*CHRM3*), and the identified bitter taste receptors of impact *TAS2R1*, *TAS2R38*, *TAS2R31*, and *TAS2R43*[21] were also studied.

MATERIALS AND METHODS

Chemicals. All the chemicals, including sodium cyclamate, acesulfame potassium, D-threonine, and histamine, were purchased from Sigma-Aldrich (Austria), unless stated otherwise. GIV3727 and lactisole (99%) were provided by Symrise (Germany). Substances insoluble in water were dissolved in 0.1% (v/v) DMSO.

Cell Culture. The human gastric cells HGT-1 (passage number 56) were obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France) and were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 4 g/L of glucose, supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin/streptomycin and cultivated applying standard conditions of 37 °C temperature in a humidified atmosphere of 95% air with 5% CO_2, as described previously.[22]

Cell Viability. Analysis of cytotoxic effects of the selected compounds was performed by staining the cells with 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reagent, as described earlier.[22] Briefly, cells were seeded in a 96-well culture plate 24 h before use. Cells were treated with the test compounds dissolved in DMEM for up to 60 min. Absorbance was measured at 550 nm and the reference wavelength at 690 nm by means of an Infinite 200 Pro Plate Reader (Infinite M200 Plate Reader, Tecan, Switzerland). As a consequence, the cell viability was determined in relation to the medium-only treated control cells (untreated controls = 100%). Only the treatments exceeding 90% viability in comparison to untreated control were used.

Intracellular pH Measurement in HGT-1 Cells. The intracellular pH in HGT-1 cells was quantitated as proxy for proton secretion using the fluorescent pH indicator 1,5-carboxy-seminaphtorhodafluor acetoxymethyl ester SNARF-1-AM (SNARF, Invitrogen, Austria).[20−26] This assay in HGT-1 cells has been described previously.[21−24] Briefly, 1×10^5 HGT-1 cells were seeded in a black 96-well plate. After 24 h, cells were washed once with Krebs−Ringer-HEPES−buffer (KRHB; 10 mM HEPES, 4.7 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4, adjusted to a pH of 7.4 with 5 M KOH) and stained with 3 μM SNARF-1 in KRHB for 35 min under standard conditions. Later cells were treated for 10 min with test substances dissolved in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g L^{-1} of glucose. For the experiments investigating the effect of glucose, substances were dissolved in non-nutritive DMEM. Histamine (1 mM) was used as a positive control. Control cells were treated with DMEM only. Additionally, DMEM containing 0.1% DMSO was used as solvent control. Fluorescence was measured at 580 and 640 nm emission after excitation at 488 nm by means of an Infinite 200 Pro plate reader. After analyzing the intracellular pH values using a calibration curve, the concentration of intracellular H^+ was calculated. The ratio between treated samples and control cells (DMEM only or DMEM containing 0.1% DMSO) was calculated. After $\log_2$ conversion, the intracellular proton index (IPX) was calculated. The lower the IPX values, the more H^+ ions have been secreted by the HGT-1 cells.[20−24]

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). HGT-1 cells, at a density of 7×10^5, were seeded in 6-well culture plates 24 h before the experiment. Cells were washed once with phosphate-buffered saline (PBS) and treated with 50 mM Cycl, AceK, D-thr, glucose, and 10 μM forskolin or 0.1% DMSO (control) for 10, 20, or 60 min under the standard conditions described above. After washing with ice-cold PBS on ice, cells were lysed, and total RNA content was collected using the peqGOLD Total RNA Isolation Kit (Peqlab) following the manufacturer's protocol. RNA quantity and quality were spectrophotometrically analyzed at 260 and 280 nm, followed by calculating the ratio of the two wavelengths using the NanoQuant Plate by means of the Infinite 200 PRO Plate Reader. Isolated RNA samples showed a ratio ranging between 1.8 and 2.2. A total of 2 μg of RNA was used for transcription into cDNA by applying the High Capacity cDNA Kit (Applied Biosystems, Austria) according to the manufacturer's protocol. Real-time PCR was performed with 100 ng of cDNA amplified with Fast SYBR Green Master Mix (Applied Biosystems, Austria). The sequences of the reverse and forward primers are shown in Table 1. Primer pairs for human *TAS1R1* (Assay ID: qHsaCID0013443), human *TAS1R2* (Assay ID: qHsaCID0016106), and human *TAS1R3* (qHsaCED0002321) were purchased from BIO-RAD (Austria). The remaining sequences were designed with NCBI Primer Blast, and the following oligonucleotide primers for human *SUR1*, *Kv11.1*, and *SLC2A1* (encoding GLUT1) were purchased from Sigma: for human *SUR1*, 5′-GCTGTCCAAAGGCACCTACT-3′ (forward) and 5′-TGAATGTCCTTCCGCACCTG-3′ (reverse); for human *Kv11.1*, 5′-CACCTTCCTGGACACCATCA-3′ (forward) and 5′-AAGCCGTCGTTGCAGTAGAT-3′ (reverse); for human *SLC2A1*, 5′-ATTGGCTCCGGTATCGTCAAC-3′ (forward) and 5′-GCTCAGATAGGACATCCAGGGTA-3′ (reverse). Analyses were carried out in triplicate by means of the StepOnePlus Real-Time PCR System (Applied Biosystems, Austria). The measured mRNAs (N_0 values) and efficiencies were calculated for each reaction setup with LinRegPCR v.12.8. Results of target genes were normalized to the geometric mean of two internal standard genes TATA-box binding protein (*TBT*) and peptidylprolyl isomerase A (*PPIA*).[22] The effects of treatments on gene expression were analyzed in comparison to non-treated control cells. Cycling conditions in the system were: 20 s/93 °C (activation), 3 s/95 °C (denaturation), 30 s/60 °C (annealing), and 15 s/72 °C (elongation).

Immunocytochemical Staining of HEK-293T-Gα16gust44 and HGT-1 Cells. Transient transfection of HEK-293T-Gα16gust44 cells was conducted as described before.[27] Briefly, HEK-293T-Gα16gust44 cells were seeded onto glass coverslips in 24-well plates 24 h before transiently transfecting the cells by using Lipofectamine 2000 (Invitrogen, Germany) with cDNA coding for human sweet receptor subunits *TAS1R2* and *TAS1R3*. HGT-1 cells were seeded onto coverslips 24 h before the immunocytochemical detection. Cells were fixed with methanol:acetone (1:1, v/v) and stained as described previously,[27] using antibodies against the C-terminal epitope tags (anti-FLAG antiserum for T1R2 (1:2000) and anti-HSV antiserum for T1R3 (1:15000) (Novagen, Germany)), goat anti-T1R2 (1:100), and rabbit anti-T1R3 (1:100) (Santa Cruz Biotechnology, USA). Detection was performed as reported previously,[27] and preabsorption of the anti-T1R2 antibody with the specific blocking peptide (1:100)

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

Table 1. Sequences of All Primer Pairs Used in the RT-qPCR Experiments[21,23,24a]

target	amplicon size	5′ → 3′ sequence	target	amplicon size	5′ → 3′ sequence
PPIA	144	F:CCACCAGATCATTCCTTCTGTAGC	*TAS2R1*	172	F:AAATGGCTCCGCTGGATCTC
		R:CTGCAATCCAGCTAGGCATGG			R:GTGGCAAGCCAAAGTTCCAA
TBT	130	F:CCCGAAACGCCGAATATAATCC	*TAS2R38*	216	F:CCCAGCCTGGAGGCCCACATT
		R:GACTGTTCTTCACTCTTGGCTC			R:TCACAGCTCTCCTCAACTTGGCA
ATP4A	176	F:CGGCCAGGAGTGGACATTCG	*TAS2R31*	218	F:TTGAGGAGTGCAGTGTACCTTTC
		R:ACACGATGGCGATCACCAGG			R:ACGGCACATAACAAGAGGAAAA
CHRM3	117	F:AGCAGCAGTGACAGTTGGAAC	*TAS2R43*	148	F:ATATCTGGGCAGTGATCAACC
		R:CTTGAGCACGATGGAGTAGATGG			R:CCCAACAACATCACCAGAATGAC
HRH2	154	F:TGGGAGCAGAGAAGAAGCAACC			
		R:GATGAGGATGAGGACCGCAAGG			
SSTR2	189	F:TCCTCCGCTATGCCAAGATGAAG			
		R:AGATGCTGGTGAACTGATTGATGC			

[a]F: Forward, R: Reverse.

(Santa Cruz Biotechnology) was carried out. The FLAG and HSV epitopes were detected by means of IgG-conjugated antimouse and antigoat antibodies (1:2000) (Molecular Probes, USA), respectively, biotin-conjugated concanavalin A (1:2000) with Streptavidin Alexa Fluor 633 (1:1000) (Molecular Probes), T1R2 with Alexa Flour 488 donkey antigoat IgG (1:2000) (Molecular Probes), and T1R3 with Alexa Fluor 488 goat antirabbit IgG (1:2000) (Molecular Probes). To visualize the nuclei, cells were stained with DAPI (4′,6-diamidino-2-phenylindole). Finally, immunofluorescent pictures were taken by confocal laser-scanning microscopy (Leica TCS-SP8, Germany).

Small Interfering RNA (siRNA) Knockdown of *TAS1R3* Gene Expression in HGT-1 Cells. *TAS1R3* expression levels were reduced in HGT-1 cells by treatment with siRNA. Gene-specific siRNA targeting of *TAS1R3* (5′-GCCUGAAGAUCCGCUGGCA-3′) was purchased from Sigma (Austria). 3×10^4 cells were seeded 24 h prior to transfection with the HiPerFect Transfection Reagent (Qiagen, Austria) using mock transfection or a transfection reagent containing either 1−10 nM *TAS1R3* siRNA (toxicity was excluded by MTT) or All-stars Negative Control siRNA (Qiagen, Austria), according to the manufacturer's protocol (Qiagen). Transfections were performed at 37 °C, 5% CO_2, in a humidified atmosphere. After 48 h transient transfection of *TAS1R3* siRNA, the knockdown efficiencies were assessed by means of RT-qPCR (Supporting Information Figure 1).

cAMP Measurement. Cells were seeded in 96-well plates at a density of 1×10^5 per well under standard conditions 24 h before the experiment. Prior to performing the experiment, the culture medium was discarded, washed once with PBS, and treated with test substances dissolved in KRHB or KRHB containing 0.1% DMSO under standard conditions at 37 °C in a humidified atmosphere of 95% air with 5% CO_2 for 10 min. Cyclic AMP levels were determined by means of a cAMP assay kit, cAMP-Glo (Promega, Germany). The assay was performed according to the manufacturer's instructions. Cyclic AMP values were calculated from the external standard curve.

Statistical Analysis. Sigma Plot software 11.0 (Sytat Software) was used for all statistical analysis. Data are shown as the mean ± standard error of the mean (SEM). A minimum of three biological and two technical replicates were conducted in all cell culture experiments. The statistical analyses are stated in the legend of the corresponding table or figure, applying a one-way ANOVA Holm-Sidak post hoc test for comparison between control and various concentrations of the tested substance, and two-way ANOVA for comparison between control and time-dependent treatments. Treated overcontrol values are demonstrated as T/C (C = 100%). P-values ≤0.05 were considered as statistically significant.

RESULTS

Effects of Cycl, AceK, and D-Thr on H^+ Release in HGT-1 Cells. Treatment of HGT-1 cells with Cycl, AceK, or D-thr in a concentration of 50 mM for 10 min resulted in a stimulation of proton secretion, as shown by reduced IPX values of −0.69 ± 0.08 (Cycl) or an inhibition as shown by increased IPX values 0.60 ± 0.05 (AceK) and 0.80 ± 0.04 (D-thr) in relation to nontreated control cells (0.02 ± 0.08) ($P \leq 0.05$, Figure 1).

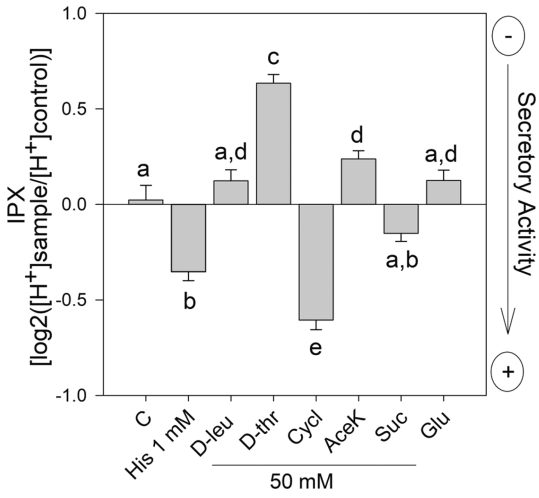

Figure 1. IPX after treatment of the cells with positive control histamine (His, 1 mM), D-leucine (D-leu, 50 mM), D-threonine (D-thr, 50 mM), cyclamate (Cycl, 50 mM), acesulfame K (AceK, 50 mM), sucrose (Suc, 50 mM), or glucose (Glu, 50 mM) for 10 min. Results are displayed as IPX and illustrated in comparison to untreated cells (Control, C) as the mean ± SEM, n = 3−4; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significance differences are marked with the letters, $P \leq 0.05$).

Treatment with 50 mM D-leucine (D-leu), sucrose (Suc), and glucose did not affect the intracellular pH ($P > 0.05$, Figure 1). Concentration-dependent treatments revealed the highest concentration (50 mM) of test compounds to elicit the highest impact on proton release in HGT-1 cells (Figure 2).

Impact of Glucose, Cycl, AceK, D-Thr, and Forskolin on mRNA Expression of Targeted Genes Involved in Mechanisms Regulating Proton Secretion in HGT-1 Cells. The cDNA transcripts generated from HGT-1 RNA by gene-specific oligonucleotide primers for *TAS1R1*, *TAS1R2*, and *TAS1R3*, as well as the primers mentioned in Table 1, were amplified by means of RT-qPCR.

Glucose had no impact on the targeted genes of interest ($P > 0.05$, Table 2). Apart from *SSTR2*, *ATP4A*, and *TAS1R1* ($P > 0.05$), Cycl induced the mRNA expression of *TAS1R3* and *HRH2* at all three treatment times, whereas *CHRM3* mRNA was reduced after 10 min of Cycl exposure ($P \leq 0.05$, Table 2). Cycl and AceK are known to have a bitter aftertaste in addition to their

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

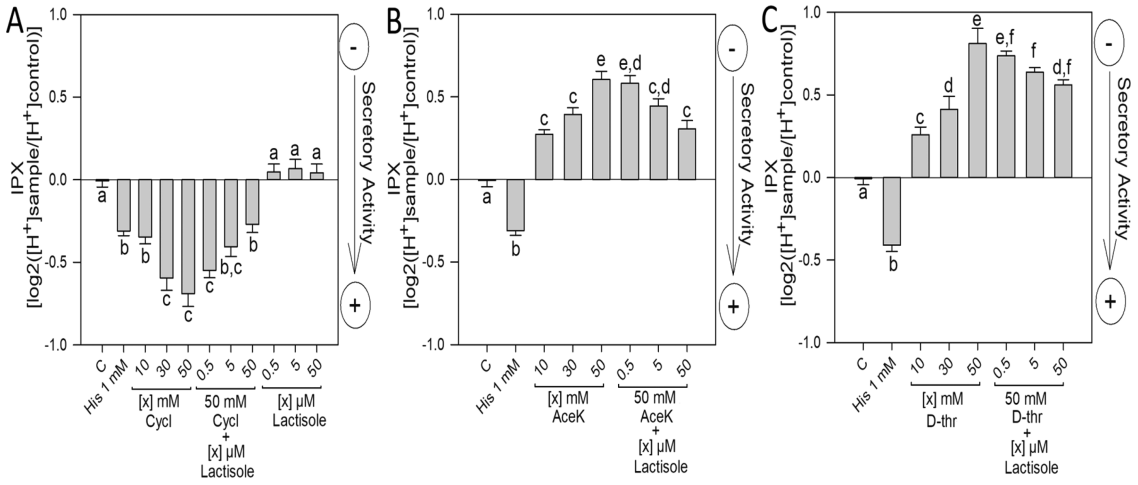

Figure 2. IPX of HGT-1 cells after treatment with (A) cyclamate (Cycl, 50 mM), in the presence or absence of lactisole, or lactisole alone, (B) acesulfame K (AceK, 50 mM), in the presence or absence of lactisole, and (C) D-threonine (D-thr, 50 mM), in the presence or absence of lactisole for 10 min. Findings are depicted as IPX and demonstrated in comparison to the untreated cells (Control, C) as the mean ± SEM, n = 3−4; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are shown with the letters, $P \leq 0.05$).

sweet-tasting character. The identified bitter taste responsive receptors for Cycl are T2R1 and T2R38,[28] while those are T2R31 and T2R43 for AceK.[29] We, therefore, studied the potential involvement of these respective T2Rs in proton release. Cycl down-regulated mRNA expression of the bitter receptors *TAS2R1* and *TAS2R38* after 20 and 60 min exposure, respectively ($P \leq 0.05$, Table 2). Treatment of the cells with AceK also regulated *ATP4A*, *HRH2*, *SSTR2*, *TAS1R1*, *TAS1R3*, *TAS2R31*, and *TAS2R43* expression ($P \leq 0.05$, Table 2). Apart from *ATP4A*, D-thr also demonstrated an impact on the targeted genes at various times of treatment ($P \leq 0.05$, Table 2). Likewise, except for *SSTR2*, forskolin caused changes of the mRNA expression level of the genes shown in Table 2. Notably, forskolin (10 µM), dissolved in 0.1% DMSO (v/v), did not show a regulative effect when compared to cells treated with the solvent control, consisting of cell culture media with 0.1% DMSO (v/v) only (data not shown, $P > 0.05$). HGT-1 gene expression of *TAS1R2* was below the limit of detection, with ΔcT_{TAS1R2} values above 35 (RT-qPCR amplification, running 40 cycles; N_o values of *TAS1R2* calculated from LinRegPCR v.12.8 were normalized over the N_o values of *PPIA*; $3.6 \times 10^{-3} \pm 5.1 \times 10^{-4}$ (as the mean ± SEM, n = 3; tr = 3)).

Immunocytochemical Detection of the T1R2 and T1R3 Receptor Proteins in HGT-1 Cells. To test the specificity of the antibodies directed against T1R2 or T1R3 receptor proteins, HEK 293T-Gα16gust44 cells were transiently transfected with cDNA of *TAS1R2* and *TAS1R3* (Figure 3). Antibodies (FLAG-tag, green) selective for the T1R2 receptor protein (red) and (HSV-tag, green) the T1R3 receptor protein (red) were used to stain transfected HEK 293T-Gα16gust44 cells (Figure 3). Both T1R2 and T1R3 receptor proteins were detected by using the anti-FLAG and anti-HSV antisera indicating the specificity of the antisera (Figure 3). Subsequently, HGT-1 cells were analyzed with the validated antiseras against T1R2 and T1R3 receptor protein (green) and concanavalin A (ConA, red) directed toward cell surface glycoproteins. Signals gained for T1R2 were relatively weak in comparison to that for T1R3 (Figure 3, green). In addition, preincubation with the specific T1R2/T1R3 antisera reduced the staining signals (Figure 3).

Impact of Lactisole on Cycl- and AceK-Induced Proton Release in HGT-1 Cells. IPX values were analyzed after treating the cells with Cycl, AceK, or D-thr in the presence or absence of T1R3 antagonist lactisole.

Coincubation of 50 mM Cycl, AceK, D-thr, and 50 µM lactisole for 10 min most effectively reduced the effects of Cycl, AceK, and D-thr, revealing IPX values of −0.27 ± 0.05; −0.69 ± 0.08, 0.30 ± 0.05; 0.60 ± 0.05, and 0.56 ± 0.06; 0.80 ± 0.04, respectively, in relation to control cells (0.02 ± 0.08) ($P \leq 0.05$, Figure 2). Treatment of the HGT-1 cells with lactisole alone showed no significant effect on proton release ($P > 0.05$, Figure 2A).

Impact of Cycl and AceK on Proton Release in *TAS1R3* Knock-Down HGT-1 Cells. In order to clarify the involvement of T1R3 in Cycl- and AceK-induced changes in proton secretion, the mRNA expression of *TAS1R3* was knocked down by treatment of the cells with *TAS1R3* siRNA (10 nM). Mean knock-down efficiency, assessed by means of RT-qPCR, was 51.0 ± 3.1% (Supporting Information, Figure 1). Moreover, the effects of Cycl and AceK on proton release were reduced by 39.0 ± 5.1% and 51 ± 3.8% in *TAS1R3* knocked-down cells compared to mock transfected, nontargeted control cells ($P \leq 0.05$, Figure 4).

Role of cAMP in Cycl, AceK, and D-Thr-Induced Changes in Proton Release in HGT-1 Cells. In order to determine the role of T1R3 downstream signaling on Cycl-, AceK-, and D-thr-induced changes in proton secretion, and to elucidate whether these effects are mediated via elevated $[cAMP]_i$, the cAMP activator forskolin and its inhibitor NKY80 were used. Treatment of HGT-1 cells with 10 µM forskolin for different time points from 10 to 55 min resulted in a stimulation of proton secretion ($P \leq 0.05$), which was decreased by 3 µM NKY80 as indicated by $AUC_{10-55\ min}$ values (n = 4; tr = 5 statistics: one-way ANOVA Holm-Sidak post hoc test, $P \leq 0.05$, data not shown). HGT-1 exposure to NKY80 and 0.1% DMSO had no effect on proton release in HGT-1 cells ($P > 0.05$, data not shown).

Treatment of the cells with 50 mM Cycl, AceK, or D-thr in the presence of 3 µM NKY80 reduced the effect of Cycl and AceK ($P \leq 0.05$, Figure 5), whereas for D-thr, the level of a statistical significant difference was not reached ($P = 0.127$, Figure 5).

Impact of Cycl, AceK, Glucose, and Forskolin on the Intracellular cAMP Concentration ($[cAMP]_i$) in HGT-1 Cells. Apart from glucose, Cycl, AceK, and forskolin induced an increase in $[cAMP]_I$, which was reduced by treatment of the cells

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

Table 2. RT-qPCR Results of HGT-1 Cells Treated with 50 mM Glucose, Cyclamate, Acesulfame K, D-Threonine, or 10 μM Forskolin[a]

target	treatment	10 min	20 min	60 min
ATP4A	glucose	1.12 ± 0.07	1.20 ± 0.08	0.97 ± 0.09
	DMSO	1.00 ± 0.07	0.90 ± 0.04	0.96 ± 0.08
	cyclamate	1.00 ± 0.04	1.06 ± 0.05	1.06 ± 0.09
	acesulfame K	**0.80 ± 0.04***	**1.31 ± 0.08***	0.88 ± 0.07
	D-threonine	1.00 ± 0.08	1.10 ± 0.05	0.93 ± 0.03
	forskolin	0.96 ± 0.08	**0.75 ± 0.02***	1.04 ± 0.05
HRH2	glucose	0.88 ± 0.10	0.96 ± 0.06	0.89 ± 0.03
	DMSO	0.87 ± 0.08	0.84 ± 0.04	0.97 ± 0.05
	cyclamate	**1.11 ± 0.03***	**1.14 ± 0.08***	**1.20 ± 0.10***
	acesulfame K	1.07 ± 0.10	**1.21 ± 0.05***	1.15 ± 0.11
	D-threonine	**1.24 ± 0.07***	**1.24 ± 0.05***	1.08 ± 0.08
	forskolin	0.97 ± 0.04	**0.80 ± 0.03***	**1.10 ± 0.05***
CHRM3	glucose	1.11 ± 0.06	0.93 ± 0.09	0.86 ± 0.04
	DMSO	1.10 ± 0.06	1.01 ± 0.05	0.90 ± 0.06
	cyclamate	**0.73 ± 0.09***	1.01 ± 0.23	0.96 ± 0.23
	acesulfame K	**0.65 ± 0.07***	**1.77 ± 0.06***	0.87 ± 0.09
	D-threonine	**0.70 ± 0.05***	1.22 ± 0.20	1.30 ± 0.36
	forskolin	**1.51 ± 0.08***	1.10 ± 0.10	**0.64 ± 0.14***
SSTR2	glucose	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
	DMSO	1.03 ± 0.02	1.05 ± 0.02	0.94 ± 0.02
	cyclamate	0.98 ± 0.04	1.01 ± 0.05	0.98 ± 0.02
	acesulfame K	0.92 ± 0.03	**0.88 ± 0.04***	1.03 ± 0.04
	D-threonine	**0.81 ± 0.03***	0.96 ± 0.03	1.02 ± 0.01
	forskolin	1.02 ± 0.03	1.02 ± 0.02	1.02 ± 0.04
TAS1R1	glucose	0.89 ± 0.07	1.11 ± 0.10	1.10 ± 0.11
	cyclamate	1.16 ± 0.06	0.90 ± 0.03	0.98 ± 0.07
	acesulfame K	1.13 ± 0.06	**0.71 ± 0.14***	1.12 ± 0.12
	D-threonine	1.12 ± 0.06	**0.70 ± 0.13***	0.98 ± 0.07
TAS1R3	glucose	1.15 ± 0.10	1.19 ± 0.06	1.19 ± 0.22
	cyclamate	**1.34 ± 0.15***	**1.45 ± 0.16***	**1.51 ± 0.20***
	acesulfame K	**0.74 ± 0.08***	**1.34 ± 0.08***	**1.52 ± 0.18***
	D-threonine	1.05 ± 0.06	**1.35 ± 0.10**	1.40 ± 0.25
TAS2R1	cyclamate	0.87 ± 0.20	**0.65 ± 0.11***	0.67 ± 0.23
TAS2R38	cyclamate	0.99 ± 0.19	0.55 ± 0.18	**0.36 ± 0.07***
TAS2R31	acesulfame K	0.96 ± 0.06	0.88 ± 0.10	0.96 ± 0.04
TAS2R43	acesulfame K	1.07 ± 0.10	1.03 ± 0.06	**1.32 ± 0.06***

[a]Data are indicated as average ± SEM in comparison to untreated control (set to 1), and forskolin results were analyzed over DMSO (0.1%, v/v) treatment. Calculations were made from at least three biological replicates. Statistics; two-way ANOVA Holm-Sidak post hoc test; significant difference between treatments over control are depicted as *; $P \leq 0.05$.

with the adenylate cyclase inhibitor NKY80. In addition, lactisole attenuated the impact of Cycl and AceK on $[cAMP]_i$ ($P \leq 0.05$, Table 3), whereas neither lactisole nor NKY80 demonstrated a significant effect on $[cAMP]_i$ ($P > 0.05$, Table 3).

Impact of Glucose on Cycl- and AceK-Induced Proton Release in HGT-1 Cells. In order to further elucidate the mechanisms of T1R2/T1R3, we studied caloric sweetener glucose in NCS-induced proton release. Cells treated with 50 mM Cycl and 5 or 10 mM glucose for 10 min showed IPX values of −0.24 ± 0.07, −0.35 ± 0.06, and −0.46 ± 0.04, respectively, in relation to the control cells (0.05 ± 0.05) ($P \leq 0.05$, Figure 6A). Cotreatment with 50 mM AceK and 5 or 10 mM glucose resulted in IPX values of 0.52 ± 0.13, 0.70 ± 0.19, and 0.84 ± 0.08, respectively, in relation to the control cells (0.09 ± 0.21) ($P \leq 0.05$, Figure 6B). The effect of 10 mM glucose coincubated with 50 mM Cycl or AceK was significantly reduced by addition of 30 μM glibenclamide, a K_{ATP} inhibitor, resulting in IPX values of −0.18 ± 0.06; −0.46 ± 0.04, and 0.51 ± 0.09; 0.84 ± 0.08, respectively, compared to nontreated control cells (0.05 ± 0.05 and 0.09 ± 0.21, respectively) ($P \leq 0.05$, Figure 6A, 6B). Cotreatment of 1 mM histamine and with 10 mM glucose resulted in an IPX value of −0.80 ± 0.05 in relation to the control cells (−0.05 ± 0.03) ($P \leq 0.05$, Figure 6C). Glucose did not change the histamine-induced proton secretion ($P > 0.05$, Figure 6C). Histamine-induced proton release was suppressed by glibenclamide with IPX values of −0.80 ± 0.05 and −0.36 ± 0.06 in relation to the control cells (−0.05 ± 0.03) ($P \leq 0.05$, Figure 6C). Additionally, 30 μM glibenclamide alone inhibited the H^+ release, with an IPX value of 0.18 ± 0.04 in relation to the control cells (−0.05 ± 0.03) ($P \leq 0.05$, Figure 6).

Impact of Glucose, Cycl, and AceK on mRNA Expression of *SUR1*, *Kv11.1*, and *SLC2A1* in HGT-1 Cells. After exposing the cells to 50 mM glucose, Cycl, or AceK for 10, 20, and 60 min, mRNA expression profiles of *SUR1*, *Kv11.1*, and *SLC2A1* were measured by means of RT-qPCR.

Glucose did not show any effect on the expression level of all targeted genes ($P > 0.05$, Table 4), except for *SLC2A1* at the time points of 10 and 20 min ($P \leq 0.05$, Table 4). Cycl revealed a

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

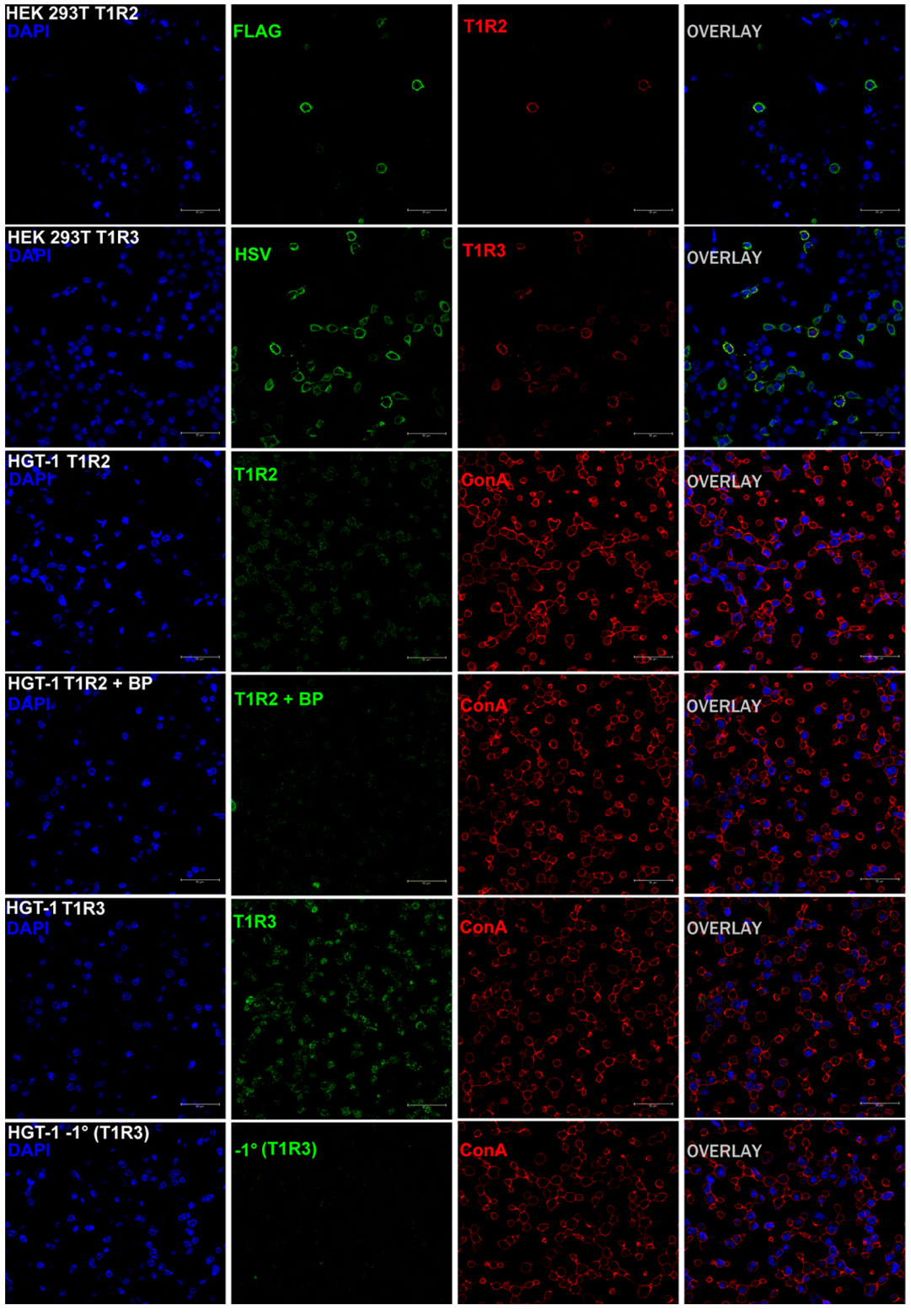

Figure 3. Specific immunostaining of the transiently transfected HEK 293T-Gα16gust44 cells expressing human T1R2 and T1R3 is exhibited by the epitope (green) tagged specific anti-T1R2 and -T1R3 antibodies (red). Nuclei of the cells are visualized with DAPI (blue). In HGT-1 cells, T1R2 and T1R3 receptor proteins are visualized by the anti-TAS1R2 and -TAS1R3 antiserums (green). Anti-T1R2 antibody preincubated with the specific blocking peptide (T1R2+BP). As a negative control for the anti-T1R3 staining, −1° (T1R3) represents the treatment of HGT-1 cells without anti-T1R3 antibody. Cell surface labeling is demonstrated with concanavalin A (ConA, red). Scale bar: 50 μm.

regulatory influence on the mRNA expression level of *SUR1*, *Kv11.1*, and *SLC2A1* at the time points of 10, 10, and 60 min, respectively ($P \leq 0.05$, Table 4). AceK most effectively regulated *SUR1*, *Kv11.1*, and *SLC2A1* ($P \leq 0.05$, Table 4).

DISCUSSION

The secretion of gastric acid is essential for protein digestion and absorption of calcium, iron, and vitamin B12, preventing bacterial overgrowth and enteric infections, and helping to reduce or

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J. Agric. Food Chem. 2018, 66, 4842−4852

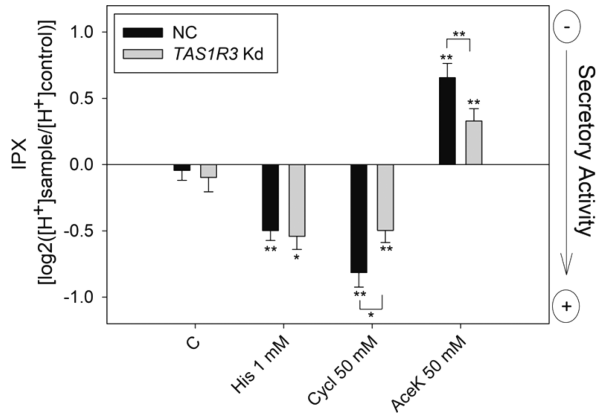

Figure 4. Effect of Cycl and AceK on H^+ release in *TAS1R3* knocked-down cells (*TAS1R3* kd). *TAS1R3* was knocked down 51.0 ± 3.1% by using siRNA (10 nM). IPX of HGT-1 cells transfected with non-targeting negative control siRNA (NC) or HGT-1 cells with knockdown of *TAS1R3* by siRNA, treated with histamine (His, 1 mM), cyclamate (Cycl, 50 mM), or acesulfame K (AceK, 50 mM) for 10 min. Results are calculated in comparison to nontargeting negative control siRNA (C, NC) as the mean ± SEM, $n = 3$; tr = 5−6. (Statistics: Student's *t* test; significant differences are expressed with **; $P \leq 0.001$,*; $P \leq 0.05$).

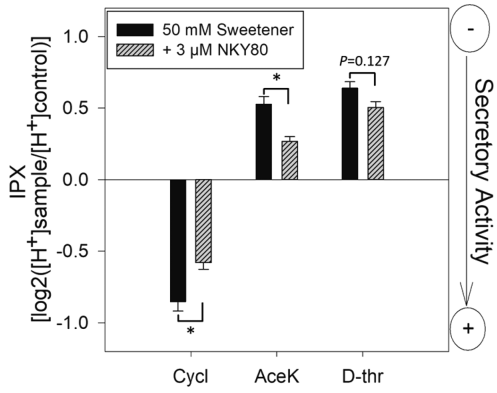

Figure 5. IPX of HGT-1 cells after coincubation of cyclamate (Cycl, 50 mM), acesulfame K (AceK, 50 mM), D-threonine (D-thr, 50 mM), and NKY80 for 10 min. Findings are exhibited as IPX and demonstrated in comparison to untreated cells (control cells, set to 0) or 0.1% DMSO-treated control cells (solvent control for NKY80, set to 0) as the mean ± SEM, $n = 3$−4; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are expressed with *, $P \leq 0.05$).

eliminate the allergenicity of foods and is among the key factors regulating food intake. The control of acid secretion is accomplished by a complex interaction of neurocrine, paracrine, and hormonal pathways.[30] One of our recent studies has demonstrated that activation of oral and gastric bitter receptors by caffeine stimulates the secretion of gastric acid in healthy subjects and that this effect is reduced by the bitter masking compound homoeriodictyol. In vitro studies using a CRISPR-Cas9 approach in HGT-1 cells demonstrated a key role for the bitter receptor *TAS2R43* targeted by caffeine and homoreiodictyol, respectively.[21] Apart from its reducing effects on caffeine-stimulated GAS, we also showed that the bitter-masking/bitter receptor (T2R43) inhibiting compound homoeriodictyol increased food intake in healthy subjects when administred as a bolus of 30 mg.[31]

The impact of NCS on food intake and mechanisms regulating satiety is still controversial. In addition, how NCS affects GAS is unknown. Here, we demonstrate that Cycl and AceK are modulators of GAS in human parietal cells (HGT-1 cells).

Table 3. cAMP Formation after Treatment with Cyclamate, Acesulfame K, Glucose, or Forskolin in the Presence and Absence of NKY80 or Lactisole for 10 min[a]

		NKY80	Lactisole
		3 µM	50 µM
control	100 ± 2.4[a]		94.3 ± 6.4[a]
cyclamate 50 mM	181 ± 9.6[b]	128 ± 5.4[c]	151 ± 3.3[d]
acesulfame K 50 mM	195 ± 8.7[b]	113 ± 2.8[c]	154 ± 6.7[d]
glucose 50 mM	110 ± 8.1[a]	105 ± 3.2[a]	107 ± 6.1[a]
0.1% DMSO control	100 ± 3.5[a]	87.2 ± 5.6[a]	
forskolin 10 µM	266 ± 38[b]	136 ± 8.2[c]	

[a]Data are displayed as [%T/C], average ± SEM in relation to control (set to 100%, marked with "a") or control containing 0.1% DMSO for forskolin and NKY80 (set to 100%), $n = 3$; tr = 2. Statistics: one-way ANOVA followed by Holm−Sidak post hoc test was employed, and significant differences between treatment over control are calculated and compared to their respective measurement and marked with letters; $P \leq 0.05$.

HGT-1 cells have been established as an in vitro model for studying mechanisms of GAS.[18−24,32,33] These cells express all functional proteins responsible for proton secretion[19] which is analyzed by quantitating the intracellular concentration of protons using a fluorescent dye that shows a characteristic pH-dependent fluorescence.

In this mechanistic study, treatment of HGT-1 cells with 50 mM Cycl increased proton secretion, whereas the same concentrations of AceK and D-thr showed a reducing effect compared to untreated control cells. At low concentrations (≤1 mM), neither NCS evoked proton release in HGT-1 cells (data not shown). RT-qPCR analyses demonstrated a regulation of mRNA expression of prosecretory gene encoding for the H^+/K^+-ATPase (*ATP4A*), the histamine (*HRH2*), and the acetylcholine (*CHRM3*) receptor and the antisecretory somatostatin receptor (*SSTR2*) for Cycl, AceK, and D-Thr, whereas treatment of HGT-1 cells with glucose had no effect, either on the mRNA expression of the selected genes or on proton secretion. Coincubations of Cycl and AceK with lactisole, a known antagonist for T1R3,[4] diminished the effect of both NCS compounds. To clarify whether the T1R2/T1R3 is involved in this response, mRNA expression of *TAS1R2* and *TAS1R3* as elements of the heterodimer sweet taste receptor[2] were analyzed by RT-qPCR. Whereas the mRNA level of *TAS1R2* was below the limit of quantification, Cycl and AceK regulated the mRNA expression level of *TAS1R3*. Overall, a down-regulation or an up-regulation of a prosecretory gene does not necessarily result in a decreased proton release or vice versa, although it provides some evidence that these proteins play a role in the cellular response. To follow up on this hypothesis, protein expression of T1R2 and T1R3 in HGT-1 cells was analyzed by immunocytochemistry, using commercially available antibodies which were validated in transfected HEK-293T cells. Staining signals for T1R3 dominated over those for T1R2. Here, we hypothesize that the structure of the T1R2/T1R3 heterodimer in parietal cells is different in the tongue taste cells[2] and in HGT-1 cells, although data accurately characterizing the protein structure are lacking. Thus, we cannot exclude that the receptor in HGT-1 cells is not a heterodimer but rather a T1R3 homodimer. This might be an actual possibility in view of the fact that such homodimers have been found and recognize sugars, as demonstrated for insulating secreting-pancreatic β-cells,[6] adipocytes 3T3-L1 cells,[7] and GLP-1-secreting Hutu-80 cells.[8] On the other hand, another possibility is that HGT-1

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

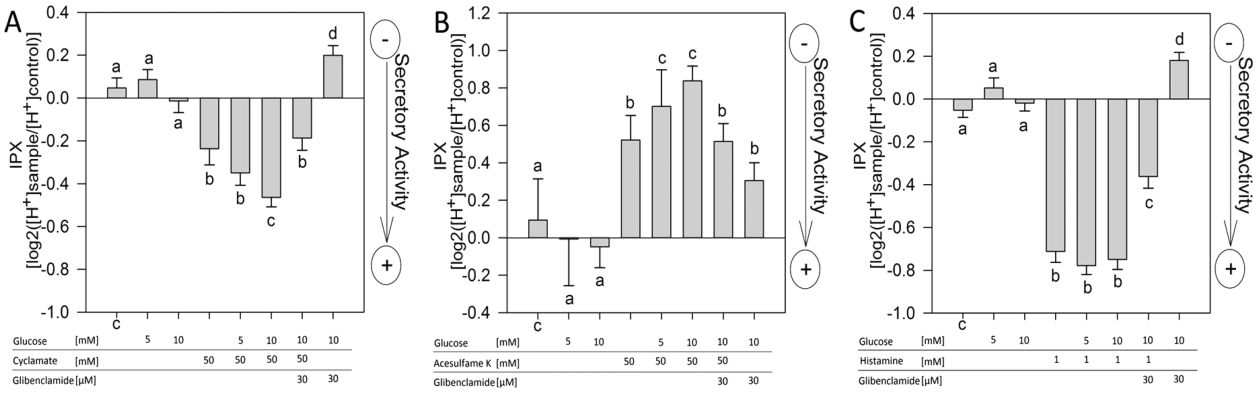

Figure 6. Enhancing effect of 5 and 10 mM glucose on 50 mM cyclamate-, acesulfame K-, or 1 mM histamine-induced H^+ secretion. IPX of HGT-1 cells treated with (A) cyclamate, (B) acesulfame K, or (C) histamine in the presence or absence of glibenclamide for 10 min. Data are presented as IPX and expressed treated over untreated control cells as the mean ± SEM, $n = 3$; tr = 5−6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are shown with the letters, $P \leq 0.05$).

Table 4. RT-qPCR Results of HGT-1 Cells Treated with 50 mM Glucose, Cyclamate, or Acesulfame K[a]

target	treatment	10 min	20 min	60 min
SUR1	glucose	1.03 ± 0.10	0.90 ± 0.08	0.84 ± 0.08
	cyclamate	**0.65 ± 0.09***	0.96 ± 0.18	1.18 ± 0.21
	acesulfame K	**1.68 ± 0.38***	**1.46 ± 0.27***	**1.31 ± 0.23***
Kv11.1	glucose	1.00 ± 0.13	1.12 ± 0.12	1.15 ± 0.22
	cyclamate	**1.29 ± 0.19***	0.96 ± 0.12	**1.61 ± 0.22***
	acesulfame K	**1.60 ± 0.27***	**1.70 ± 0.20***	1.34 ± 0.25
SLC2A1	glucose	**1.20 ± 0.10***	**1.21 ± 0.13***	1.00 ± 0.11
	cyclamate	0.98 ± 0.14	1.18 ± 0.16	**1.53 ± 0.15***
	acesulfame K	**1.35 ± 0.18***	**1.30 ± 0.12***	**1.54 ± 0.20***

[a]Data are indicated as average ± SEM in comparison to untreated control (set to 1). Calculations were made from at least three biological replicates. Statistics: two-way ANOVA Holm−Sidak post hoc test; significant difference between treatments over control are depicted as *; $P \leq 0.05$.

cells express both hetero- and homodimers, with a prevalence of homodimers. Future studies are warranted to elucidate the protein structure of the T1R2/T1R3 receptor in HGT-1 cells and in gastric tissues. However, our hypothesis is confirmed by results from the *TAS1R3* siRNA knockdown approach: in HGT-1 cells showing a 51% lower *TAS1R3* mRNA level compared to nontransfected cells, the effect of Cycl and AceK on proton secretion was significantly reduced. This suggests the involvement of T1R3 in NCS-induced proton release, although it also indicates additional cell surface proteins are involved in this response. This hypothesis is supported by our data showing opposite effects of Cycl and AceK on proton secretion. Cycl, reported to also target the bitter receptors T2R1 and T2R38,[28] stimulates the secretion of protons, whereas AceK, an agonist of T2R31 and T2R43,[29] shows a reducing effect compared to control cells. We demonstrated involvement of T2R43 by using the T2R43 antagonist 4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV3727)[34] which decreased the AceK-evoked response (Figure S2). A sensory study by Świąder et al.[35] demonstrated that the sweet taste intensity of Cycl and AceK in water solutions increased with higher concentrations in comparison to equi-sweet concentrations of sucrose, whereas at lower concentrations, up to about 0.5 ppm, AceK demonstrated a less intense bitter off-taste compared to Cycl. The human T1R2/T1R3 heterodimer possess various ligand-binding sites for sweeteners. AceK, e.g., binds to the amino-terminal domain of T1R2,[5] whereas Cycl acts on the transmembrane helical domain of T1R3 as an agonist.[36] There is, however, evidence demonstrating that a T1R3 homodimer is functional in response to AceK in Hutu-80 cells.[8] Nevertheless, we do not exclude the potential involvement of T1R2. Future studies are needed to discriminate between the efficacy of T1R and T2R by means of T1R and T2R targeted knock-down approaches. The outcome from a sensory study by Galindo-Cuspinera et al.[37] suggests that the transmembrane helical domain of the T1R3 section of the sweet taste receptor also functions as an allosteric binding site for saccharin for which lactisole is a known antagonist. The authors also demonstrated that saccharin at high concentrations (>25 mM) elicits a bitter taste character and suppresses the sweet response, whereas Cycl at high concentration exhibits a bitter and sweet character. Another explanation for the opposite effects of Cycl and Acek apart from the involvement of additional cell surface receptors and concentration-dependent responses might be antagonistic effects: A recent publication by Behrens et al.[38] demonstrated antagonistic activities for Cycl and saccharin on each other's bitter responsive T2Rs. However, we can not explain the opposite effects by Cycl and AceK on proton secretion in HGT-1 cells yet, neither by our current nor by previous findings where the stimulating effect of the bitter tasting caffeine, targeting T2R7, T2R10, T2R14, T2R31, and T2R43[32] on proton secretion, was reduced by the T2R43 antagonist homoeriodictyol.[21] Although the 50 mM concentrations of Cycl and AceK applied to HGT-1 cells in this mechanistic work are comparatively high, it can not be excluded that in gastric cells the density of taste receptors is lower than that on the tongue, requiring higher concentrations of NCS to elicit a similar response. Future studies are needed to clarify whether antagonistic activities of Cycl and AceK on TAS2Rs, beyond their role as agonists for T2R1/T2R38[32] and

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

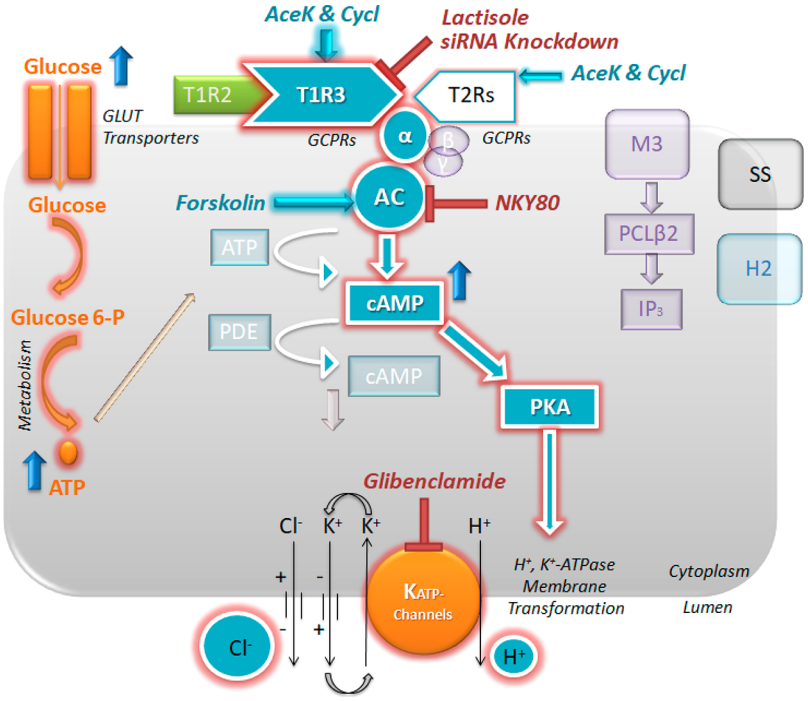

Figure 7. Illustrative proposed mechanism of sweetener-induced H^+ secretion in HGT-1 cells. The main T1R3 stimulatory pathway as well as T2Rs is elicited in dark blue and glowed with red. The glucose-dependent mechanism is demonstrated in orange. T1R3 agonists and AC activator forskolin are shown in blue. Inhibitors, as well as *TAS1R3* siRNA knockdown, are depicted in red. Activation of T1R3 is followed by induction of the secondary signaling cascade through cAMP. cAMP activates protein kinase A (PKA) which subsequently stimulates the H^+, K^+-ATPase that is accompanied by H^+ Cl^- secretion. Other signaling mechanisms, which might presumably be activated during H^+ secretion induced by NCS, are illustrated in the background, such as histaminic (light blue, H2), cholinergic (purple, M3), and somatostatin (black, SS) receptors.

T2R31/T2R43,[33] respectively, might help to explain the contrary effects of these NCSs on proton secretion. In addition, involvement of other cell-surface proteins cannot be excluded. Therefore, the role of T1R3 was further elucidated by analyzing NCS-associated downstream signaling pathways.[6−8] First, the impact of Cycl and AceK on intracellular cAMP was studied by using the adenylate cyclase activator forskolin[39] and its inhibitor NKY80.[40] Increased cAMP concentrations analyzed after exposure of HGT-1 cells to forskolin and either of the NCSs tested were reduced by NKY80 cotreatment. In addition, glucose did not evoke a response. Involvement of T1R3 was verified by lactisole, which also lowered the cAMP increase by Cycl and AceK. Although the opposite response of HGT-1 cells to Cycl and AceK on proton secretion is not reflected by comparable effects of these compounds on cAMP, our results indicate a link between T1R3 activation and proton secretion. This hypothesis is supported by effects of NKY80, which reduced the Cycl and AceK-evoked response on proton secretion.

T1R3-expressing cells have been shown to also express glucose transporters (GLUTs) and K_{ATP}-sensitive K^+ channels (K_{ATP}) which are considered cellular "glucose sensors".[41] Conducting RT-qPCR experiments, we observed a regulation of the mRNA expression level of these genes by Cycl and AceK in HGT-1 cells. Since Sidani et al.[42] demonstrated that glibenclamide inhibits H^+, K^+-ATPase-mediated and histamine-induced acid secretion in isolated gastric glands from wild-type mouse, we also tested the impact of the K_{ATP} inhibitor glibenclamide[41] which markedly diminished the effect of Cycl and AceK on proton secretion in HGT-1 cells. Since the transport of K^+ is directly linked to H^+ exchange, these results support our hypothesis of Cycl and AceK as modulators of GAS. Assuming that the cations gained from cyclamate (Na^+) and acesulfame (K^+) would also contribute to this opposite effect on proton release, the cells treated with 50 mM NaCl and KCl showed no effect on Cycl- and AceK-induced proton secretion (data not shown). To elucidate whether Cycl and AceK not only act through binding to the sweet taste receptor but also via activation of GLUTs, we studied glucose in coincubation experiments as suggested by Yee et al.[41] who described that a mixture of NCSs and a caloric sweet tastant (glucose) augment the cellular NCS response. This hypothesis is supported by our data demonstrating that glucose did enhance the impact of Cycl and AceK on proton secretion, while not showing an effect when cells where treated with glucose solely. Consistent with our results, Li et al.[43] demonstrated that a much higher concentration of glucose (>50 mM) is required to stimulate the canonical sweet taste receptor in taste buds. Moreover, it has been hypothesized that the sweet taste receptor in β-cells is not identical to the canonical sweet taste receptor expressed in the taste buds of the tongue, as a relatively high concentration of NCS was required to stimulate the sweet taste receptor in β-cells.[44] In addition, the heterodimeric sweet taste receptor is known to detect the sweet taste of caloric sugars, for example sucrose at the EC_{50} = 100 mM,[43] whereas it senses the sweet taste of Cycl at EC_{50} = 2.2 mM and AceK at EC_{50} = 120 μM.[45] Since the 10 min 50 mM glucose exposure in this work also did not alter intracellular cAMP concentrations nor mRNA expression of *TAS1R3*, future experiments will be needed to elucidate the different pathway kinetics of NCS and glucose. Although glucose is a known agonist of the sweet taste receptor[2] and a substrate for ATP formation upon cellular uptake by GLUTs,[46] these cellular responses might follow different kinetics as compared to those of NCS, which are many times sweeter than glucose and may have, therefore, significantly different T1R3 binding kinetics than glucose. Glucose is assumed to bind to the amino-terminal

DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

domain of both subunits, T1R2 and T1R3, with distinct binding affinities.[47] Cycl has been shown to bind to the transmembrane domain of T1R3,[36] while the amino-terminal domain of T1R2 is the binding site for AceK.[5] Therefore, we hypothesize that the enhancing effect of glucose on the cellular response to Cycl and AceK predominantly depends on an initial T1R3 activation by the NCS compounds. However, we cannot exclude an enhancing effect on cAMP concentrations via metabolic transformations of glucose through gylcolysis, the Krebs cycle, and oxidative phosphorylation reactions, finally increasing ATP as the primary substrate for the adenylate cyclase to form cAMP[48] (Figure 7).

The contrary effects of Cycl and AceK on proton secretion in HGT-1 cells might also be explained by different kinetics. In our mechanistic studies, which were intended to prove the hypothesis that NCSs have an impact on mechanisms regulating GAS via sweet taste receptor, both NCS compounds were used at equal concentrations, although AceK is about 7 times sweeter than Cycl (in relation to sucrose),[3] which are higher than those commonly added to foods and beverages. The European Union (EU) allows maximum doses for Cycl in various foods from 250 mg/L to 2500 mg/kg, with typical carbonated soft drinks containing up to 1.4 mM. For AceK, the doses range from 25 mg/L to 2500 mg/kg.[49]

In summary, our study demonstrates that (i) T1R3 is a major functional part of the T1R2/T1R3 receptor and that (ii) Cycl and AceK are T1R3-dependent modulators of proton secretion in HGT-1 cells. However, future studies are needed to identify their efficacy of Cycl and AceK on GAS in vivo when applied in food-representative concentrations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b00658.

Results demonstrating the *TAS1R3* siRNA knockdown efficiencies determined by means of RT-qPCR and the effect of GIV3727 and AceK-induced proton release (PDF)

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Funding

Financial support was provided by the Austrian Federal Ministry of Economy, Family and Youth, the Austrian National Foundation for Research, Technology and Development, and the Austrian Science Fund (grant FWF P23797).

Notes

The authors declare the following competing financial interest(s): The authors Jakob P. Ley and Joachim Hans are employees at Symrise AG, Holzminden, Germany.

■ ABBREVIATIONS USED

Cycl, sodium cyclamate; AceK, acesulfame potassium; D-thr, D-threonine; NKY80, 2-amino-7-(2-furanyl)-7,8-dihydro-5(6*H*)-quinazolinone; SNARF, 1,5-carboxy-seminaphtorhodafluor acetoxymethyl ester; IPX, intracellular proton index; HGT-1, human gastric parietal tumor cells; NCS, noncaloric sweeteners; GAS, gastric acid secretion; AC, adenylate cyclase

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DOI: 10.1021/acs.jafc.8b00658
J. Agric. Food Chem. 2018, 66, 4842−4852

Supporting Information

Human Sweet Receptor T1R3 is Functional in Human Gastric Parietal Tumor Cells (HGT-1) and Modulates Cyclamate and Acesulfame K-Induced Mechanisms of Gastric Acid Secretion

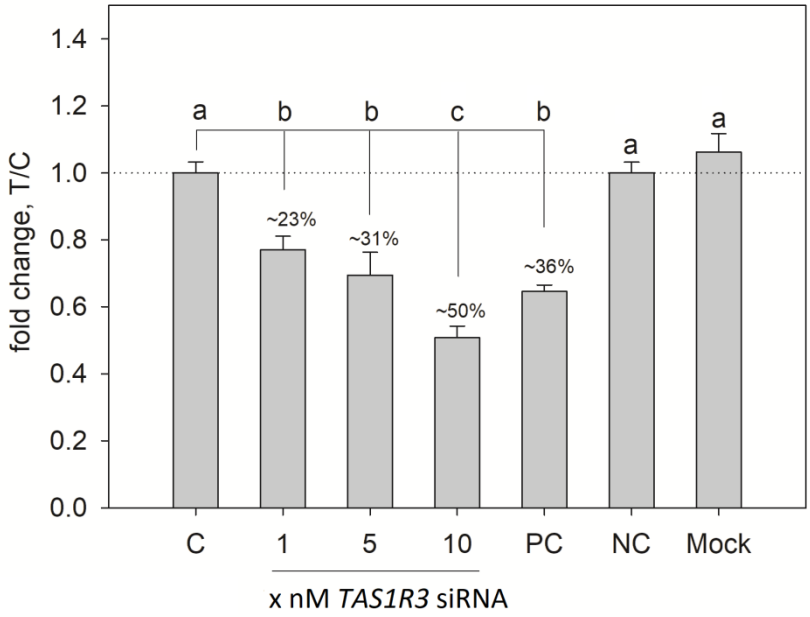

Figure S1. siRNA knockdown of *TAS1R3* gene expression in HGT-1 cells

HGT-1 cells were transiently transfected with the transfection reagent (Mock), combined with either *TAS1R3* siRNA (1, 5, or 10 nm), or positive control Hs/Mm_MAPK1 siRNA (PC, 5 nM), or all-stars negative control siRNA (NC, 10 nM) for 48 h. The transfection efficiencies were analyzed by means of qRT-PCR. Results were normalized to the mean of the internal standard gene PPIA. Findings are presented in comparison to non-treated cells (C) as fold change T/C ± SEM, n = 3; tr=3. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are shown with the letters; $P \leq 0.05$).

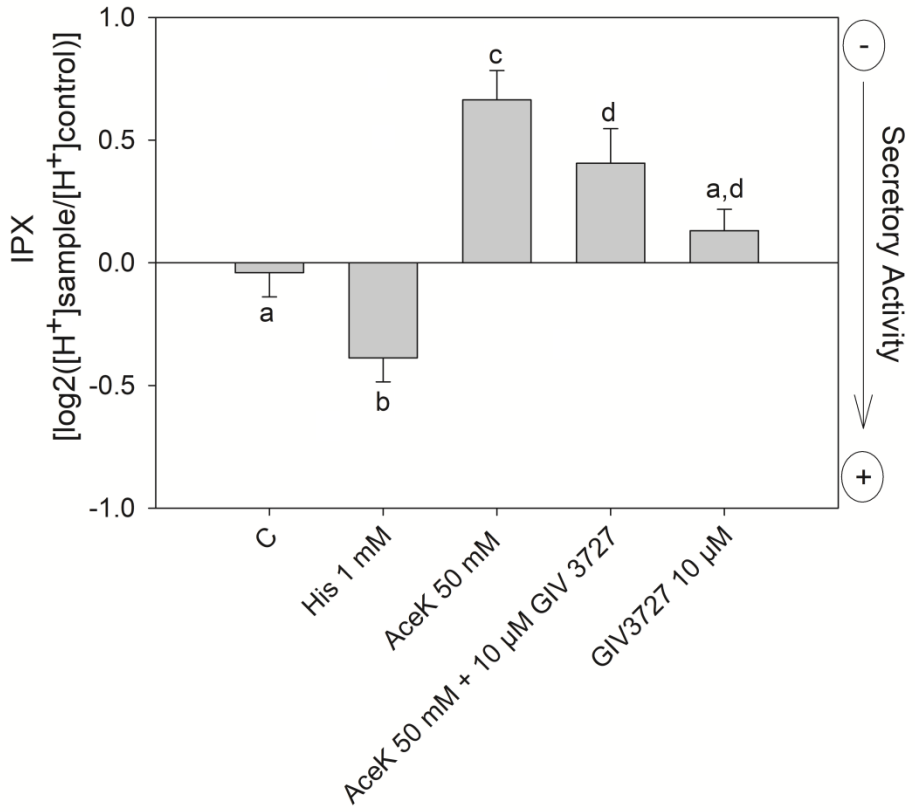

Figure S2. Impact of T2R43 antagonist GIV3727 on AceK-induced H^+ secretion in HGT-1 cells

IPX of HGT-1 cells after treatment with histamine (His, 1mM), acesulfame K (AceK, 50 mM) in the presence or absence of GIV3727 (10 µM) for 10 min. Findings are depicted as IPX and demonstrated in comparison to the untreated cells (Control, C) as the mean ± SEM, n = 3; tr = 5-6. (Statistics: one-way ANOVA Holm-Sidak post hoc test; significant differences are shown with the letters; $P \leq 0.05$).

(2) "Serotonin biosynthesis and release from human gastric adenocarcinoma cells and its functional role in arginine-induced proton secretion"

Ann-Katrin Holik[1], Verena Stöger[2], Barbara Lieder[1,2], Angelika Reiner[3], **Muhammet Zopun**[1], Julia K. Hoi[2], Nicole Kretschy[4], Gareth J. Sanger[5], Mark M. Somoza[4], Marc Pignitter[1], Veronika Somoza[1,2]

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Submitted to Journal of Gastroenterology

This study investigated the 5-HT secretory characteristics of HGT-1 cells and the link between 5-HT and mechanisms of GAS. Experiments carried out on transcriptional and functional levels showed that HGT-1 cells are able to secrete 5-HT. L-arginine induced both 5-HT and proton secretion in HGT-1 cells, which was reduced by the 5-HT_3 antagonist granisetron, implied the involvement of 5-HT_3 receptor in both actions in response to L-arginine. Moreover, *ex-vivo* data suggested the presence of 5-HT in the human stomach and L-arginine to effect gastric motility.

I participated in the immunostaining of 5-HT in HGT-1 cells, LCMS/MS method development, planning and execution of analysis with LC-MS/MS methods for characterization of 5-HT in cell supernatant and AADC activity assay in HGT-1 cells.

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A.K.H., V. Stöger, B.L., M.Z., M.P., J.H., A.R., G.J.S., and V. Somoza designed research; A.K.H., V. Stöger, B.L., M.Z., M.P., J.H., A.R., performed research; G.J.S. and V. Somoza contributed new reagents/analytic tools; A.K.H., V. Stöger, B.L., M.Z., M.P., J.H., A.R., G.J.S., and V. Somoza analyzed data; and A.K.H., B.L., G.J.S., V.Stöger, and V. Somoza wrote the paper.

Funding

Financial support was provided by the Austrian Federal Ministry of Economy, Family and Youth, the Austrian National Foundation for Research, Technology and Development, and the Austrian Science Fund (grant FWF P23797).

The authors thank for the financial support by Symrise AG and Christian Doppler Gesellschaft and gratefully acknowledge Dr. C. L. Laboisse (Inserm 94-04, Facultè de Medicine, Nantes) for providing the HGT-1 cells, clone6.

Declaration of Interests

The authors declare no conflict of interest. GJS is currently in receipt of funding from Takeda Pharmaceuticals, BBSRC together with GlaxoSmithKline, Benevolent and the Dunhill Foundation. He acts as an advisor to Takeda Pharmaceuticals and to Zealand Pharma.

Abstract

Among mammals, most of the body's serotonin is located in the gastrointestinal tract where it plays a role in a number of different pathways, including nausea and vomiting. We investigated whether HGT-1 cells, derived from an adenocarcinoma of the stomach, are capable of serotonin synthesis and whether stimulation of serotonin release plays a role in proton secretion as key mechanism of gastric acid secretion. First, HGT-1 cells were immunostained showing the presence of serotonin. Using 100 µM of the tryptophan hydroxylase inhibitor p-chlorophenylalanine, the signal intensity decreased by 27%, supporting the hypothesis of serotonin biosynthesis by HGT-1 cells. Treatment of HGT-1 cells with 30 mM L-Arg increased extracellular serotonin to 147 ± 18 % compared to control cells. The L-Arg-induced increase in serotonin release was inhibited by co-incubation with the 5-HT_3 receptor antagonist granisetron. Furthermore, the impact of L-Arg on proton secretion was reduced by granisetron, suggesting the serotonin receptor subtype HTR3 to be involved in serotonin release and proton secretion in response to treatment with L-Arg. Finally, gastric antral mucosa specimen stained positive for serotonin and treatment of human stomach strips with 30 mM L-Arg resulted in an increase in muscle tension to 149.9 ± 55.29 % (control set to 100%) *ex vivo*. This *ex vivo* data showed serotonin to be present in the human stomach and L-Arg to influence gastric motility. The *in vitro* data from HGT-1 cells suggests L-Arg to impact serotonin release and proton secretion in a likely HTR3-related mechanism.

Keywords: Human gastric tumour cells, serotonin release, immunofluorescence

Abbreviations:

5HT: serotonin, AADC: aromatic amino acid decarboxylase, HGT-1: human gastric cancer cell line, KRHB: Krebs-Ringer-HEPES buffer, PCPA: p-chlorophenylalanine, SERT: serotonin transporter, TPH: tryptophan hydroxylase

Introduction

Within the central nervous system (CNS) the monoamine serotonin (5HT), synthesised from the essential amino acid L-tryptophan, is an important neurotransmitter associated with a manifold of different disorders such as schizophrenia [1] ,depression [2] in the regulation of satiety [3, 4]. Serotonin is also synthesised in large amounts outside the CNS, especially within the enterochromaffin cells in the mucosa of the gastrointestinal (GI) tract [5]. Choi et al. [6] carried out investigations into the distribution of different cell lineages on three healthy human donor stomachs. Their analysis revealed distinct patterns of enteroendocrine cells, showing serotonin-positive cells in the proximal stomach and in the antrum. In addition, the presence of the serotonin transporter (SERT) in the antrum was demonstrated. The release of serotonin from the upper GI tract has long been known to play a key role in the mechanism of certain forms of nausea and vomiting. Clinically, serotonin may also be of importance in a number of gastrointestinal diseases. In a study on constipation-predominant IBS patients, diarrhea-predominant IBS patients and healthy controls, diarrhea-predominant IBS patients were shown to have increased platelet-depleted plasma serotonin concentrations under fed and fasting conditions [7]. Consequently, serotonin receptor antagonists have been suggested in the treatment of IBS. The serotonin type 3 receptor antagonist alosetron has been described to relieve abdominal discomfort and pain in a multicentre, randomized, double-blind, placebo controlled study on women suffering from diarrhea-predominant IBS [8]. Serotonin is thought to mostly act locally, stimulating and sensitising abdominal vagal nerve terminals which project to the brainstem; in some circumstances plasma concentrations of serotonin may also be increased, sufficient to pass through the blood-brain-barrier [9]. While serotonin has been studied in the context of gastrointestinal diseases, less information is available on the influence of peripheral serotonin, released postprandially from the enterochromaffin cells, on gastrointestinal mechanisms regulating satiety. Two studies carried out in the early eighties suggest peripheral administration of serotonin to induce hypophagia [10, 11]. Recently, peripheral serotonin has been indicated to influence glucose and lipid metabolism [12]. A role for 5-HT released from endocrine cells by the presence of acid within the lumen [13] and entering the blood circulation has also been to an ability to inhibit gastric acid secretion [14]. It is important to understand how serotonin is released from endocrine cells in response to stimulation with different compounds. For this purpose, several cell line models have been established. These include neuroblastoma cells (SH-SY5Y) [15] and enterochromaffin cells (QGP-1) [16], but also the human colon carcinoma cell line Caco-2 has been reported to synthesize and

degrade serotonin [17]. Thus, we aimed at investigating the capacity of a stomach-derived cell line model, human gastric tumour cells (HGT-1,) to synthesise and release serotonin after nutrient-stimulation and to investigate serotonin's functional role for gastric acid secretion.

The human gastric cell line HGT-1 was established by Laboisse and co-workers [18] from a poorly differentiated adenocarcinoma on the posterior wall of the body of a patient's stomach in 1982. HGT-1 cells have been shown to express histamine H_2 receptors, which have been demonstrated to mediate adenylate cyclase activation and cAMP production [18]. Furthermore, this cell line has been described to express acetylcholine receptors, the H^+/K^+ ATPase proton pump, an omeprazol-binding site possessing K^+ channel [19], angiotensin 1-converting enzyme [20], and transporters required for gastric acid secretion [21]. HGT-1 cells have consequently been established as a valuable cell model for assessing the effects of different components, e.g. compounds encountered in coffee beverages, on proton secretion by measuring the intracellular pH value [22, 23]. Here, we demonstrate this cell model's capacity of synthesizing serotonin and its subsequent secretion in response to exogenous stimuli by incubating with L-Arg, an amino acid which as recently reported to influence food intake [24], delay and inhibit gastric emptying and enhance gastric adaptive relaxation [25]. As 5-HT3 receptors have been suggested to be involved in gastric acid release [26] and HGT-1 cells are capable of proton secretion, we carried out co-incubation experiments with the 5-HT_3 inhibitor granisetron [27].

Materials and Methods

Materials

All reagents were obtained from Sigma-Aldrich (Austria) unless stated otherwise. Anti-5HT and Anti-TPH2 antibodies were purchased from Merck-Millipore (Austria).

Cell culture

The human gastric tumour cell line (HGT-1, Dr. C. Laboisse, Nantes, France) was cultivated using Dulbecco's modified Eagle medium (DMEM) containing 4.5 g L^{-1} glucose. The medium was supplemented with 4 mM L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin (100 units penicillin, 171 µM streptomycin). Cells were maintained in a humidified incubator at 5% CO_2 and sub-cultured at 90% confluence, using cells between passage 60 and 80 in experiments. Caco-2 cells were obtained from ATCC (American Type Culture Collection) and maintained in DMEM containing 4.5 g L^{-1} glucose, 10% fetal bovine serum and 1% penicillin/streptomycin. QGP-1 cells

were cultivated in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cell Viability Assay

HGT-1 cells were seeded in 96-well plates at a density of $1x10^5$ cells per well and left to settle for 24 h. Next, the cells were incubated with Krebs-Ringer-HEPES buffer (KRHB, supplemented with 0.1% ascorbic acid, pH 7.4) or serum-free DMEM for 5 min. Then the incubation solution was replaced with MTT working solution (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, 100 µL, 1 mg mL^{-1}) and incubated for 15 min at 37°C in a humidified incubator. Finally, the MTT working solution was removed, 150 µL DMSO added and the absorbance read at 570 nm.

qPCR

HGT-1 cells were seeded in 24-well plates at a density of $1.5x10^5$ cells per well. The cells were treated with L-Arg for 3 h prior to RNA isolation using the PeqLab total RNA kit (Peqlab, VWR, Austria). Then, the isolated RNA was reversely transcribed with the high capacity cDNA kit (Applied Biosystems, Thermo Fisher Scientific). The synthesized cDNA was added to a qPCR mix containing fast SYBR green master mix (Applied Biosystems, Thermo Fisher Scientific) and the amplification assessed on a StepOnePlus device (Applied Biosystems, Thermo Fisher Scientific). Tata-box binding protein (TBP), glyeraldehyde 3-phosphate dehydrogenase (GADPH) and peptidylprolyl isomerise A (PPIA) were used as endogenous controls. The sequences of all primers are provided in Table S1. Primers have either been designed using PrimerBlast or have been taken from PrimerBank. LinReg v2013.0 was used in the data analysis.

DNA Microarrays

Microarray analysis was carried out as described previously [28], using a reaction chamber allowing for the simultaneous synthesis of two microarrays [29]. HGT-1 cells were seeded in 6-well plates and left to settle for 24 h. Thereafter, cells were incubated with 50 mM L-Arg for 3 h prior to RNA isolation (RNeasy Mini Kit, Qiagen). Microarray labelling, hybridization and analysis were carried out as described previously [15], however an Axon GenePix 4400A was used for scanning the microarrays.

TPH2 expression

TPH2 expression was determined by Western Blot. Briefly, HGT-1 cells were seeded in 6-well plates, left to grow for 24h, washed with ice-cold PBS and lysed with RIPA buffer supplemented with

protease inhibitor cocktail, PMSF and, sodium vanadate. The lysate was passed through a 22-gauge needle and centrifuged at full speed and 4°C for 15 min. Next, the protein concentration in the supernatant was assessed by Bradford assay. A total of 20 µg protein or 3 µL pre-stained protein standard (New England Biolabs) were loaded per lane of a 16% polyacrylamide SDS-gel. After electrophoresis, the proteins were transferred onto an activated (2 min methanol, 5 min blotting buffer) PVDF membrane. The membrane was blocked using 5% BSA dissolved in TBS-T for 1 h at room temperature. Then, primary antibody (rabbit anti-TPH2, 1:200, Merck Millipore or rabbit anti-β-actin, 1:2000, Santa Cruz) diluted in blocking solution was added and incubated over night at 4°C. After washing four times with TBS-T, secondary antibody (goat anti-rabbit, 1:2000, CST cell signalling) diluted in blocking solution was added and incubated for 1 h at room temperature. Afterwards, four washing steps with TBS-T were carried out prior to pipetting 500 µL Signal Fire Chemiluminescence reagent (New England Biolabs) onto the membrane. Finally, after 5 min incubation at room temperature an image was recorded using a Fusion FX7 device (Vilber). Image analysis was carried out using ImageJ.

Aromatic Amino Acid Decarboxylase (AADC) activity

The method used in the assessment of the enzymatic activity of the aromatic amino acid decarboxylase was modified from Vieira-Coelho et al. [17] Briefly, HGT-1 cells grown to confluence in a T175 flask were washed with HBSS and detached from the flask by scraping after addition of 1 mL HBSS. The cells were then subjected to three freeze-thaw cycles in liquid nitrogen, homogenised using a Potter-Elvehjem device, passed through a small-bore needle, and centrifuged at 4°C (full speed, 15 min). A total of 100 µL of the supernatant was added to incubation medium (100 µL) and incubated for 15 min at room temperature. The incubation medium consisted of: tolcapone (4 µM), paragyline (300 µM), pyridoxal phosphate (240 µM), sodium borate (110 µM), Na_2HPO_4 (150 µM), KH_2PO_4 (350 µM) and HBSS. Next, 200 µL 5-HTP (0 – 9 mM) and ascorbic acid, giving a final concentration of 0.1%, were added. After 15 min incubation in the dark, 60 µL 6 M hydrochloric acid were added and centrifuged at 4°C for 15 min. The supernatant was passed through a 0.2 µm filter prior to injecting 50 µL onto the HPLC column. The analytical method used is described in the LC-MS/MS section of the supplemental information. The total protein content of the cell lysate was determined by Bradford assay.

Serotonin staining

Immunofluorescence (HGT-1 cells)

For serotonin staining, HGT-1 cells were seeded onto glass coverslips located in a 24-well plate. p-chlorophenylalanine (100 µM) [30] was added to one part of the cell suspension during seeding. After two days, the cells were washed once with ice-cold PBS and fixed with a mixture of acetone and methanol (50:50 % v/v) for 2 min at -20°C. The fixed cells were stained using a primary anti-serotonin antibody. The detailed procedure is described in the supplemental information.

Immunohistochemistry (HGT-1 cells, tissue)

Histological specimens were obtained from two patients from the Pathologisch-Bakteriologisches Institut, Sozialmedizinisches Zentrum Ost – Donauspital, Vienna, Austria after signing a written informed consent that covers all diagnostic procedures on the extracted tissue. One specimen derived from a 79-year old female patient undergoing distal partial gastrectomy for benign peptic ulcer with perforation. The other specimen derived from a 47-year old male patient with proximal high-grade adenocarcinoma. 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 using ultraView Universal DAB Detection Kit (Ventana Medical Systems). The primary antibody M0758 (clone 5HT-H209, Agilent DAKO) was applied at 1:5 for 32 minutes at 37°C. No pretreatment was performed. All counterstaining was performed with hematoxylin. Blocking experiments in order to control for unspecific staining were performed using normal mouse IgG1 serum replacing the primary antibody. All other steps were performed similar to the staining procedure as described above.

Serotonin release

Serotonin ELISA

Serotonin release was carried out as described previously [31]. A more detailed procedure is given in the supplemental information.

Gastric motility

Stomach specimens from bariatric surgery were used for this study. Tissue collection was approved by the local ethical committee in the UK (REC 15/LO/2127) and patients gave written informed consent. The proximal region of the stomach was separated in the mucosa and muscularis mucosa by blunt dissection. Afterwards, 5-8 x 15 mm muscle strips were mounted in 10 mL organ bath chambers equipped with isometric force transducers (MLT0201, AD Instruments, Chalgrove, UK) and

filled with Krebs' solution (gassed with 95 % O_2, 5 % CO_2), maintained at 37°C. After 1 h of tissue equilibration under 2 g tension and changing bath solution every 15 minutes, nerves were excited by electrical field stimulation (EFS). This was given every 10 s for 1 min at 5 Hz, 0.5 ms bipolar pulse duration and 200 mA with a stimulator (STG2008, Scientifica, Uckfield, UK). When the responses to EFS, usually consisting of cholinergically-mediated muscle contraction [32], were continuously consistent a frequency response curve was constructed by application of EFS at 1,2,5,10,15 and 20 Hz, followed by changing bath solutions and switching back to 5 Hz. Strips were then treated with 30 mM L-Arginine for 15 minutes (afterwards precipitation in the Krebs' solution occurred). Changes in baseline tension were determined before and 5 min after application on the top of the peak for at least 3 EFS-induced responses. Data acquisition was done by Biopac Inc. CA, USA.

Proton secretory activity

Proton secretory activity and corresponding data analysis were determined as described previously [22, 23]. HGT-1 cells were treated with 0.1 – 10 µM serotonin, 30 mM L-arginine. Furthermore, co-incubation experiments with 5-HT_3 receptor inhibitor granisetron (10 µM) or SERT inhibitor fluoxetine were carried out.

LC-MS/MS

Briefly, tryptophan, 5-hydroxytryptophan and serotonin in the cellular supernatants collected were analysed in a single run by LC-MS/MS in positive MRM mode. The detailed method is described in the supplemental information.

Statistical analysis

SigmaPlot 11 was used for statistical analysis. Data are presented as average ± SEM or standard deviation. The number of biological replicates is stated in legend of the corresponding figure or table. Treatments are shown in relation to control cells set to 1 or 100%, labelled as treated over control in the figures. Image J was used for reading out the fluorescence of single cells. Proton secretion data was subjected to Nalimov's outlier test prior to data analysis.

Results

Cell viability

Cell viability was assessed using the MTT assay. None of the treatments led to a significant decrease in metabolically active HGT-1 cells compared to the corresponding control cells. ($p > 0.05$, data not shown).

Serotonin release

The serotonin-sensitive ELISA detected serotonin in the supernatants collected from cells treated with Krebs-Ringer HEPES buffer (+0.1% ascorbic acid). Compared to Caco-2 cells ($3.74 \times 10^{-5} \pm 8.27 \times 10^{-6}$ pg serotonin corrected for cell number and incubation volume) and QGP-1 cells ($9.56 \times 10^{-6} \pm 4.81 \times 10^{-6}$ pg serotonin corrected for cell number and incubation volume), the highest serotonin concentration was detected in HGT-1 cells ($2.76 \times 10^{-4} \pm 1.49 \times 10^{-6}$ pg serotonin corrected for cell number and incubation volume).

Serotonin biosynthesis

The presence of the two enzymes required in the biosynthesis of serotonin was analyzed. The expression of TPH2 was determined by Western blotting. This yielded a single band at approx. 61 kDa on average, fitting the expected molecular weight of approx. 56 kDa (Figure 3A-B). β-Actin was used as a loading control. Likewise, this showed one band (Figure 3A). The expression of AADC was tested by the capability of cellular lysate to react 5-HTP provided in excess to serotonin. This experiment showed a rise in the determined serotonin with increasing 5-HTP concentrations (Figure 3C). From this graph, a Lineweaver-Burk plot was derived which led to a value of 0.28 mM for K_m and 85 nmol mg protein^{-1} h^{-1} for v_{max}.

Effects of L-Arg on gene expression, serotonin release, proton release and gastric motility

Gene expression

First, the effects of 50 mM L-Arg on the gene expression of HGT-1 cells were assessed in a genome wide screening using customized cDNA microarrays. The scatter plot shown in Figure S2 shows a broad distribution of probes, suggesting a strong impact of L-Arg treatment on gene expression. Pathway analysis using the freeware DAVID, suggested the expression of serotonin receptors to be altered (Table 1A, B). As a result, we first assessed the expression of *HTR1A, 2A, 1B, 3A, 3B, 3C, 3D, 3D, 4* and *7*, in addition to the genes encoding the enzymes required in serotonin synthesis and the serotonin reuptake transporter *SLC6A4*. This showed *SLC6A4, TPH1, TPH2, HTR3C, HTR3D* and *HTR7* to be consistently expressed over 4-5 biological replicates. However, *HTR1B, HTR3B* and *HTR3E* were only detectable in some of the samples. *HTR1A, HTR2A, HTR3A* and *HTR4* were detected in none of the samples tested. Treatment of HGT-1 cells with L-Arg for 3 h regulated the gene expression of *SLC6A4, TPH2, HTR3C* and *HTR7* (Table 2).

Serotonin staining

Figure 1 shows a typical result of HGT-1 cells stained using an anti-serotonin antibody. Figure S1 shows an additional HGT-1 staining experiment using another antibody.

In a second set of experiments, HGT-1 cells were seeded in either DMEM or DMEM containing 100 µM PCPA. In both cases, positive serotonin staining was observed (Figure 1D-E). However, cells that received PCPA at seeding, exhibited less pronounced serotonin staining (Figure 1E). After image analysis with Image J, a fold-change of 0.73 was calculated, indicating a reduction in serotonin staining by 27% in the PCPA treated group. In the absence of a blocking peptide, the primary anti-5HT antibody was pre-incubated with pure serotonin for 30 min prior to the incubation. In this experiment, control cells receiving the pre-blocked version of the antibody showed a reduction in serotonin staining by 23%, suggesting that part of the antibody was prevented from binding to cellular serotonin by the pre-incubation. Figure 2 shows characteristic staining for serotonin in human benign and carcinoma stomach samples with positivity in scattered single cells.

Serotonin release

Figure S3A shows a chromatogram of the supernatant collected from HGT-1 cells. Both L-Trp and 5-HT were detected in the supernatant. However, the 5-HTP concentration was too low to be detected by LC-MS/MS. The incubation with 30 mM L-Arg increased the serotonin concentration in the cellular supernatant to 147 ± 18 % compared to cells treated with KRHB only (+0.1% ascorbic acid; Figure S3B). This result was verified by LC-MS/MS where an increase to 162 ± 48 % was detected (Figure S3C). No significant difference between the neutral pH control and the control adjusted to pH 9.5 was found (data not shown in figure). Similarly, no difference between L-Arg simply added to KRHB and the pH adjusted L-Arg solution was detected (data not shown in figure).

In the experiment using 10 µM granisetron, the L-Arg induced serotonin release was prevented by the 5-HT_3 antagonist, reducing the increase to 124 ± 23% observed after L-Arg treatment to 95 ± 16%, compared to the control set to 100%. Granisetron alone did not impact the serotonin release compared to control cells treated with KRHB only (Figure 4).

Proton secretion

After a 10 min incubation time with 0.1 µM serotonin, HGT-1 cells showed an inhibition of the proton secretion in comparison to the untreated control analysed by means of intracellular proton index [IPX]: 0.21 ± 0.03 vs. control> -0.00 ± 0.02) (Figure 5A). Statistics were done for 3-6 biological and 6 technical replicates with Kruskal-Wallis one Way ANOVA on Ranks with Holm-Sidak post hoc

test vs. non-treated control, $p < 0.05$. Incubation with 10 µM of the 5-HT_3receptor antagonist granisetron reduced the L-Arg induced proton secretion from IPX -3.48 ± 0.13 to -3.00 ± 0.53 ($p < .0.05$, Figure 5B), while incubation with SERT inhibitor fluoxetine showed no impact (data not shown).

Gastric motility

After 15 minutes treatment of the stomach strips with 30 mM L-Arg, traces showed no consistent changes in responses to EFS (data not shown) but an increase of the baseline gastric muscle tension up to 150 ± 55 % in comparison to the 100 % vehicle control (Figure 5C).

Discussion

The indole amine serotonin has been described to exert a wide variety of actions in the human body in health and disease, reaching from its role in gastrointestinal function and the regulation of nausea, vomiting and satiety to several brain disorders such as Schizophrenia. Several cell lines have been shown to release serotonin upon stimulation, among which enterochromaffin QGP-1 cells and enterocyte-like Caco-2 cells represent cell models of peripheral serotonin release. As several reports indicate the presence of serotonin in the stomach, we investigated the capacity of a cell line originating from an adenocarcinoma of the stomach to synthesize and consequently release serotonin into the cellular supernatant upon stimulation. Moreover, a functional role of serotonin on mechanisms regulating gastric acid secretion was elucidated.

First, we analyzed the expression of the enzymes involved in the biosynthesis of serotonin on the functional level. In an attempt to assess the activity of TPH in HGT-1 cells, we could not see a robust increase in 5-HTP with increasing substrate concentrations of L-Trp. In Caco-2 cells, a similar challenge was described by Nakamura [30] and co-workers, who opted to use living cells rather than a cell-free enzyme assay and were successfully showing *de novo* synthesis of serotonin by using the TPH inhibitor p-chlorophenylalanine. In order to still show the presence of TPH on the protein level, we carried out Western blot experiments using an antibody directed against TPH2. This revealed a single band with the expected molecular weight of the targeted protein. The isoform TPH2 has been reported to be primarily expressed in the brain, while TPH1 has been shown to be abundantly expressed in the periphery. However, TPH2 expression was shown in the intestine, although to a weaker extent than TPH1 and more restricted in its distribution [30]. Similar to the study by Vieira-Coe et al. [17], reporting the ability of Caco-2 cells to synthesize and degrade serotonin, we determined the

activity of AADC based on their method. In this experiment, we showed an increase in the serotonin concentration with increasing substrate concentrations of 5-HTP, thereby indicating the presence of functional AADC. Taken together, the protein expression of TPH and the presence of functional AADC suggest HGT-1 cells to be capable of synthesising serotonin. To conclusively show that serotonin is in fact present in HGT-1 cells, we performed immunostaining experiments, using two different antibodies directed against serotonin. In both cases, HGT-1 cells stained positive for serotonin. In an experiment using the TPH inhibitor PCPA, we observed a decrease in the signal intensity in cells being treated with the inhibitor compared to cells receiving culture medium only. Furthermore, human adenocarcinoma excised from the proximal stomach showed scattered single cell staining positive for serotonin. This pattern is similar to the expression pattern of serotonin-cells in healthy stomach tissue, in which only a low number of serotonin expressing cells was found compared to other cell types present [6]. This finding is also concordant with the literature demonstrating only a low number of scattered carcinoma cells with serotonin expression. In addition, only a very low percentage of serotonin expression in gastric adenocarcinomas has been described [33]. Regardless of the number of cells staining positive, this suggests serotonin to be present in proximal stomach samples of both healthy and diseased donors. Next, we compared the serotonin content of HGT-1 cells to cell lines which have been described to release serotonin upon stimulation in previous studies. In the cellular supernatants of the enterochromaffin cell line QGP-1 and differentiated Caco-2 cells, serving as cell model of enterocytes, a serotonin-sensitive ELISA detected smaller quantities than in the supernatant collected from HGT-1 cells. L-Tryptophan (L-Trp) has to be present in the cells in order to synthesise serotonin. However, the antibody used in the ELISA may cross-react with L-Trp when it is present in high concentrations. As the actual L-Trp level in the supernatant collected from HGT-1 cells was unknown, we analysed L-Trp, 5-HTP and 5-HT by LC-MS/MS to exclude the Trp level in the sample exceeds the level reported to cross-react by the ELISA manufacturer. This showed L-Trp to be present, but not in concentrations high enough to interfere with the ELISA. Interestingly, we detected both L-Trp and serotonin, but not 5-HTP. This fits with the conversion of L-Trp to 5-HTP being the rate limiting step, and hence formed 5-HTP being reacted to serotonin immediately.

In addition to showing the enzymes required in the biosynthesis of serotonin on a functional level, we assessed the mRNA expression of the cultured cells, including several serotonin receptors and the serotonin reuptake receptor SERT by qPCR. This demonstrated *TPH1, TPH2, SLC6A4, HTR3C/D* and *HTR7* to be expressed. However, we failed to detect the second enzyme required in the

synthesis of serotonin, *AADC*. As we were able to show AADC activity, and AADC is a prerequisite in the biosynthesis of serotonin, it seems likely that the AADC mRNA level was below the limit of detection of our qPCR method. In addition, mRNA expression of the receptor subtypes *HTR1A, HTR2A* and *HTR3A* was below the limit of the detection. However, genes encoding for serotonin receptors have been described in stomach tissue previously. A study by Van Lelyveld [34] reported very low gene expression levels of *HTR3A* and *HTR3B* in the stomach. Furthermore, the authors showed expression of *HTR3C, HTR3E* and *HTR4* in both antrum and fundus of healthy donor samples [34]. Among the currently known seven serotonin receptor families, 5-HT_3 receptors are the only ones belonging to the family of cys-loop ligand-gated ion channels [35]. Serotonin has been suggested to modulate the secretion of gastric acid *via* this subtype of serotonin receptors [26]. Furthermore, a link between intragastric pH and serotonin release has been shown using perfused rat stomach [36]. In this study, at an intragastric pH of 2, the basal serotonin release into the vasculature was reported to be ten times higher than that into the gastric lumen [36]. This may be of particular interest in HGT-1 cells as they have previously been used to study the influence of a number of compounds on proton secretion [22]. Hence, we tested the effect of serotonin on proton secretion from HGT-1 cells, showing incubation with 0.1 µM serotonin to significantly reduce proton secretion, thus suggesting serotonergic pathways may influence proton secretion in this cell line similarly as suggested by Lai et al. [26]. The 5-HT_3 receptors are also of interest in the context of gastric emptying. An *in vivo* study on male Wistar rats carried out by Doihara et al. [37] reported a delay in gastric emptying by TRPA1 agonists involving a serotonergic pathway. In this study, a delay in gastric emptying was observed after administration of 1 mg x kg^{-1} serotonin. The effect of TRPA1 agonists on gastric emptying was abolished in experiments using either the TPH inhibitor PCPA or the 5-HT_3 receptor inhibitor granisetron. Additionally, serotonin has been suggested to play a role in emesis [38 35].

As a HTR3-involving delay in gastric emptying after serotonin administration has been described previously[37], we assessed the effect of L-Arg, an amino acid recently shown to delay and inhibit gastric emptying in a study on male Sprague-Dawley rats [25] and to enhance gastric adaptive relaxation, on gastric motility on human stomach strips. This indicated L-Arg to increase baseline gastric muscle tension. Next, we tested the effect of L-Arg on serotonin release from HGT-1 cells. This showed, both by ELISA and LC-MS/MS, treatment with L-Arg to increase the serotonin content in the cellular supernatant collected. The increase could either be due to a rise in serotonin release or due to an impact on the serotonin transporter SERT. *SERT* expression has been reported in the stomach [39] and could also be detected by qPCR in our study. Our microarray experiment revealed

treatment with L-Arg to have a strong impact on gene expression of HGT-1 cells. Pathway analysis with the free online tool DAVID showed the cluster with the second highest enrichment score to consist predominantly of genes involved in serotonin release. As a result, we carried out qPCR experiments, showing L-Arg to alter the gene expression of *HTR3C*, *HTR7*, *SERT*, and *TPH2*. Interestingly, even though TPH2 has been described to be most abundant in the brain, we could detect this isoform by Western blot and treatment with L-Arg influenced only the expression of *TPH2*, not however, *TPH1*. As L-Arg influenced the expression of *HTR3* and the *HTR3C* subunit has previously been described in the modulation of gastric acid secretion [26], we carried experiments into a possible link between L-Arg induced serotonin release and the 5-HT_3 receptor by performing co-incubation experiments with the 5-HT_3 receptor antagonist granisetron. The increase in extra-cellular serotonin after treatment with L-Arg was prevented by co-incubation with granisetron, indicating a 5-HT_3 receptor-mediated pathway may be involved. A similar control of serotonin release by 5-HT_3 autoreceptors has been shown for enterochromaffin cells from isolated intestinal segments of guinea pigs [40]. Moreover, a study showing delayed gastric emptying after serotonin administration, revealed this effect to be abolished by either inhibiting the 5-HT_3 receptor or TPH as well [37]. In the present study, the effect of L-Arg on proton secretion was decreased by granisetron co-incubation, pointing to involvement of 5-HT_3 receptors not only in the regulation of serotonin release, but also of the proton secretion HGT-1 cells.

In order to test whether the results obtained from HGT-1 cells may point to a possible regulation *in vivo* and may thus be used as a screening system for identifying potentially interesting compounds, will need to be addressed in either human intervention studies or animal trials. In addition, the mechanism linking gastric acid and serotonin release may be analysed further in HTR3 knock-out models and by identifying cellular mediators involved such as Ca^{2+} mobilization, cAMP or ERK activation.

In conclusion, this study shows serotonin synthesis and release from HGT-1 cells as model system for peripheral serotonin in the stomach. Furthermore, experiments using the 5-HT_3 receptor antagonist granisetron suggest this serotonin autoreceptor to be involved in both proton and serotonin secretion, although, to elucidate the clinical impact serotonin may have after release from endocrine cells of the stomach, animal trials and human intervention studies need to be conducted.

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Table 1: Clusters generated by DAVID functional annotation clustering software using the microarray data gathered after 3h incubation with L-Arg or DMEM only.

A:cluster 1, enrichment score: 3.41		
	p-value	Benjamini
disulfide bond	1.14E-05	3.75E-03
glycoprotein	4.90E-04	3.30E-02
glycosylation site: N-linked	1.00E-03	2.80E-01
signal peptide	1.90E-03	3.90E-01
signal	3.40E-02	5.20E-01

B: cluster 2, enrichment score: 3.14		
	p-value	Benjamini
serotonin receptor signaling pathway	2.30E-07	4.60E-04
G-protein coupled serotonin receptor activity	2.30E-06	1.30E-03
serotonin binding	5.00E-05	1.40E-02
serotonergic synapse	1.70E-04	1.30E-02
5-hydroxytryptamine receptor family	8.10E-04	4.90E-01
neurotransmitter receptor activity	2.70E-03	3.10E-01
release of sequestered calcium ion into cytosol	1.50E-02	8.90E-01
adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway	2.40E-02	9.20E-01
vasoconstriction	5.80E-02	9.60E-01
dendrite	2.10E-01	9.30E-01

Table 2 qPCR data obtained after treating HGT-1 cells with 30 mM L-Arg for 3h. Data are shown as average ± SEM; n = 4-5, tr= 2-3; Statistics: Mann-Whitney Rank Sum Test

	control	L-Arg	p-value
TPH1	1.00 ± 0.04	1.50 ± 0.16	0.078
TPH2	1.00 ± 0.11	10.0 ± 2.0	<0.001
SLC6A4	1.00 ± 0.05	10.8 ± 1.2	<0.001
HTR7	1.00 ± 0.06	2.88 ± 0.57	0.034
HTR3D	1.00 ± 0.11	2.83 ± 0.75	0.138
HTR3C	1.00 ± 0.09	2.62 ± 0.45	<0.001

Figure legends

Figure 1 Immunofluorescence of HGT-1 cells. Overlay of nuclear staining and serotonin staining (A), serotonin staining only (B), nuclear staining only (C). A representative image is shown; at least three biological replicates and multiple technical replicates have been stained. Immunofluorescence of HGT-1 cells treated either with DMEM only (D) or tryptophanhydroxylase inhibitor PCPA (E). n=1, tr=2

Figure 2 Immunohistochemical staining for serotonin in human stomach samples. Gastric antral mucosa demonstrating cytoplasmic reactivity in scattered individual enterochromaffin-like cells within gastric glands, overview (A), detail (B).

Gastric adenocarcinoma with cytoplasmic reactivity in scattered individual carcinoma cells, overview (C), detail (D).

Figure 3 Western blot analysis of the protein expression of TPH2. (A) Three biological replicates have been tested for both TPH2 expression and the endogenous control β-actin. (B) Representative image of the full-length blot showing the protein ladder and the TPH2 band. (C) Graph of the serotonin formed normalized to protein concentration and incubation time *vs.* the substrate concentration. Data were collected from 3-4 biological replicates with 1-2 technical replicates.

Figure 4 Influence of 10 µM of the 5-HT_3 receptor antagonist granisetron on L-Arg induced serotonin release. Data are shown as average ± standard deviation calculated in relation to L-Arg (set to 100%). n = 3-4, tr = 2; Statistics: One-Way ANOVA *vs.* control followed by Holm-Sidak *post hoc* test.

Figure 5 (A) Impact of incubation with 0.1 – 1 µM serotonin on proton secretion in HGT-1 cells, n= 3-6, tr= 6; Statistics: one-way ANOVA on Ranks followed by Dunn's *post hoc* test *vs*. control, (B) Impact of 10 µM 5-HT_3 receptor antagonist granisetron on L-Arg induced proton secretion, n= 3, tr= 3-6, Statistics: one-way ANOVA followed by Holm-Sidak *post hoc* test, (C) Impact of incubation with 30 mM L-Arg on gastric motility of human stomach strips. Representative traces for a vehicle control (H2O) and a 30 mM L-Arg treatment

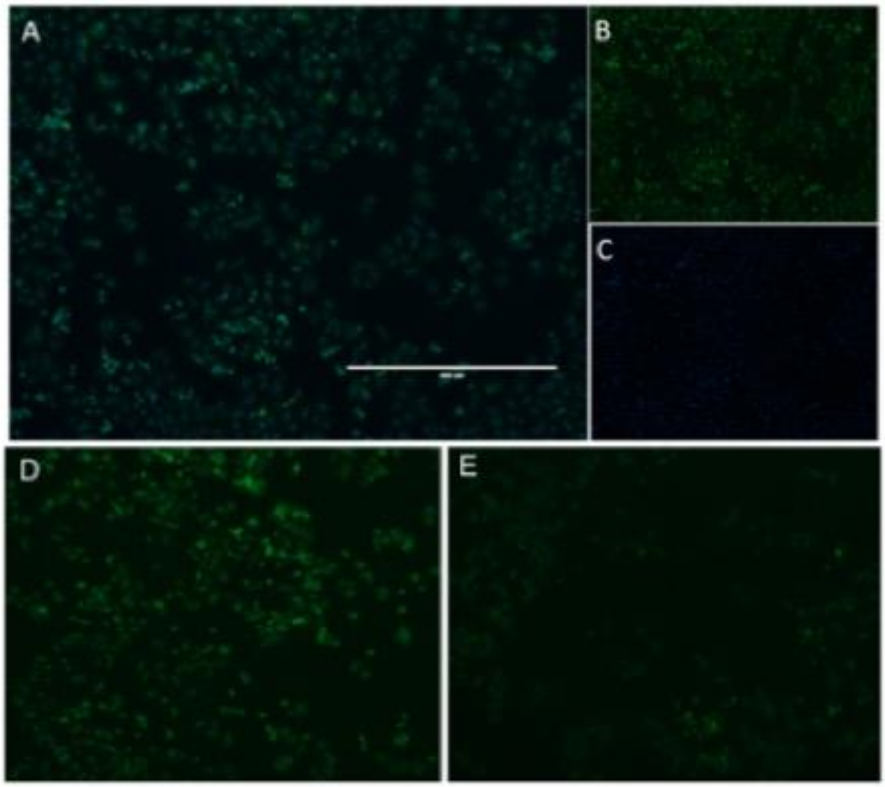


Figure 1

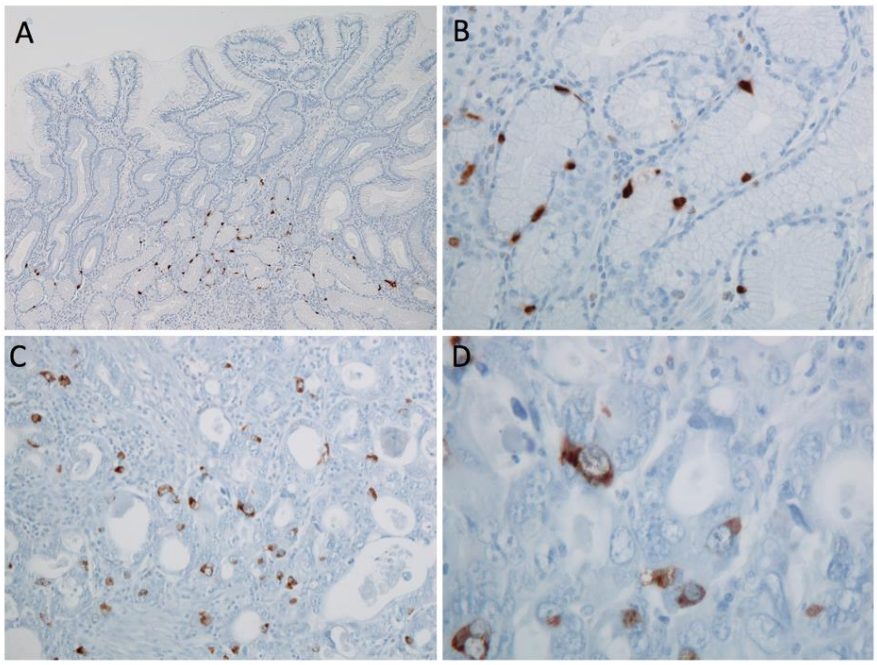
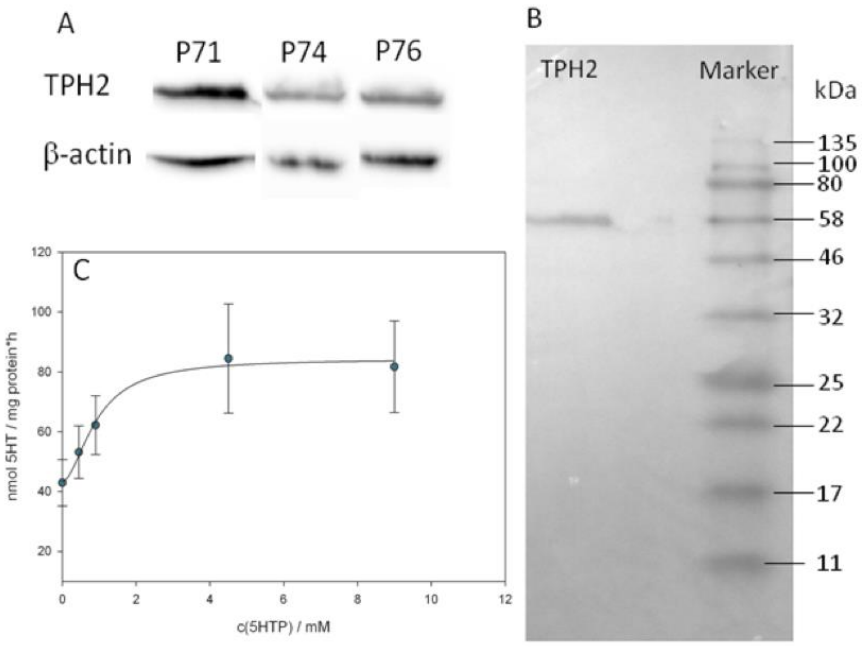


Figure 2


A
P71
P74
P76
TPH2
β-actin
B
TPH2
Marker
kDa
135
100
80
58
46
32
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22
17
11
C
nmol 5HT / mg protein*h
c(5HTP) / mM
120
100
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60
40
20
0
2
4
6
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10
12

Figure 3

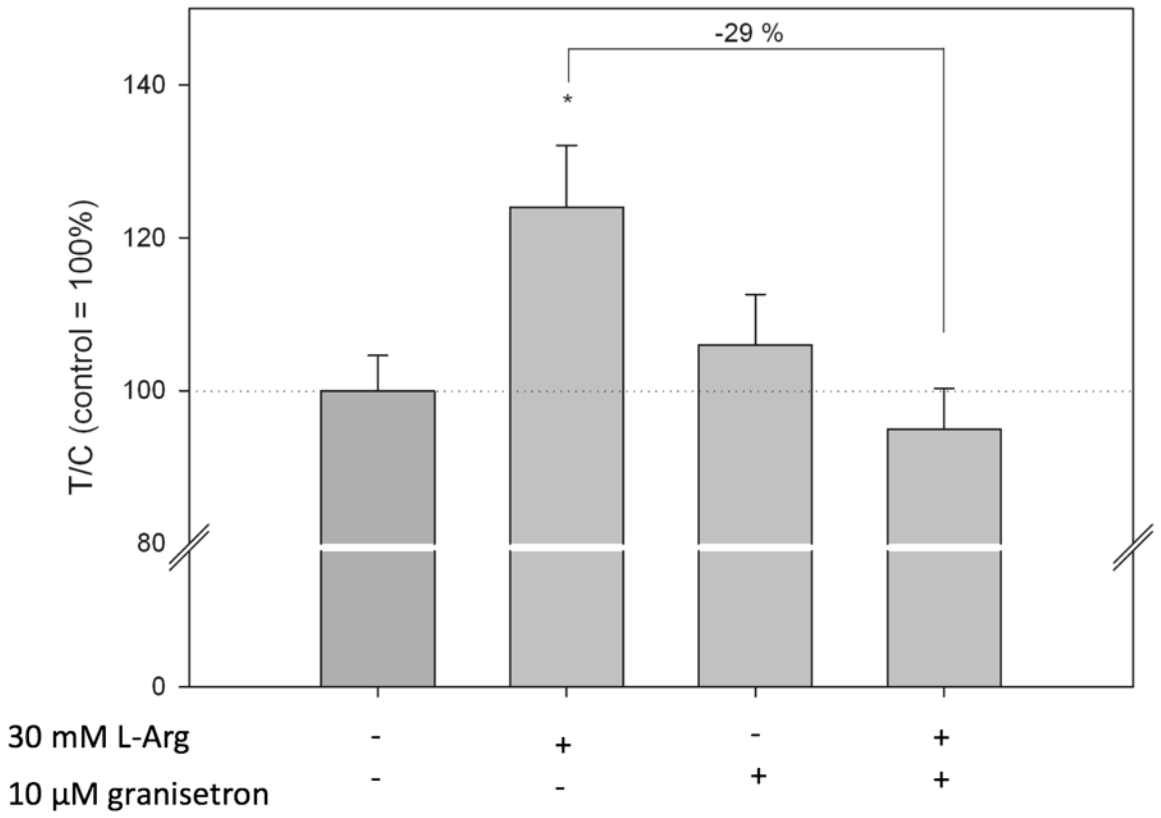

Figure 4

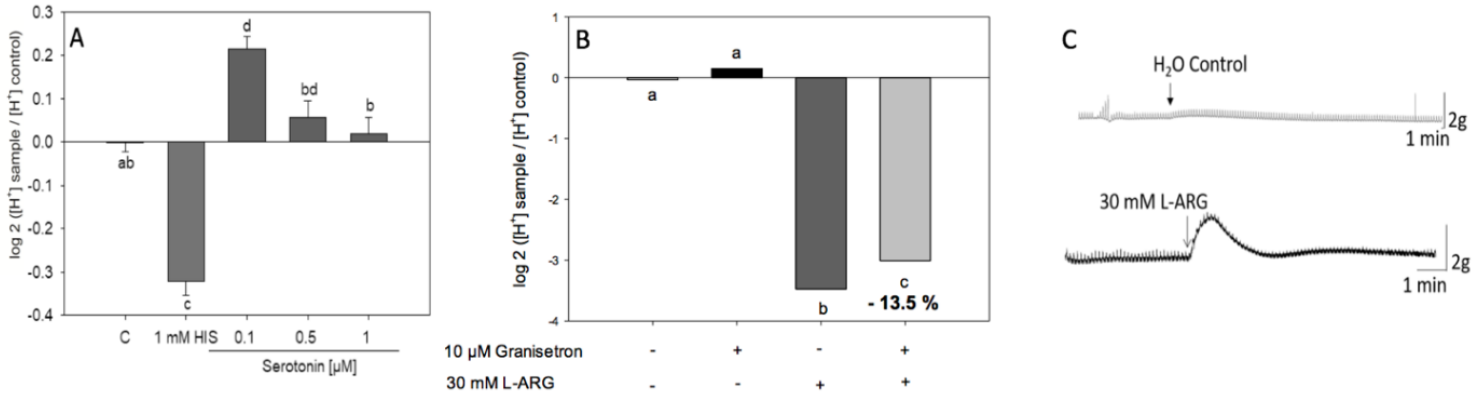
A
log 2 ([H+] sample / [H+] control)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
ab
c
d
bd
b
C
1 mM HIS
0.1
0.5
1
Serotonin [µM]
B
log 2 ([H+] sample / [H+] control)
1
0
-1
-2
-3
-4
a
a
b
c
- 13.5 %
10 µM Granisetron - + - +
30 mM L-ARG - - + +
C
H2O Control
2g
1 min
30 mM L-ARG
2g
1 min

Figure 5

Supplemental information

Methods and materials

Immunofluorescence (HGT-1 cells)

Next, the fixed cells were washed twice with PBS and blocked for 1 h with 5% FBS and 0.5% Triton X-100 in PBS (blocking solution) at room temperature and gentle agitation. Then, the solution was exchanged with primary antibody (1:100, anti-serotonin, raised in rabbit, Merck Millipore) diluted in blocking solution and incubated first at 37°C prior to incubation for 15 min at room temperature with gentle agitation. Thereafter, the cells were washed four times with PBS for five minutes each. Then, secondary antibody (1:1000, Alexa-488, goat anti-rabbit (Molecular Probes, Thermo Fisher Scientific), diluted with PBS containing 5% FBS and 0.2% Triton X-100) was added and incubated for 1 h at room temperature with gentle agitation. Afterwards, the cells were washed four times with PBS for 5 min each. During the last wash, 5 µL of DAPI stain (fixed cells nucBlue, Molecular Probes, Thermo Fisher Scientific) were added. Finally, the coverslip was briefly dipped into ddH_2O and mounted using Immu-Mount solution (Life Technologies, Thermo Scientific). Fluorescent images were taken using an EVOS FL microscope (Thermo Fisher Scientific) equipped with an Olympus 10x objective. For control purposes, images of untreated cells, cells treated with primary antibody only and cells treated with secondary antibody only were taken. For testing the specificity of the primary antibody, some coverslips were incubated with primary antibody, which was exposed to pure serotonin for 30 min prior to the experiment. Data analysis was carried out using Image J, assessing the fluorescence of at least 150 cells per image of two technical replicates.

Serotonin ELISA

Briefly, HGT-1 cells were seeded in 24-well plates at a density of $1.5x10^5$ cells per well. One day after seeding, the cells were washed with pre-warmed PBS and subsequently incubated with 200 µL KRHB (supplemented with 0.1% ascorbic acid, pH 7.4) or 30 mM L-Arg for 5 min in a humidified incubator at 37°C in the dark. Since L-Arg induced a change in the pH, both a pH control and a pH adjusted L-Arg solution were tested in addition. The incubation solutions having a pH of 9.5 were passed through a 0.22 µm filter prior to incubation to remove insoluble material. To assess whether 5-HT_3 receptors are involved, co-incubations with 10 µM 5-HT_3 antagonist granisetron were carried out. After incubation, the supernatant was collected, diluted 1:5 with KRHB and the serotonin concentration assessed using a serotonin-sensitive ELISA (DLD Diagnostika, Hamburg, Germany). Caco-2 cells were seeded in 12-well plates and differentiated for 21 days, within which period the

culture medium was exchanged every two to three days. On day 21, the cells were washed with pre-warmed PBS, incubated for 5 min with 150 µL KRHB (+0.1% ascorbic acid), the supernatant collected and used in the ELISA without further dilution. Similarly, QGP-1 cells were seeded at a density of 250 000 cells per well in 24-well plates, settled for 72 h, and incubated with 250 µL KRHB (+0.1% ascorbic acid) and the collected supernatant centrifuged for 5 min at 1,000x g at 4°C and tested for its serotonin content without further dilution.

LC-MS/MS

The cells were seeded and incubated as described above. However, the collected supernatants were acidified with 0.1% formic acid, and passed through a 0.2 µm filter prior to LC-MS/MS analysis. A total of 50 µL was injected. The analysis was carried out on a Shimadzu LC-MS 8040 equipped with a Synergi 4 µm Fusion-RP 80 A column (150 x 2 mm, Phenomenex) and a security guard cartridge (Phenomenex). A binary gradient of water (+0.1% formic acid, A) and acetonitrile (+0.1% formic acid, B) was used in the chromatographic separation: 0-3 min 0% B, 3-10 min change to 90% B, 13-18 min 90% B, 18-23 min change to 0% B, 23-30 min 0% B. In order to protect the MS from contamination, the eluent was directed to the waste outlet from 0 to 2 min and 8.7 to 30 min, while everything eluting between 2 and 8.7 min was directed to the MS. The MRM settings chosen are shown in Table 2. The MS interface was set as follows: nebulizing gas flow 3 L min^{-1}, DL temperature 150°C, heat block temperature 350°C and drying gas flow 17 L min^{-1}. The calibration range used for the quantification of serotonin in HGT-1 supernatants was 0.4 – 8.3 ng mL^{-1}.

Tables

Table S1 Sequence and product size of the primers used for qPCR experiments

Target	Forward primer	Reverse primer	Product size
TPH1	TAAGACCTGGGGAACCGTATT	TGGAAAAACCTGTACGCTCTTT	173
TPH2	ATCTCGGCGAAGAAGTTCTGA	CAGGGCACATCCTCTAGCTC	165
AADC	TGGGGACCACAACATGCTG	TCAGGGCAGATGAATGCACTG	121
HTR1A	TCATCGTGGCTCTTGTTCTG	CGGGGTTAAGCAGAGAGTTG	108
HTR1B	CTGGTGTGGGTCTTCTCCAT	AGAGGATGTGGTCGGTGTTC	109
HTR2A	GTTGCTTACTCGCCGATGATA	TGCCAAGATCACTTACACACAAA	144
HTR3A	GAAGCCAACCACCGTATCCAT	CCACATCCACGAACTCATTGAT	218
HTR3B	TCTCCCTACCTCTAAGTGCCA	CTCAATGGTCCCAGATGAGTTC	116
HTR3C	TTCCGGTCTCACTGCCTATATC	AAGGTGAAGGTACAGTTCTGTTG	129
HTR3D	CCCTACGTGGTAAACTTTCTGG	TGTGATGAAGTGCTAGTGGCT	179
HTR3E	AGACGCATCCCGGAACATC	GGCACGAGAAGGTTTATGACA	165
HTR7	CTCCATCACCTTACCTCCACTC	ATGCCACTGCGGTAGAGTAAAT	110
SERT	ACGGAGTTCTACAGAAGGTTGT	ATAGAGTGCCGTGTGTCATCT	118
TBP	CCCGAAACGCCGAATATAATCC	GACTGTTCTTCACTCTTGGCTC	130
GAPDH	AGGTCGGAGTCAACGGATTTG	GGGGTCATTGATGGCAACAATA	95
PPIA	CCACCAGATCATTCCTTCTGTAGC	CTGCAATCCAGCTAGGCATGG	144

Table S2 MRM settings for the simultaneous detection of L-Trp, 5-HTP and serotonin by LC-MS/MS

Target	Transition (m/z)	Dwell time (ms)	Q1 pre bias (V)	CE (V)	Q3 pre bias (V)
L-Trp	205 -> 146	400	-12	-19	-13
5-HTP	221 -> 204	400	-12	-13	-13
	221 -> 162	400	-12	-19	-13
5-HT	177 -> 160	400	-12	-13	-13
	177 -> 115	400	-12	-30	-13

Figures

Figure S1 (A) Serotonin staining of HGT-1 cells; (B) Serotonin staining of HGT-1 cells at higher magnification

Figure S2 Scatter plot of log2 transformed intensities of control and treatment with L-Arg for 3h. The diagonal line represents a fold change of 1, i.e. equal intensity in control and treatment.

Figure S3 Typical chromatogram of the cellular supernatant collected from HGT-1 cells, showing the traces of L-Trp, 5-HTP and 5-HT (A). In the absence of 5-HTP, its retention time is shown with an arrow. Serotonin in the cellular supernatant after treatment of HGT-1 cells with 30 mM L-Arg for 5 min analysed by ELISA (B, n=5) or LC-MS/MS (C, n=4). Data are shown in relation to the control (set to 100%). (D) Typical chromatogram obtained after injection of a mixed standard of 5-HT, 5-HTP and L-Trp. The chromatogram shows 5-HTP at a retention time of 5 min.

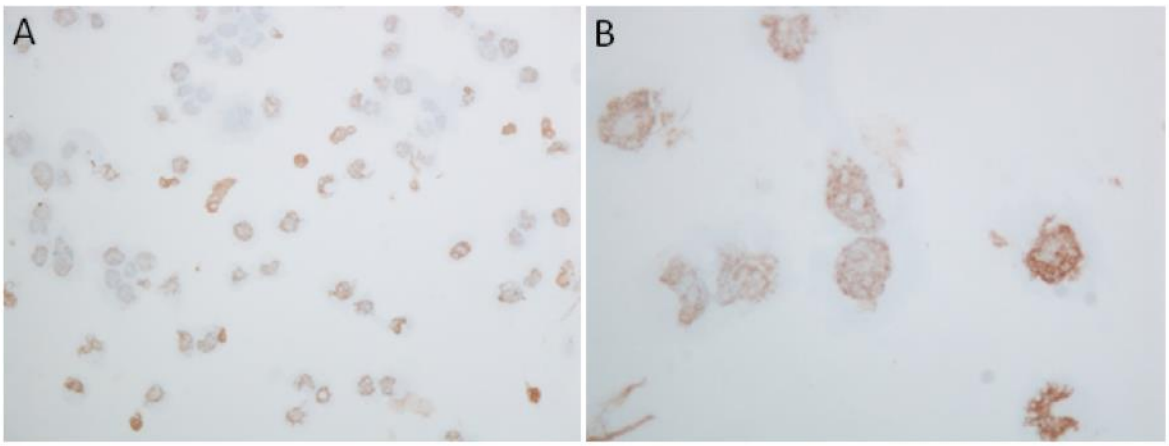

Figure S1

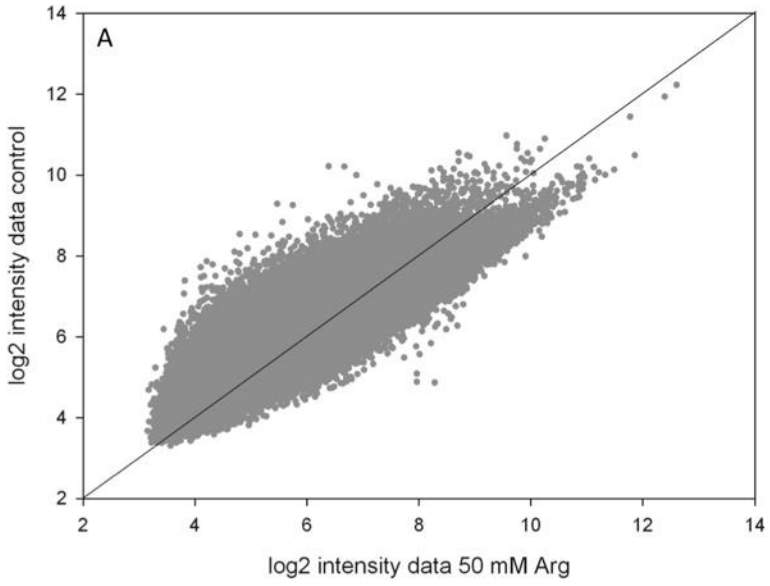
A
log2 intensity data control
log2 intensity data 50 mM Arg
14
12
10
8
6
4
2
2
4
6
8
10
12
14

Figure S2

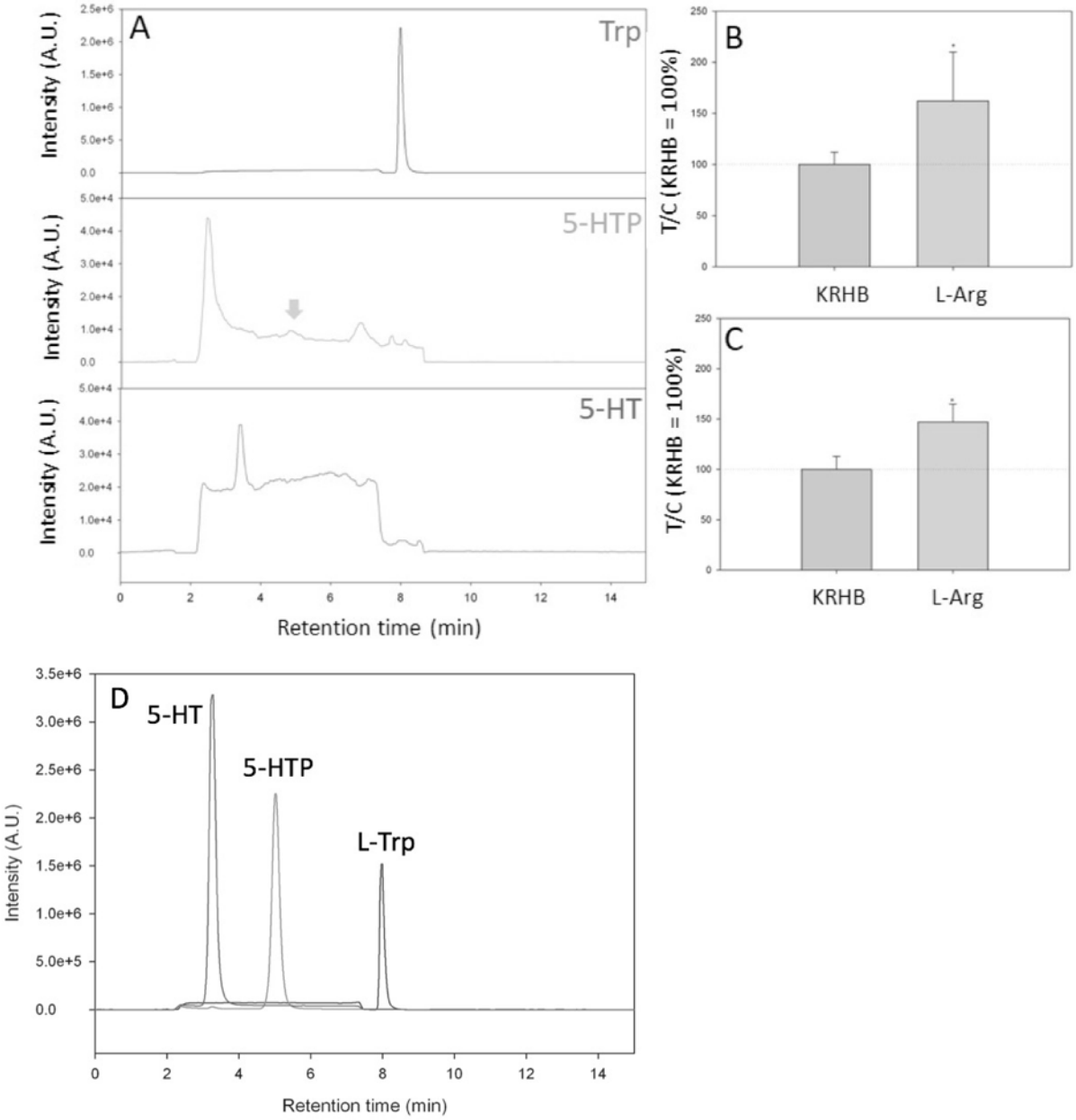
A
Trp
5-HTP
5-HT
Intensity (A.U.)
Retention time (min)
B
C
T/C (KRHB = 100%)
KRHB
L-Arg
D
5-HT
5-HTP
L-Trp
Intensity (A.U.)
Retention time (min)

Figure S3

(3) "Non-caloric sweeteners induce peripheral serotonin secretion via a T1R3-dependent pathway in human gastric cells in culture"

Muhammet Zopun[1], Barbara Lieder[1], Ann-Katrin Holik[1], Jakob P. Ley[3], Joachim Hans[3] and Veronika Somoza[1,2]

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Publised in Journal of Agricultural Food Chemistry 2018, 66, 7044-7053.

This study investigated the effect of NCSs on 5-HT secretion from HGT-1 cells. The secretion of 5-HT was regulated by cyclamate, acesulfame K, saccharin, sucralose, and NHDC, effects of whose were reduced by T1R3 receptor inhibitor lactisole. In addition, the impact of cyclamate and acesulfame K on 5-HT release was decreased in *TAS1R3*-knocked down cells. Besides, an AC inhibitor NKY80, an IP_3 inhibitor neomycin, and a MEK inhibitor PD98059 diminished the effects of all the selected NCSs on 5-HT release. Furthermore, glucose augmented the effect of cyclamate, acesulfame K, saccharin and sucralose on 5-HT secretion, which was diminished by a GLUT inhibitor cytochalasin B.

I participated in the experimental plan and analyzed the effect of NCSs on 5-HT release, cAMP and cell viability in HGT-1 cells. Also, I carried out the *TAS1R3* siRNA knockdown and qPCR experiments of genes relevant for 5-HT secretion induced by NCSs. Furthermore, I did the statistical analysis and prepared the manuscript of draft.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 7044−7053

pubs.acs.org/JAFC

Noncaloric Sweeteners Induce Peripheral Serotonin Secretion via the T1R3-Dependent Pathway in Human Gastric Parietal Tumor Cells (HGT-1)

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Supporting Information

ABSTRACT: The role of sweet taste in energy intake and satiety regulation is still controversial. Noncaloric artificial sweeteners (NCSs) are thought to help reduce energy intake, although little is known about their impact on the satiating neurotransmitter serotonin (5-HT). In the gastrointestinal (GI) tract, 5-HT regulates gastric acid secretion and gastric motility, both part of the complex network of mechanisms regulating food intake and satiety. This study demonstrated a stimulating impact compared to controls (100%) on 5-HT release in human gastric tumor cells (HGT-1) by the NCSs cyclamate (50 mM, 157% ± 6.3%), acesulfame potassium (Ace K, 50 mM, 197% ± 8.6%), saccharin (50 mM, 147% ± 6.7%), sucralose (50 mM, 194% ± 11%), and neohesperidin dihydrochalcone (NHDC, 1 mM, 201% ± 13%). Although these effects were not associated with the sweet taste intensity of the NCSs tested, involvement of the sweet receptor subunit T1R3 in the NCS-evoked response was demonstrated by mRNA expression of *TAS1R3*, co-incubation experiments using the T1R3 receptor antagonist lactisole, and a *TAS1R3* siRNA knockdown approach. Analysis of the downstream signaling revealed activation of the cAMP/ERK/Ca^{2+} cascade. Co-treatment experiments with 10 mM glucose enhanced the 5-HT release induced by cyclamate, Ace K, saccharin, and sucralose, thereby supporting the enhancing effect of glucose on a NCS-mediated response. Overall, the results obtained identify NCSs as potent inducers of 5-HT release via T1R3 in human gastric parietal cells in culture and warrant *in vivo* studies to demonstrate their efficacy.

KEYWORDS: *artificial noncaloric sweeteners, serotonin, sweet taste receptor, HGT-1 cells*

INTRODUCTION

Overweight and obesity and their comorbidities are major health threats in Western countries. Evidence-based factors for undesired weight gain are an inactive lifestyle combined with overconsumption of calories.[1] Sugar-sweetened foods and beverages contribute largely to the overall caloric intake in industrialized countries. One solution to overcome excessive energy intake is the use of noncaloric sweeteners (NCSs) instead of sugar. However, the impact of NCSs on biomarkers of satiety such as the neurotransmitter serotonin is still controversial.[2] NCSs such as cyclamate, acesulfame potassium (Ace K), sucralose, saccharin, and neohesperidin dihydrochalcone (NHDC) are categorized as high-intensity or high-impact sweeteners, since their sweetness potencies are described to be multiple times higher than that of sucrose in lower concentrations. In contrast, glucose is rated 30% less sweet than sucrose.[2]

The sweet taste of sugars and NCSs is mediated by activation of the human sweet taste receptor, a heterodimer consisting of two G-protein coupled receptor subunits, T1R2 and T1R3. Downstream signaling of the T1R2/T1R3 receptor involves the subunits of α, β, and γ of gustducin, phospholipase Cβ2 (PLCβ2), Ca^{2+} as second messenger, and transient receptor potential channel subunit M5 (TRPM5).[3−5]

For the heterodimer T1R2/T1R3, multiple binding sites for structurally diverse ligands have been reported: Whereas the amino-terminal domain of T1R2 is essential for the sweet taste of Ace K, saccharin, and sucralose,[6,7] cyclamate and NHDC bind to the transmembrane domain of T1R3,[8,9] and the natural sugars sucrose and glucose are assumed to bind to the amino-terminal domain of both subunits, T1R2 and T1R3, with distinct binding affinities.[10] For cyclamate, Ace K, and saccharin, a bitter off-taste has been described, which has been traced back to activation of bitter taste receptors (T2Rs). For example, cyclamate activates T2R1 and T2R38,[11] whereas Ace K and saccharin activate T2R31 and T2R43.[12]

Received: April 20, 2018
Revised: June 2, 2018
Accepted: June 6, 2018
Published: June 6, 2018

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

Several years ago, it was reported that T1R receptors are expressed not only in taste buds on the tongue but also in extra-oral tissues, such as the brain[13] and the gastrointestinal (GI) tract.[14] However, presence of T1R1, forming a heterodimer with T1R3, which mediates umami taste, and T1R2 is minor in comparison to that of T1R3 in brush cells and ghrelin-producing cells of the murine stomach.[15] In conjunction with these studies, we recently reported that human gastric parietal tumor cells (HGT-1), which are a well-established *in vitro* model to study mechanisms of gastric acid secretion,[16−19] do express the subunits of the sweet taste receptor, T1R2 and T1R3.[20] In accordance with the results from murine brush and ghrelin-producing stomach cells,[15] the subunit T1R3 is predominantly present in HGT-1 cells, whereas only low levels of T1R2 were detected. In addition, we demonstrated a functional role for T1R3 in proton release induced by cyclamate and Ace K in HGT-1 cells.[20] Moreover, previous studies revealed that the HGT-1 cell line is a suitable model to study peripheral serotonin (5-HT) release, as HGT-1 cells synthesize and secrete 5-HT upon stimulation by arginine, for example.[21] The monoamine neurotransmitter 5-HT is largely localized in the gastrointestinal (GI) tract of mammals, where it regulates a variety of GI functions, including gastric acid secretion[22,23] and GI motility.[24] Sucralose, one of the NCSs, has been demonstrated to increase 5-HT secretion in human normal and neoplastic enterochromaffin cells (EC) *in vitro* via the cAMP/ERK/Ca^{2+} signaling cascade.[25] However, the effect of other NCSs than sucralose on 5-HT release has not been studied so far. Therefore, we hypothesized that NCSs regulate 5-HT secretion in HGT-1 cells and aimed at clarifying if the sweet taste receptor T1R2/T1R3 is involved in this response by using the T1R3 antagonist lactisole and a knock down approach. Furthermore, involvement of the downstream signaling of T1R2/T1R3 was mechanistically investigated.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich unless specified otherwise. Lactisole (99%) was kindly provided by Symrise. Compounds insoluble in water (forskolin, NHDC, cytochalasin B, PD98059) were predissolved in dimethyl sulfoxide (DMSO), with 0.1% (v/v) final concentration during the incubations.

Cell Culture. Human gastric cancer cells, HGT-1 (passage number 56), obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes, France), were cultured as reported previously[26] and kept in growth medium (Dulbecco's modified Eagle medium, DMEM) containing 4 g/L glucose supplemented with 10% FBS, 2% L-glutamine, and 1% penicillin/streptomycin (100 units penicillin, 171 μM streptomycin) under the standard conditions at 37 °C in a humidified atmosphere with 5% CO_2.

Cell Viability Test. MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide) experiments were carried out to exclude a negative influence of the test substances in the chosen concentrations on the cell viability. The assay procedure has previously been described.[20] Briefly, after treatment of the cells according to the subsequent 5-HT release assays with selected compounds dissolved in Krebs−Ringer buffer (KRB, containing 10 mM HEPES, 4.7 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 26 mM $NaHCO_3$, and 1.2 mM KH_2PO_4, pH of 7.4) or KRB with 0.1% DMSO (solvent control), cells were incubated with MTT working solution (0.83 mg/mL MTT) until formation of the purple formazan salt. The remaining MTT was removed from the cells, and the formazan salt was dissolved in DMSO before absorption was determined at 570 nm by means of a Tecan infinite 200 plate reader. Viability of the cells was calculated in percent of control treated cells. Test concentrations that resulted in more than 90% viability in comparison to untreated control were used in following experiments.

Quantitation of 5-HT Secretion. 5-HT secretion was basically carried out as previously described for SH-SY5Y[27] and Caco-2 cells.[28] In order to assess the 5-HT release in HGT-1 cells, 7.5×10^5 cells were seeded in 24-well plates 24 h before use. Cells were washed with PBS once before they were stimulated with 50 mM cyclamate, Ace K, saccharin, or sucralose or 1 mM NHDC in the presence or absence of 50 μM lactisole, 3 μM NKY80, 100 μM neomycin, 10 μM PD98059, or 10 mM glucose or glucose containing 20 μM cytochalasin B dissolved in KRB or KRB containing 0.1% DMSO (solvent control) at 37 °C in a humidified atmosphere of 95% air with 5% CO_2 for 20 min. Forskolin was used as a positive control for 5-HT release.[29] After incubation, the supernatants of treated cells were collected and either used directly or stored at −20 °C for a maximum of 1 week. Quantitation of 5-HT in the supernatant as a measure for 5-HT secretion was performed by using the serotonin high sensitive enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (DLD Diognostika). The 5-HT concentration in the supernatant was calculated by using an external standard curve. Results are provided as % treatment over control ($[\text{control}]_{5\text{-HT}}$ = 1.62 ± 0.42 ng/mL, $[\text{solvent control}]_{5\text{-HT}}$ = 1.20 ± 0.20 ng/mL].

Isolation of RNA, Production of cDNA, and Quantitative Real-Time PCR (qPCR). In order to determine the impact of sweeteners on the mRNA expression level of the targeted genes, qPCR was conducted as described before.[20] Briefly, cells were incubated with 50 mM saccharin or sucralose or 1 mM NHDC dissolved in FBS-free DMEM or FBS-free DMEM containing 0.1% DMSO (solvent control for NHDC) for 10, 20, and 60 min prior to the RNA isolation using the peqGOLD Total RNA Isolation Kit (Peqlab). A total of 2 μg of purified RNA from each sample was subsequently reverse-transcribed using the high capacity RNA to cDNA Kit (Thermo Fisher). The primer pairs for the *TAS1R1* (qHsaCID0013443) and *TAS1R3* (qHsaCED0002321) were purchased from Bio-Rad. The reverse and forward sequences of the specific primers were designed using NCBI Primer Blast (Table 1).

Table 1. Sequences of All Primer Pairs Used in the RT-qPCR Experiments

target	amplicon size	5′→3′ sequence[a]
PPIA[18]	144	F: CCACCAGATCATTCCTTCTGTAGC
		R: CTGCAATCCAGCTAGGCATGG
TBP[27]	130	F: CCCGAAACGCCGAATATAATCC
		R: GACTGTTCTTCACTCTTGGCTC
TAS2R31[26]	218	F: TTGAGGAGTGCAGTGTACCTTTC
		R: ACGGCACATAACAAGAGGAAAA
TAS2R43[26]	148	F: ATATCTGGGCAGTGATCAACC
		R: CCCAACAACATCACCAGAATGAC
SLC2A1[20]	197	F: ATTGGCTCCGGTATCGTAAC
		R: GCTCAGATAGGACATCCAGGGTA

[a]F, forward; R, reverse.

PCR was carried out using Fast SYBR green master mix (Thermo Fisher) on a StepOnePlus (Thermo Fisher) system. The hypothetical starting concentration of mRNA (N_0) was calculated from the amplification curve with LinReg v.12.8, normalized to the geometric mean of the expression of two reference genes, peptidylprolyl isomerase A (PPIA) and TATA-Box binding protein (TBP) (Table 1). The impact of the treatments on gene regulatory level is displayed relative to control cells.

Small Interfering RNA (siRNA) Knockdown of *TAS1R3*. The detailed procedure of the reduction in the mRNA expression of *TAS1R3* by siRNA knockdown and the transient transfection efficiencies measured by means of RT-qPCR have previously been described.[20] Briefly, a number of 3×10^5 cells was seeded in a 24-well plate 24 h prior to the transient transfection of gene-specific siRNA targeting *TAS1R3* (5′-GCCUGAAGAUCCGCUGGCA-3′), pur-

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

chased from Sigma. Cells were transfected with HiPerFect Transfection Reagent (Qiagen, Austria) using mock transfection or transfection reagent containing either 1−10 nM *TAS1R3* siRNA (toxicity was excluded by MTT) or All-stars Negative Control siRNA (Qiagen, Austria), according to manufacturer's protocol (Qiagen, Austria). After 48 h of transfection, the knockdown efficiencies measured using qPCR were approximately 51.0% ± 3.1%.[20]

Quantitation of Intracellular Cyclic AMP Concentration ([cAMP]$_i$). Intracellular cAMP concentration was determined using a commercial cAMP ELISA (R&D system) following manufacturer's recommendation as described previously.[28] Cells were seeded in 6-well plates at a density of 2.5 × 10^6 per well 24 h prior to the experiment. The culture medium was discarded, cells were washed with PBS and subsequently treated for 5, 10, and 20 min with 0.1, 1, and 10 μM forskolin in KRB or KRB containing 0.1% DMSO (solvent control) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Incubation was stopped by placing the culture plates on ice for a couple of minutes before KRB solution was aspirated and cells were washed three times with ice-cold PBS. Cells were lysed in the provided cell lysis buffer (1 × 10^7 cells/mL), and the obtained lysates were collected and frozen at −20 °C until analysis. cAMP concentrations were calculated using an external standard curve. Results are given as % of control treated cells ([control]$_{cAMP}$ = 10.5 ± 2.08 pmol/mL, [solvent control]$_{cAMP}$= 11.0 ± 3.30 pmol/mL]).

Quantitation of Intracellular Total ERK1/2 Concentration ([ERK1/2]$_i$). Analysis of total intracellular ERK1/2 in HGT-1 cells was conducted as described previously,[30] using the ERK1/2 ELISA Kit (ENZO, Enzo Life Sciences (ELS) AG) following the manufacturer's protocol. A total of 7.5 × 10^5 cells per well were seeded in a 24-well plate 24 h prior to a 20 min exposure of the cells to 50 mM cyclamate, Ace K, saccharin, or sucralose or 1 mM NHDC with or without 10 mM glucose or 10 μM PD98059 dissolved in KRB or KRB containing 0.1% DMSO (solvent control). HGT-1 cells were then harvested and centrifuged (1400 rpm for 7 min at 4 °C). After discarding the supernatant, cell pellets were briefly washed with Hank's Balanced Salt Solution, and the cells were pelleted again followed by lysis. Afterward, cell lysate was centrifuged (16 000*g* for 20 min at 4 °C), and supernatant was analyzed for the total ERK1/2 content using the total ERK1/2 ELISA Kit (ENZO, Enzo Life Sciences (ELS) AG) according to manufacturer's protocol. The absorbance was measured at 450 nm by means of an Infinite M200 Plate Reader (Tecan, Switzerland). Calculation of [ERK1/2]$_i$ in the sample was analyzed from four-parameter logistic fitting curve in comparison to the control, as % of untreated control cells ([control]$_{ERK1/2}$ = 14.3 ± 2.24 pg/mL, [solvent control]$_{ERK1/2}$ = 15.4 ± 3.87 pg/mL]).

Statistics. Values are expressed as the mean fold change ± standard error of the mean (SEM) calculated from at least three biological replicates unless indicated otherwise. Comparison of two treatments was done by Student's *t* test, whereas one-way ANOVA followed by Holm−Sidak post hoc test was applied for comparison of multiple treatments. A two-way ANOVA was applied for analyzing time- and compound-specific effects. All calculations were carried out using Sigma Plot software 11.0v (Systat Software), and a *P* value ≤0.05 was considered statistically significant.

RESULTS

Effects of Sweeteners on 5-HT Secretion. In a first set of experiments, the effects of 50 mM glucose, cyclamate, sucralose, Ace K, or saccharin or 1 mM NHDC on 5-HT secretion in HGT-1 cells were analyzed. NHDC was tested at a lower concentration of 1 mM due to its limited solubility in water. All tested NCSs significantly stimulated 5-HT secretion in comparison to control treated cells (100% ± 3.2%) up to 200% ± 13% for treatment with 1 mM NHDC or 157% ± 6.3%, 197% ± 8.6%, 147% ± 6.7%, or 194% ± 11% for treatment with 50 mM cyclamate, Ace K, saccharin, or sucralose, respectively (Figure 1A, each compound vs control,

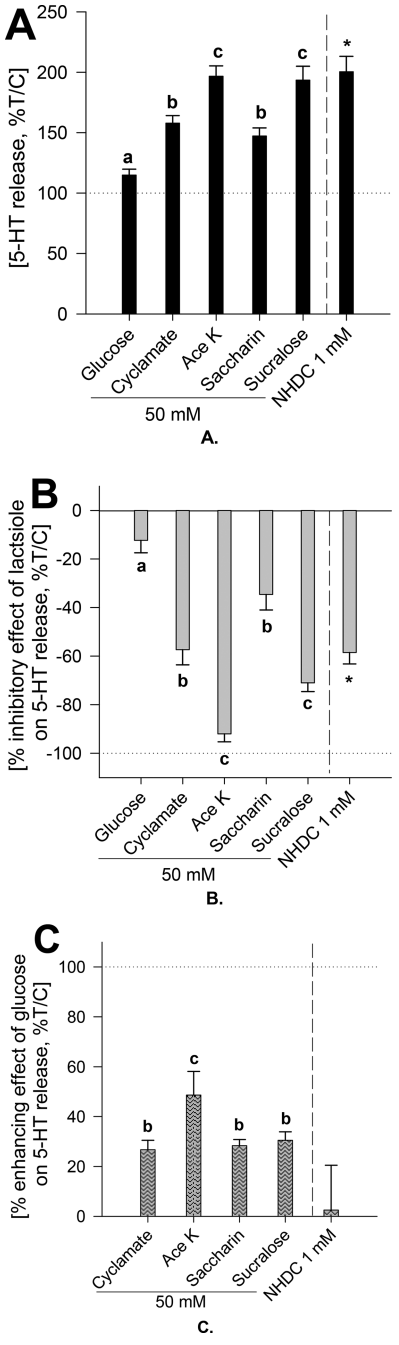

Figure 1. Impact of sweet tastants on 5-HT release in HGT-1 cells. (A) Cells were treated with glucose, cyclamate, Ace K, saccharin, sucralose, or NHDC for 20 min. (B) Inhibitory effect of T1R3 inhibitor lactisole on sweetener-induced 5-HT release. Cells were treated with sweeteners in the presence of 50 μM lactisole for 20 min. (C) Effect of glucose on 5-HT release induced by NCSs. Cells were incubated with cyclamate, acesulfame K, saccharin, sucralose, or NHDC in the presence of 10 mM glucose for 20 min. All results are displayed as [%T/C] and illustrated in comparison to control cells (=100%) or DMSO control 0.1% v/v, solvent control for NHDC (=100%) as the mean ± SEM, n = 3−6. Statistics: one-way ANOVA Holm−Sidak post hoc test; significances are marked with letters (control cells = a, not shown); $P \leq 0.05$. Statistics: Student's *t* test for (A) control vs NHDC, (B) NHDC in the presence of lactisole vs NHDC, or (C) NHDC in the presence of glucose vs NHDC, and significances are marked with the *; $P \leq 0.05$. Treatment with 50 μM lactisole or 10 mM glucose alone did not modify 5-HT release (Student's *t* test, $P > 0.05$, data not shown).

DOI: 10.1021/acs.jafc.8b02071
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Table 2. mRNA Regulation of Target Genes after Treatment of HGT-1 Cells with 50 mM Saccharin, Sucralose, or 1 mM NHDC[a]

target	treatment	10 min	20 min	60 min
TAS1R1	saccharin	1.01 ± 0.10	1.08 ± 0.06	1.04 ± 0.05
	sucralose	1.12 ± 0.07	1.02 ± 0.07	0.99 ± 0.07
	NHDC	1.09 ± 0.08	0.87 ± 0.06	0.84 ± 0.06
TAS1R3	saccharin	1.32 ± 0.06*	1.41 ± 0.09*	1.25 ± 0.06*
	sucralose	0.36 ± 0.04*	0.45 ± 0.08*	0.70 ± 0.02*
	NHDC	0.72 ± 0.03*	0.64 ± 0.07*	1.10 ± 0.05
TAS2R31	saccharin	0.82 ± 0.07	0.79 ± 0.06	0.63 ± 0.05*
TAS2R43	saccharin	0.96 ± 0.06	0.85 ± 0.05	0.90 ± 0.05
SLC2A1	saccharin	1.46 ± 0.05*	1.33 ± 0.05*	1.50 ± 0.05*
	sucralose	1.23 ± 0.05*	1.29 ± 0.06*	1.18 ± 0.04*
	NHDC	1.02 ± 0.04	0.95 ± 0.04	0.90 ± 0.04

[a]Data are represented as fold change average ± SEM in comparison to untreated control (set to 1) or 0.1% DMSO control (solvent control for NHDC, set to 1), $n = 3$. Statistics: two-way ANOVA Holm−Sidak post hoc test; significant difference between treatments and control are illustrated with *; $P \leq 0.05$.

$P \leq 0.05$). Treatment with Ace K or sucralose showed a more pronounced effect on 5-HT release in comparison to that of cyclamate or saccharin, whereas 50 mM glucose had no effect (Figure 1A, $P \leq 0.05$).

To analyze the involvement of the sweet taste receptor subunit T1R3, the specific T1R3 inhibitor lactisole was applied at various concentrations, ranging from 5 to 50 μM. The highest inhibitory effect of lactisole was observed at a concentration of 50 μM (Figure S1). As demonstrated in Figure 1B, the presence of 50 μM lactisole reduced the 5-HT secretion evoked by cyclamate (−57.3% ± 6.3%), Ace K (−92.0% ± 3.3%), saccharin (−34.6% ± 6.4%), sucralose (−71.0% ± 3.6%), and NHDC (−58.5% ± 4.7%), compared to treatment with the NCSs solely ($P \leq 0.05$). Inhibitory effect of lactisole on either Ace K- or sucralose-induced 5-HT release was statistically more pronounced in comparison to that of cyclamate or saccharin (Figure 1B, $P \leq 0.05$).

In order to assess whether the combination of a noncaloric sweetener and a caloric sweetener enhances the reception of a noncaloric sweetener alone, the impact of an additional treatment with 10 mM glucose, as caloric sweetener, on NCS-induced 5-HT release was studied. Recently, we have demonstrated the enhancing effect of 5 or 10 mM glucose on cyclamate or Ace K induced proton release in HGT-1 cells. Treatment with 10 mM glucose elicited a higher enhancing effect on proton release in HGT-1 cells compared to a treatment with 5 mM glucose. Therefore, we studied here if a treatment with 10 mM glucose will enhance NCS-induced 5-HT release as well. As displayed in Figure 1C, 10 mM glucose increased the 5-HT release induced by cyclamate (26.7% ± 3.8%), Ace K (48.6% ± 9.5%), saccharin (23.8% ± 2.5%), and sucralose (30.5% ± 3.4%), compared to treatment with the NCSs alone ($P \leq 0.05$). In contrast, 10 mM glucose did not change the 5-HT secretion evoked by 1 mM NHDC ($P > 0.05$).

In order to determine whether glucose transporters are involved in the glucose-mediated increase of 5-HT release, the impact of the nonspecific glucose-transporter inhibitor cytochalasin B[31] was tested. Addition of 20 μM cytochalasin B reduced the enhancing effect of glucose on 5-HT release induced by cyclamate (−24.1% ± 2.8%), Ace K (−43.9% ± 11%), saccharin (−30.2% ± 2.0%), and sucralose (−50.3% ± 7.6%), in comparison to cells co-incubated with NCSs and 10 mM glucose (each $P \leq 0.05$, Figure S1). Treatment with 20 μM cytochalasin B alone did not modify 5-HT release (statistics: Student's t test, $P > 0.05$, Figure S2).

Impacts of NCSs on the mRNA Expression Level of Targeted Genes. In one of our previous studies, an impact of cyclamate and Ace K on the mRNA expression of genes encoding for the umami-taste receptor subunit (*TAS1R1*), the sweet-taste receptor subunit (*TAS1R3*), selected bitter taste receptors (*TAS2R31*, *TAS2R43*), and GLUT-1, *SLC2A1*, has been demonstrated after exposure of HGT-1 cells for 10, 20, or 60 min, whereas the mRNA expression of *TAS1R2* was below the limit of quantification, analyzed by qPCR.[20] In the current study, the impact of saccharin, sucralose, and NHDC on mRNA expression of the same target genes (Table 1) was measured by qPCR after the incubation of the cells with the compound of interest for 10, 20, or 60 min. Exposure of the HGT-1 cells to 50 mM saccharin or sucralose regulated the gene expression of *TAS1R3* and *SLC2A1* solely (Table 2). *TAS1R3* expression was down-regulated after incubating the cells with 1 mM NHDC after 10 and 20 min (Table 2). Expression of *TAS2R31* but not *TAS2R43* was down-regulated after treatment of the cells with saccharin for 60 min (Table 2).

Impact of Cyclamate and Ace K on 5-HT Release in the *TAS1R3*-Knockdown HGT-1 Cells. In order to clarify whether T1R3, the more abundant subunit of the heterodimer T1R2/T1R3 in HGT-1 cells,[20] is involved in 5-HT secretion induced by NCS, we selected cyclamate, known to bind to T1R3,[8] and Ace K, described as ligand to T1R2,[6] as model compounds in *TAS1R3* siRNA-knockdown experiments. As displayed in Figure 2, 5-HT secretion induced by 50 mM cyclamate or Ace K was reduced by 30.4% ± 5.8%, and 47.3% ± 6.4%, respectively, in *TAS1R3*-knockdown HGT-1 cells in comparison to nontreated control cells (100% ± 2.3%, each vs control $P \leq 0.05$).

The Role of cAMP and IP_3-Signaling in NCSs-Induced 5-HT Release. Previous studies with HGT-1 cells demonstrated that a cAMP-mediated pathway might play a pivotal role in taste receptor signaling in this cell line.[26] To investigate the role of cAMP in NCS-induced 5-HT release in HGT-1 cells, we first examined the impact of forskolin, a well-established stimulator of adenylyl cyclase activity that increases cAMP levels,[32] on 5-HT release.

Exposure of the cells to 1 or 10 μM forskolin increased $[cAMP]_i$ after 10 or 20 min incubation in comparison to control cells (Table 3), up to 164% ± 7.6% ($P \leq 0.05$).

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

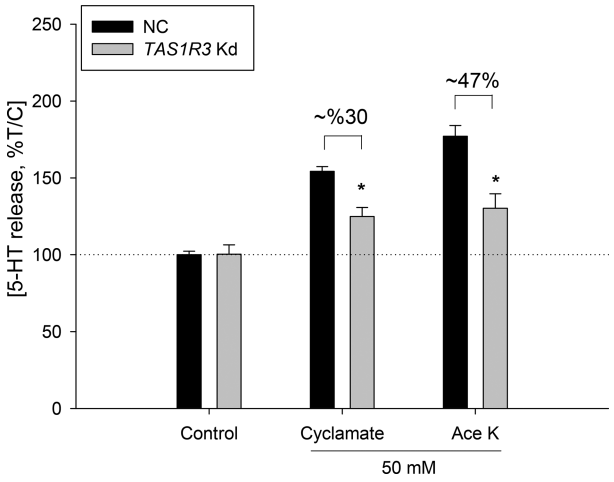

Figure 2. Impact of knockdown of *TAS1R3* on cyclamate- and Ace K-induced 5-HT release. *TAS1R3* was knocked down by 50% using siRNA. TAS1R3-knocked down cells (*TAS1R3* Kd) and nontargeted control cells (All-stars Negative Control siRNA, NC) were stimulated with 50 mM cyclamate or acesulfame K for 20 min, and changes in 5-HT release were calculated. Values are exhibited as [%T/C], mean ± SEM, $n = 4$. Statistics: Student's t test; significances are marked with the *; $P \leq 0.05$. There was no statistically significant difference between nontargeting negative control (NC) and control cells with TAS1R3 siRNA knockdown ($P > 0.05$).

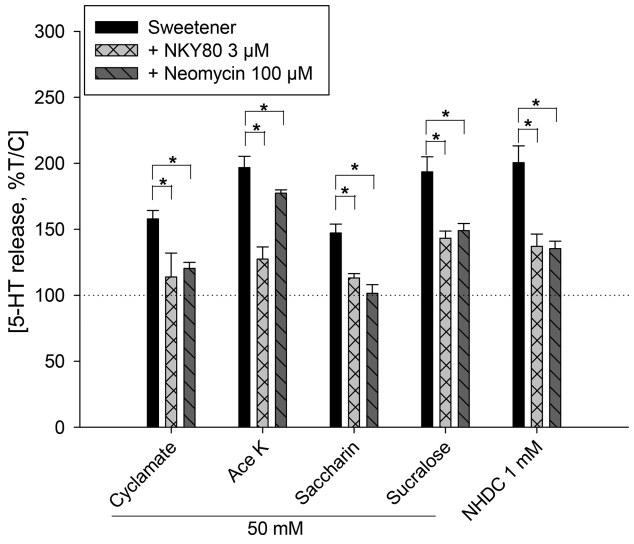

Figure 3. Effect of NKY80 or neomycin on NCS-induced 5-HT release in HGT-1 cells. Cells were exposed to cyclamate, Ace K, saccharin, sucralose, or 1 mM NHDC in the presence or absence of either NKY80 or neomycin for 20 min. Values are presented as [%T/C] and demonstrated in comparison to control (set to 100%) or 0.1% DMSO control (v/v, solvent control for NHDC or NKY80, set to 100%) as the mean ± SEM, $n = 3–6$. Statistics: Student's t test; significances are marked with the *; $P \leq 0.05$. Treatment with 3 μM NKY80 or 100 μM neomycin alone did not regulate 5-HT release (Student's t test, $P > 0.05$, data not shown).

Likewise, incubation with 10 μM forskolin for 10 and 20 min also increased 5-HT release to 123% ± 5.5% and 129% ± 3.7%, respectively. This stimulatory effect of 10 μM forskolin was reduced by co-incubation with 0.3 and 3 μM NKY80, an inhibitor of adenylyl cyclase:[33] one-way ANOVA revealed a significant ($n = 3–4$, $P \leq 0.05$) reduction of 5-HT release by 12.1% ± 2.3% or 8.11% ± 1.4%, respectively (data not shown).

To assess the impact of cAMP signaling on NCS-induced 5-HT release, the effect of the adenylyl cyclase inhibitor NKY80[33] was assessed. As shown in Figure 3, addition of 3 μM NKY80 reduced the 5-HT secretion induced by cyclamate (−44.1% ± 18%), Ace K (−69.3% ± 9.3%), saccharin (−34.1% ± 3.3%), sucralose (−50.3% ± 5.5%), and NHDC (−63.5% ± 9.3%), compared to cells treated with the NCSs solely ($P \leq 0.05$).

As a next step, we investigated the role of IP_3 signaling in NCS-induced 5-HT release as a common pathway for G-protein coupled receptor types. For this purpose, the effect of neomycin, a drug that inhibits IP_3-mediated Ca^{2+} release from intracellular stores,[34] on 5-HT release with or without stimulation by NCSs was investigated. As depicted in Figure 3, neomycin prevented the 5-HT release stimulated by cyclamate (−37.5% ± 4.6%), Ace K (−19.3% ± 2.5%), saccharin (−45.8% ± 6.7%), sucralose (−44.6% ± 5.3%), and NHDC (−65.2 %± 5.6%), compared to cells treated with the NCSs solely ($P \leq 0.05$).

The Impact of ERK1/2 in NCS-Induced 5-HT Release in HGT-1 Cells. As a next step, the impact of the kinases ERK1/2, which play a pivotal role in cAMP/PKA signaling, on NCS-induced 5-HT release was studied. As shown in Figure 4A, treatment of the HGT-1 cells with cyclamate, Ace K, saccharin, or sucralose at a concentration of 50 mM or NHDC at 1 mM increased total $[ERK1/2]_i$ compared to control-treated cells (100% ± 6.8%) ($P \leq 0.05$ vs control). Addition of 10 μM of the MEK1 inhibitor PD98059[35] reduced the effect of NCSs on total $[ERK1/2]_i$, cyclamate (−33.1% ± 6.4%), Ace K (−32.6% ± 2.2%), saccharin (−29.8% ± 4.3%), sucralose (−54.1% ± 2.0%), and NHDC (−57.2% ± 4.0%), compared to cells treated with the NCSs alone ($P \leq 0.05$). Incubation with 10 μM PD98059 alone did not alter total $[ERK1/2]_i$ ($P > 0.05$, data not shown). Moreover, treatment with 10 mM glucose alone in the absence of NCSs enhanced total $[ERK1/2]_i$ by 29.0% ± 1.7% ($P \leq 0.05$, data not shown). Likewise, glucose (10 mM) elevated the effect of NCSs on total $[ERK1/2]_i$: cyclamate (32.7% ± 2.2%), Ace K (24.8% ± 2.4%), saccharin (29.2% ± 3.9%), and sucralose (31.9% ±

Table 3. Impact of Forskolin on $[cAMP]_i$ and 5-HT Release[a]

	$[cAMP]_i$, %T/C			5-HT release, %T/C		
forskolin, μM	5 min	10 min	20 min	5 min	10 min	20 min
0.1	116 ± 11	120 ± 6.3	128 ± 21	103 ± 9.3	121 ± 11	107 ± 5.2
1	107 ± 5.0	164 ± 7.6*	139 ± 7.0*	105 ± 4.8	114 ± 9.8	115 ± 4.9
10	116 ± 10	164 ± 12*	134 ± 3.6*	102 ± 5.2	123 ± 5.5*	129 ± 3.7*

[a]Intracellular cAMP content after incubating the HGT-1 cells with forskolin for 5, 10, and 20 min. 5-HT release after the treatment of the cells with forskolin for 5, 10, and 20 min. Results are displayed as [%T/C] and illustrated in comparison to control (containing 0.1% DMSO, set to 100%) as the mean ± SEM, $n = 3–4$. Statistics: one-way ANOVA Holm−Sidak post hoc test; significances are marked *; $P \leq 0.05$).

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

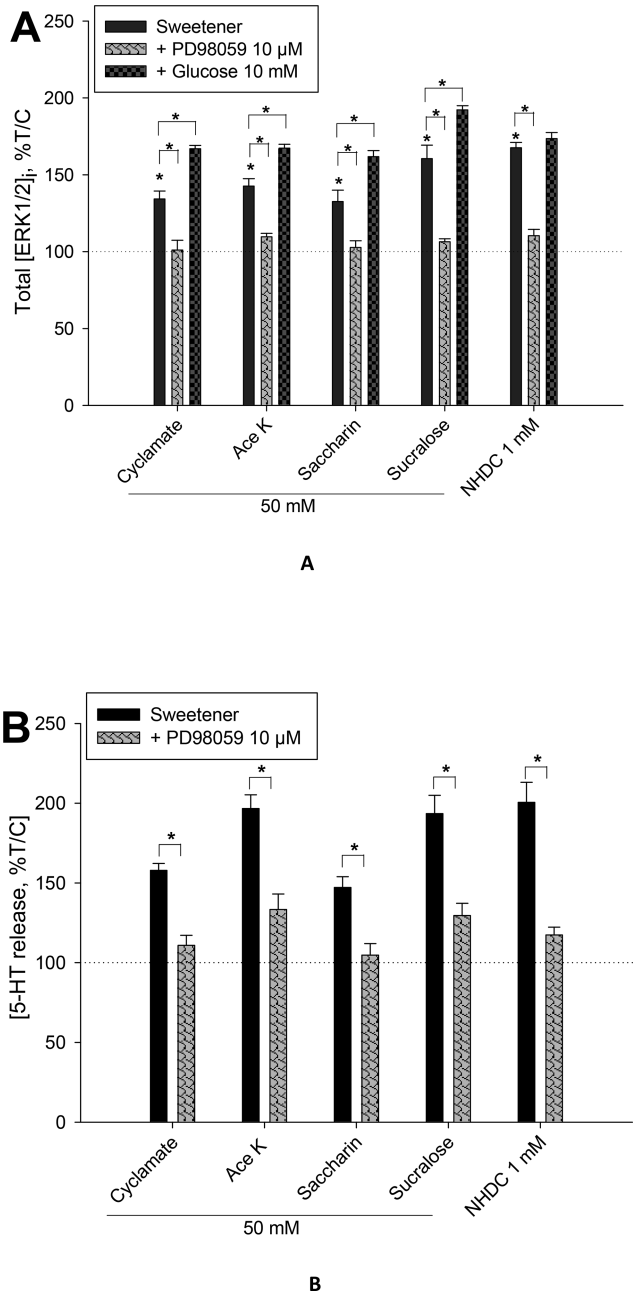

Figure 4. (A) Effect of NCSs on total $[ERK1/2]_i$ in HGT-1. Cells were exposed to cyclamate, Ace K, saccharin, sucralose, or NHDC in the presence or absence of either glucose or MEK inhibitor PD98059 for 20 min. Results are displayed as $[ERK1/2]_i$ in comparison to the control cells (C, or DMSO 0.1% control = 100%) as the mean ± SEM, n = 3−6. Statistics: one-way ANOVA Holm−Sidak post hoc test; significances are marked with the *; $P \leq 0.05$. (B) Effect of MEK inhibitor PD98059 on NCS-evoked 5-HT release in HGT-1 cells. Cells were treated with cyclamate, Ace K, saccharin, sucralose, or NHDC in the presence or absence of PD98059 for 20 min. Results are displayed as [%T/C] and illustrated in comparison to control cells (=100%) or 0.1% DMSO (v/v, solvent control for NHDC or PD98059, set to 100%) as the mean ± SEM, n = 3−6. Statistics: Student's t test; significances are marked with the *; $P \leq 0.05$. Treatment with 10 μM PD98059 alone did not modify 5-HT release (Student's t test, $P > 0.05$, data not shown).

2.7%),compared to cells treated with the NCSs alone, $P \leq 0.05$). However, addition of 10 mM glucose did not augment NHDC-stimulated $[ERK1/2]_i$ ($P > 0.05$).

As shown in Figure 4B, PD98059 reduced the 5-HT release induced by cyclamate (−47.0% ± 6.3%), Ace K (−63.3% ± 9.7%), saccharin (−42.5% ± 7.2%), sucralose (−63.9% ± 7.7%), and NHDC (−83.1% ± 4.8%), compared to cells treated with the NCSs alone ($P \leq 0.05$).

DISCUSSION

High impact sweeteners with no caloric load, also called noncaloric sweeteners (NCSs), are used as sugar substitutes in foods and beverages in order to help to reduce energy intake and maintain a healthy body weight. However, the metabolic effects of NCSs are controversially discussed in the current literature. Besides their beneficial effects on reducing caloric intake, results from metabolic studies investigating the effects of NCSs are conflicting (reviewed by Pepino[36]). Previous studies indicate that caloric and noncaloric sweeteners regulate secretion of satiety hormones via activation of extra-oral sweet taste receptors in the gastrointestinal cells.[37,38] However, less is known about the impact of NCSs and the role of the sweet taste receptor on serotonin release. Although Kidd et al.[25] demonstrated an increased release of the neurotransmitter 5-HT in human neoplastic (KRJ-I) EC cells after stimulation with sucralose, the impact of NCSs on 5-HT release in the stomach has not been studied so far. We have recently reported that HGT-1 cells do express the sweet taste receptor subunits T1R2 and, more abundantly, T1R3,[20] and synthesize and release peripheral 5-HT upon stimulation.[21] In the present study, we hypothesized that incubation with commonly used NCSs will stimulate 5-HT secretion in human gastric HGT-1 cells. In addition, we investigated the functional and mechanistic role of the sweet taste receptor and its downstream signaling in the process of 5-HT secretion by HGT-1 cells.

NCSs are categorized as high-impact sweeteners, since their sweetness potency at lower concentrations is largely increased in comparison to the household sugar sucrose. For example, cyclamate, Ace K, sucralose, and saccharin have been described to be 30, 200, 300, and 600 times sweeter than sucrose, respectively (reviewed by Edwards et al.[2]). All of the NCSs tested in this study, namely cyclamate, Ace K, saccharin, sucralose, and NHDC, stimulated 5-HT release in HGT-1 cells. Among these NCSs, Ace K and sucralose were more potent than cyclamate and saccharin, with glucose showing no effect at concentrations of 50 mM. Given the comparatively low test concentration of 1 mM, NHDC was identified as the most potent compound to stimulate 5-HT release in HGT-1 cells. With respect to glucose, this result is in accordance with previous studies that showed that higher concentrations of glucose than used in the present study (≥100 mM) are required to activate 5-HT secretion in human enterochromaffin BON cells,[39] in intestinal Caco-2 cells,[28] and in EC cells from guinea-pig colon.[40] For the NCSs tested in this study, no literature data is available for their impact on peripheral or central 5-HT release. Overall, the reported sweet taste intensities for the here tested compounds are not linked to their potential to induce 5-HT secretion in HGT-1 cells. However, involvement of the sweet taste receptor cannot be excluded. Thus, as a next step, involvement of T1R2/T1R3 and its downstream signaling has been addressed. First, gene expression analysis of *TAS1R3* after incubation with 50 mM saccharin or sucralose or 1 mM NHDC revealed a time-dependent regulation over a time span of 10 to 60 min. This is in accordance with results from a previous study that

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

demonstrated a regulation of *TAS1R3* after incubation with 50 mM cyclamate or Ace K.[20]

At functional level, however, the impact of cyclamate and Ace K on proton secretion was reversed, as cyclamate induced proton release while Ace K had an inhibitory effect in HGT-1 cells.[20] This demonstrates that an up-regulation or down-regulation of gene expression does not necessarily lead to differences in the protein level or even a functional change. However, it can be concluded that the NCSs do have an impact on the regulation of genes involved sweet-sensing mechanisms of HGT-1 cells. Moreover, *TAS1R1*, encoding for one of the subunits for umami taste receptor T1R1/T1R3, was not regulated by the here investigated NCSs, pointing to T1R3 as a potential target of NCSs in HGT-1 cells. To further investigate the involvement of the sweet taste receptor, the effect of lactisole, a well-known antagonist for the subunit T1R3,[41] was studied, and lactisole produced a reduction in 5-HT release evoked by all tested NCSs, clearly demonstrating an involvement of the T1R3. This result was also confirmed by a small interfering RNA (siRNA) knock-down approach using cyclamate and Ace K as test compounds. Cyclamate was chosen as it was shown to elicit its sweet taste via T1R3,[8] whereas Ace K binds to the N-terminal domain of T1R2.[6] In the *TAS1R3* knockdown cells, the effect size of cyclamate and Ace K on 5-HT release was significantly lowered. We hypothesize that although Ace K, saccharin, and sucralose bind to T1R2, the subunit T1R3 may be crucial for downstream-signaling in certain cell types. This outcome is supported by a previous investigation, demonstrating that the effect of cyclamate and Ace K on proton release was reduced in *TAS1R3* knockdown HGT-1 cells as well.[20] These results suggest that the subunit T1R3 is a crucial site of sweet compound recognition in HGT-1 cells and plays a key role in some subcellular processes in response to NCSs in HGT-1 cells, as it is also the case in other cell models. For example, T1R3 modulates $[Ca^{2+}]_i$ in pancreatic β-cells,[42] adipogenic differentiation in 3T3-L1 cells,[43] and GLP-1 secretion in Hutu-80 cells.[38] Nevertheless, we cannot exclude a role for T1R2 or other so far unidentified GPCRs coupled to the sweet taste receptor in HGT-1 cells. This will be addressed in further studies. In addition, in the current study, the concentrations of NCSs applied to HGT-1 cells were relatively high (50 mM), following the hypothesis that the density of T1Rs and T2Rs expressed in gastric cells is lower than that in oral taste buds, which would require higher concentrations of NCSs to induce a similar response. Likewise, an effect of Ace K, saccharin, or sucralose on GLP-1 secretion in Hutu-80 cells was observed at a concentration of 50 mM.[38] In this context, Nakagawa et al.[44] demonstrated that the sweet taste receptor system expressed in β-cells differs from the canonical sweet taste receptor on the tongue since immunoreactivities of T1R3 detected in MIN6 cells were stronger in comparison to that of T1R2, pointing the presence of T1R3 homodimers. Here, a high concentration of sucralose (50 mM) was required to stimulate insulin secretion in MIN6.

Yee et al.[45] suggested that glucose transporters are coexpressed in mouse T1R3-expressing taste cells and proposed that, at submaximal level, the combination of a NCS with a caloric sweetener would induce a stronger sweet taste response in comparison to a sweetener alone. In addition, T1R3 receptor activation by sucralose was demonstrated to regulate the expression of glucose transporter SGLT-1 and to promote glucose uptake.[14] In the current study, 50 mM glucose alone did not alter 5-HT secretion in HGT-1 cells, whereas the presence of 10 mM glucose enhanced the 5-HT secretion induced by cyclamate, Ace K, saccharin, and sucralose. Such synergism has also been reported in a clinical study by Brown et al.,[46] showing that artificial sweeteners evoked a synergistic effect with glucose to stimulate GLP-1 secretion. In addition, a previous study with HGT-1 cells demonstrated that glucose augmented the cyclamate- and Ace K-induced proton release.[20] The same study also showed that HGT-1 cells express *SLC2A1*, encoding for the glucose transporter GLUT-1, and that cyclamate and Ace K regulated the mRNA expression of *SLC2A1*.[20] Moreover, we previously demonstrated that glucose did not enhance $[cAMP]_i$ in HGT-1 cells, as well as mRNA expression of *T1R3* receptor in HGT-1 cells.[20] The current study addressed the effect of saccharin, sucralose, and NHDC on mRNA expression level of *SLC2A1*, demonstrating regulation by saccharin and sucralose but not by NHDC in HGT-1 cells. This result strengthens the hypothesis that glucose transporters might be involved in increased 5-HT release. Thus, we co-incubated cells with NCSs in the presence of glucose with the nonspecific GLUT inhibitor cytochalasin B[31] and demonstrated that cytochalasin B prevents the impact of glucose on NCS-induced 5-HT release in HGT-1 cells, supporting a role for GLUTs in 5-HT release in HGT-1 cells. This is in the line with the outcome from the study by Kim et al.,[39] demonstrating D-glucose-stimulated 5-HT release to be reduced by the glucose transporter inhibitor phloridzin in BON cells.

Regarding the signal transduction pathways involved in 5-HT release, it has been suggested that cAMP participates in vesicle-membrane docking and exocytosis in the process of 5-HT secretion,[25] and is identified to be one of the key elements in 5-HT release.[29] In accordance with the results from Ohtsu et al.[38] and Kidd et al.,[25] confirming that sweeteners increase intracellular cAMP concentration, a previous study demonstrated an induction of $[cAMP]_i$ in HGT-1 cells after stimulation with NCSs as well.[20] Moreover, the pivotal role of cAMP in 5-HT release in HGT-1 cells was confirmed in the present study by demonstrating that forskolin, a well-known activator of adenylyl cyclase (AC),[32] increased not only cAMP levels but also 5-HT release in HGT-1 cells. von Mentzer et al.[29] demonstrated an inducing effect for forskolin on 5-HT release via enhancing the $[cAMP]_i$ in EC BON cells as well. Moreover, the effect of forskolin on both $[cAMP]_i$ and 5-HT release was reduced by addition of the AC inhibitor NKY80,[33] further supporting the role of cAMP in 5-HT release in HGT-1 cells. Here, we hypothesize that activation of T1R3 receptor by NCSs in HGT-1 cells leads to an increase of cAMP, finally inducing also 5-HT release, as lactisole decreased the elevation of $[cAMP]_i$ induced by cyclamate and Ace K in HGT-1 cells.[20]

Specific downstream signaling following sweet taste receptor activation has not been entirely elucidated. However, signaling transduction cascade of the GPCRs T1R2 and T1R3 has been shown to involve the G-protein gustducin, activating the PLCβ2-dependent pathway to increase $[Ca^{2+}]_i$.[5] Intracellular Ca^{2+} mobilization is known to be involved in 5-HT secretion by facilitating vesicle−membrane docking and exocytosis.[47] Moreover, expression of transducin (*GNAT2*) and α-gustducin (*GNAT3*) has been associated with the involvement of cAMP and IP_3 in downstream signaling of bitter compound-induced proton secretion.[26] A coexistence and interaction of distinct signaling pathways has also been previously demonstrated by Hochheimer et al. in cultured taste cells, resulting in a

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

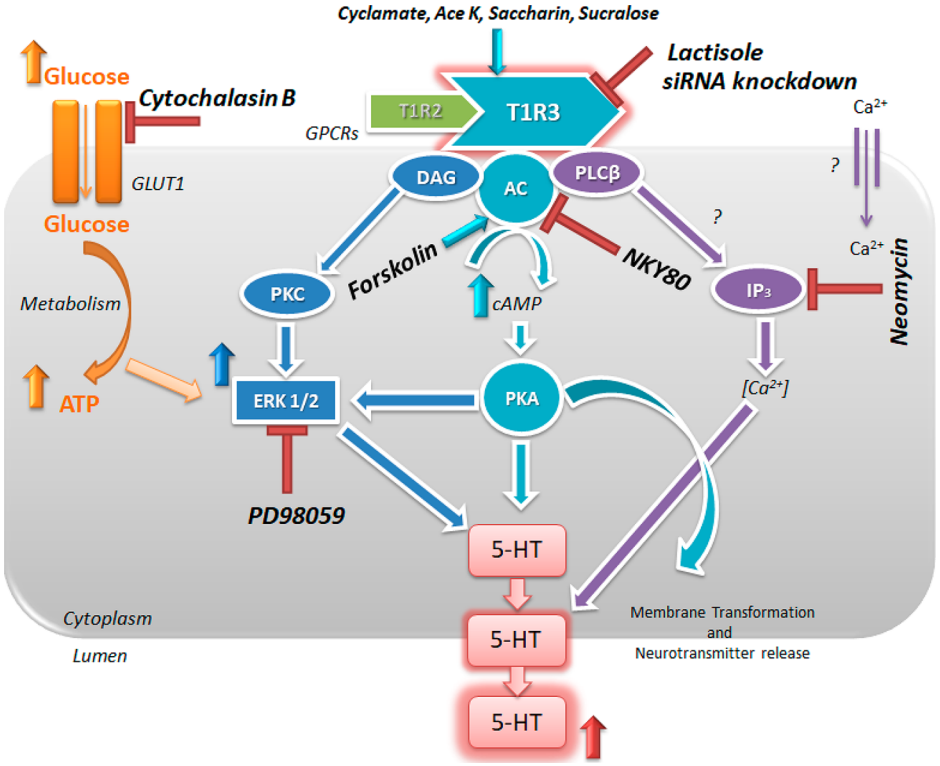

Figure 5. Proposed pathways involved in the cyclamate-, Ace K-, saccharin-, and sucralose-induced 5-HT secretion in HGT-1 cells. The crucial component of the heterodimer sweet taste receptor T1R3 is elicited in green. The glucose-dependent mechanism is displayed in orange. T1R3 agonists and AC activator forskolin are shown in blue. Inhibitors and TAS1R3 siRNA knockdown are represented in red. Activation of T1R3 evokes cAMP/ERK/Ca^{2+} signaling route through inducing AC (shown in light blue), DAG (shown in dark blue), and IP_3 (shown in purple). Glucose is carried into the cell by GLUT-1, and glucose induces the intracellular total ERK1/2. 5-HT secretion is mediated by T1R3 via cAMP-stimulated PKA (which is accompanied by membrane transformation), ERK, and Ca^{2+}.

modulation of the signaling responses to different taste stimuli dependent on the activation pattern of taste receptors.[48] Also in the present study, an involvement of the IP_3-signaling pathway in NCS-induced 5-HT release was demonstrated by addition of neomycin, an IP_3 inhibitor,[34] which led to reduced 5-HT secretion in HGT-1 cells.

Activation of GPCRs, such as T1R2/T1R3, by sweet tastants has been shown to initiate a secondary signaling cascade via cAMP/ERK/Ca^{2+} signaling through AC and IP_3 in neoplastic EC cells, resulting in 5-HT release.[25] Our results support these findings as well: Data on total $[ERK1/2]_i$ and 5-HT release signify that glucose induced total $[ERK1/2]_i$ and enhanced the effect of the cyclamate, Ace K, saccharin, and sucralose on total $[ERK1/2]_i$. Moreover, the MEK-1 inhibitor PD98059[35] prevented an increase in total $[ERK1/2]_i$ as well as 5-HT release evoked by all tested NCSs. These data on ERK1/2 imply (i) that the 5-HT release in response to the NCSs tested here is linked to $[ERK1/2]_i$ and (ii) that glucose participates in 5-HT secretion induced by cyclamate, Ace K, saccharin, and sucralose via $[ERK1/2]_i$ in HGT-1 cells. However, glucose did not enhance the NHDC-induced 5-HT release and NHDC-stimulated $[ERK1/2]_i$, and cytochalasin B did not reduce the effect of glucose on 5-HT release stimulated by NHDC, which did not regulate the mRNA expression of *SLC2A1*. Thus, we hypothesize that NHDC induced different responses in HGT-1 cells than the other tested NCSs. NHDC's hydrophobic character might interact with neighboring bilayer, accompanied by the loss of active GLUT transporter contribution to the action as it has been suggested by Johnston et al.[49] Nevertheless, this needs to be addressed in future NHDC-induced glucose uptake studies. Figure 5 summarizes the pathways involved in the NCS-induced 5-HT secretion demonstrated for HGT-1 cells. Analysis of the downstream signaling proposed that binding of agonists to the sweet taste receptor T1R3 leads to 5-HT release by activating a secondary signaling cascade via cAMP/ERK/Ca^{2+}, thereby increasing intracellular $[ERK1/2]_i$. Except for NHDC, glucose enhanced the NCS-evoked effect on 5-HT release.

For cyclamate, Ace K, and saccharin, a bitter off-taste has been reported that has been traced back to an activation of the bitter taste receptors T2R38 for cyclamate[11] and T2R31/T2R43 for Ace K and saccharin.[12] Since we have previously reported the mRNA expression of *TAS2R43* being regulated in HGT-1 cells after treatment with 50 mM Ace K, whereas cyclamate had no effect,[20] we also addressed the effects of saccharin on the mRNA expression level of the relevant bitter taste receptor genes *TAS2R31* and *TAS2R43*. Here, no regulation was observed after 10 to 60 min incubation with 50 mM saccharin. This finding contradicts a major role for the targeted bitter taste receptors in the described 5-HT release induced by NCSs, although an interaction of downstream signaling pathways mediated by bitter and sweet taste receptors cannot be excluded and has to be elucidated in future studies. In addition, future studies will also address the role of T1R2 in NCS-induced serotonin release from HGT-1 cells as well as the release of 5-HT in response to NCSs from stomach cells *in vivo* to verify the current findings.

DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02071.

Dose-dependent effects of lactisole on NCS-induced 5-HT release and impact of GLUT inhibitor cytochalasin B on the enhancing effect of glucose on 5-HT release induced by NCSs (PDF)

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Funding

Financial support was provided by the Austrian Federal Ministry of Economy, Family and Youth, the Austrian National Foundation for Research, Technology and Development.

Notes

The authors declare the following competing financial interest(s): The authors Jakob P. Ley and Joachim Hans are employees at Symrise AG, Holzminden, Germany.

■ ACKNOWLEDGMENTS

We kindly acknowledge Dr. C. L. Laboisse (Inserm 94-04, Facultè de Medicine, Nantes) for providing the HGT-1 cells, clone6. The financial support by the Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development is gratefully acknowledged.

■ ABBREVIATIONS

Ace K, acesulfame potassium; NHDC, neohesperidin dihydrochalcone; 5-HT, serotonin; NCS, noncaloric sweetener; HGT-1, human gastric parietal tumor cells; FBS, fetal bovine serum; AC, adenylyl cyclase; cAMP, cyclic-adenosine monophosphate; $[cAMP]_i$, intracellular cAMP concentration; ERK, extracellular signal-regulated kinases; $[ERK1/2]_i$, intracellular total ERK concentration; Ca^{2+}, calcium; IP_3, inositol trisphosphate; PKA, protein kinase A; GI, gastrointestinal tract

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DOI: 10.1021/acs.jafc.8b02071
J. Agric. Food Chem. 2018, 66, 7044−7053

Supporting Information

Noncaloric sweeteners Induce Peripheral Serotonin Secretion via the T1R3-Dependent Pathway in Human Gastric Parietal Tumor Cells (HGT-1)

Figure S1. Dose-dependent effect of lactisole on NCSs-induced 5-HT release. **A:** Cells were treated with 50 mM cyclamate in the presence or absence of 0.5, 5 or 50 µM lactisole for 20 min, or with 0.5-50 µM lactisole alone. **B:** Cells were treated with 50 mM Ace K in the presence or absence of 0.5, 5 or 50 µM lactisole for 20 min. All results are displayed as [%T/C] and illustrated in comparison to control cells (=100%) as the mean ± SEM, n = 3. Statistics: one-way ANOVA Holm-Sidak post hoc test; significances are marked with the letters (control cells=a, not shown); $P \leq 0.05$.

Figure S2. Impact of GLUT inhibitor cytochalasin B on the enhancing effect of glucose on 5-HT release induced by NCSs. Cells treated with cyclamate, Ace K, saccharin, sucralose, or NHDC in the presence of glucose were exposed to 20 µM GLUT inhibitor cytochalasin B for 20 min. Results are displayed as [%T/C] in comparison to control-treated cells (control=100%) as the ± SEM, n = 3. Statistics: one-way ANOVA with Holm-Sidak *post hoc* test; significances are marked with ***** ($P \leq 0.05$)

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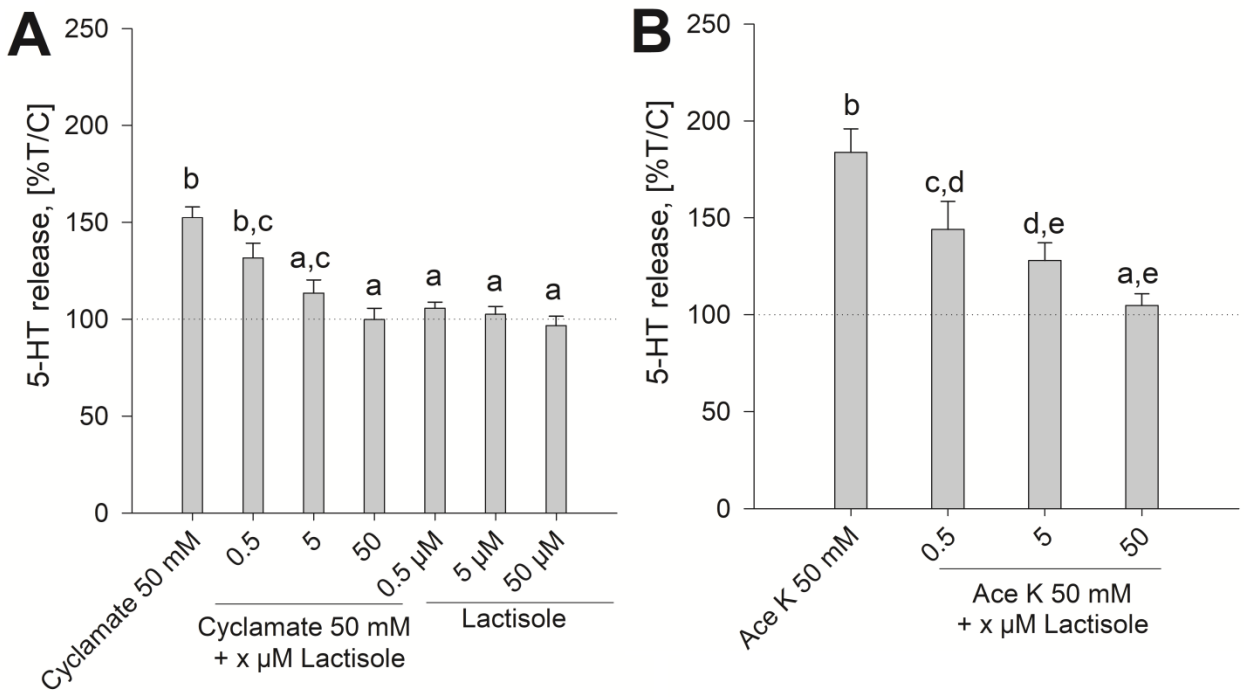
A
B
5-HT release, [%T/C]
250
200
150
100
50
0
b
b,c
a,c
a
a
a
a
c,d
d,e
a,e
Cyclamate 50 mM
0.5
5
50
0.5 µM
5 µM
50 µM
Cyclamate 50 mM
+ x µM Lactisole
Lactisole
Ace K 50 mM
0.5
5
50
Ace K 50 mM
+ x µM Lactisole

Fig. S1.

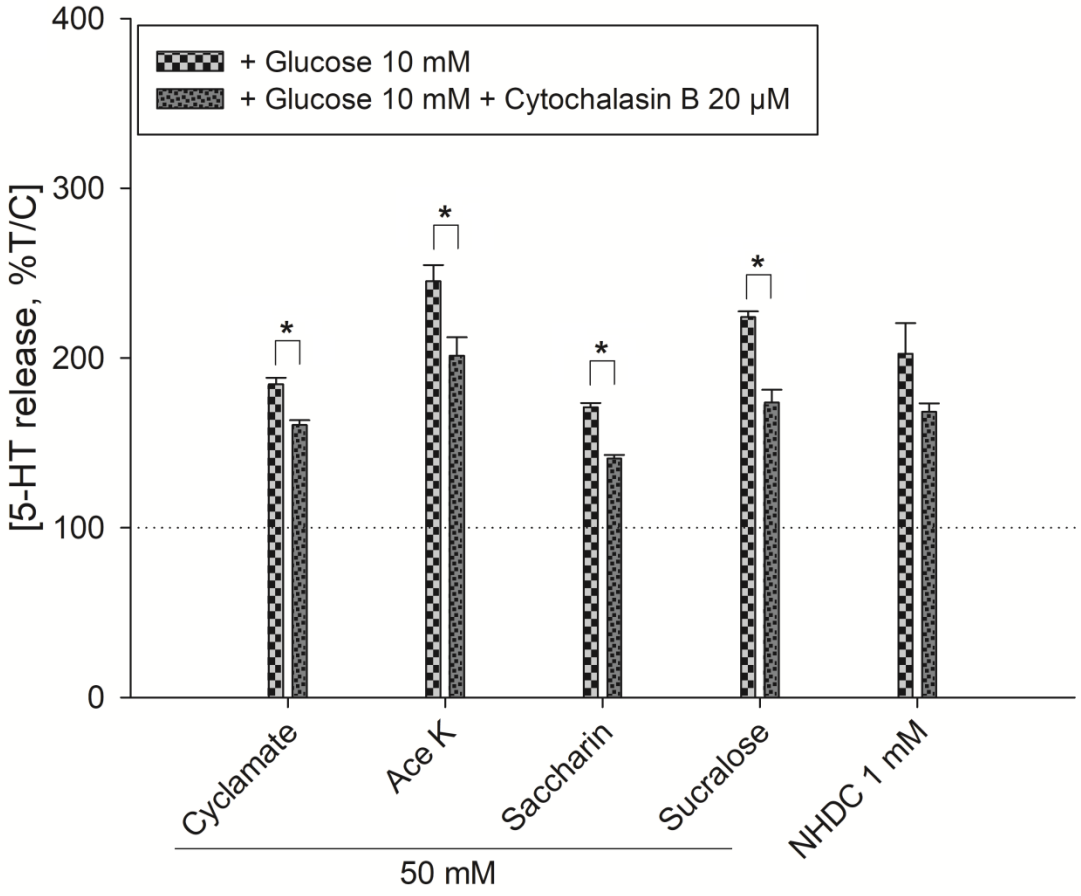
400
300
200
100
0
[5-HT release, %T/C]
+ Glucose 10 mM
+ Glucose 10 mM + Cytochalasin B 20 µM
*
*
*
*
Cyclamate
Ace K
Saccharin
Sucralose
NHDC 1 mM
50 mM

Fig. S2.

IV. Conclusion and Perspectives

Increased energy intake is assumed to be one of the key contributors to the overweight individuals and escalating rates in obesity worldwide. As a consequence, one potential way to combat the obesity issue is to reduce energy intake and increase satiation through NCSs as the sweet taste signaling mechanisms identified in the GI tract influence the regulation of satiety [1]. Therefore, NCSs have been developed and introduced as substitutes for caloric sweeteners in an attempt to control energy balance, or to aim for the maintenance or reduction in body weight. Indeed, NCSs were considered a health benefit due to the fact that they produce a sweet taste without having a caloric load or triggering glycemic effects, and that they regulate GI functions in the GI tract [45, 54–57]. Although the impact of sweet tasting substances, especially NCSs, in the body has been investigated in the GI tract, much less is known about their effects on the mechanisms of GAS and 5-HT secretion, which play a role in the control of satiety [2,3].

Therefore, the aim of the present cumulative thesis was to identify the impact of sweet tastants *in vitro* on proton and 5-HT release, common regulatory factors of GAS. By so doing, we wished to shed some light onto the underlying mechanisms behind proton and 5-HT release, and particularly on the function of the sweet taste receptor T1R2/T1R3 in those processes.

The first study (*(1) "Human Sweet Receptor T1R3 is Functional in Human Gastric Parietal Tumor Cells (HGT-1) and Modulates Cyclamate and Acesulfame K-Induced Mechanisms of Gastric Acid Secretion"*) puts into evidence the impact of NCSs cyclamate, acesulfame K with a bitter aftertaste on proton release in HGT-1 cells. Cyclamate and acesulfame K seem to play different roles in the mechanism of proton release in HGT-1 cells. While the presence of cyclamate decreased the value of intracellular proton index, the IPX, referring to the lower intracellular proton concentration and the larger amount of protons pumped out of cells, acesulfame K had the opposite effect and increased the value of IPX in HGT-1 cells. Concentration-response tests hint at the possibility of a dose-dependent relationship in proton release induced by cyclamate and acesulfame K in HGT-1 cells. Furthermore, mRNA

of *TAS1R3* was induced by cyclamate and acesulfame K, effects of whose on proton release were altered by a well-known sweet taste receptor antagonist lactisole [36], which inhibits the sweet taste in human by acting on T1R3 site of the heterodimeric complex T1R2/T1R3 [36]. Data obtained from functional and transcriptional tests implied that the T1R3 subunit is the main site of the T1R2/T1R3 receptor, which differs from the well-known heterodimeric complex of T1R2/T1R3, as in other cell types [43–45, 53]. Although the T1R3 receptor, when activated by NCSs, led to the regulation of proton release, it seemed that other mechanisms are involved in proton release in the presence of cyclamate and acesulfame K since they played opposing roles in proton release, which continue to function even in *TAS1R3*-knocked down cells. Reportedly, cyclamate binds to the bitter receptors T2R1 and T2R38 [76], whereas acesulfame K binds to the T2R31 and T2R43 [77]. Following this report, cyclamate down-regulated mRNA expression of *TAS2R1* and *TAS2R38* after 20 and 60 min exposure, respectively, while acesulfame K up-regulated mRNA of *TAS2R43* after 60 min treatment. In addition, the inhibitory effect of acesulfame K on proton release was diminished by a T2R43 antagonist 4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV3727) [78]. This outcome suggested an involvement of T2R43 in proton release. Future studies are needed to clarify the opposite effects of these NCSs on proton secretion. After having recognized the involvement of T1R3 receptor in proton release induced by cyclamate and acesulfame K, the role of T1R3 was further elucidated by analyzing NCSs-associated downstream signaling pathways. The presence of cyclamate and acesulfame K led to the elevation of cAMP concentration, which was attenuated by an AC inhibitor NKY80 [79] and T1R3 inhibitor lactisole [36]. This result suggested that proton release is triggered after the initial stimulation of T1R3 by NCSs through intracellular cAMP elevation. In addition, glucose augmented cyclamate- and acesulfame K-induced proton release, and this release is reduced by a K_{ATP} channel inhibitor glibenclamide [80]. Taken together, the glucose-course experiments suggested that proton release induced by NCSs is mediated *via* K_{ATP} channels in HGT-1 cells. In addition to NCSs, the impact of a sweet-tasting amino acid D-threonine [81] in proton release in HGT-1 cells was also tested. D-threonine was found to inhibit proton release in a concentration-dependent manner, and the presence of the T1R3 antagonist

lactisole led to a reduction in D-threonine-induced proton release. However, contrary to NCSs, D-threonine-induced proton release was not reduced by NKY80. Still, we found that the T1R3 receptor was involved in proton release induced by D-threonine in HGT-1 cells. As a result, this first study suggests that T1R3 is the chief functional subunit of the T1R2/T1R3 receptor and that cyclamate and acesulfame K are T1R3-dependent modulators of proton secretion in HGT-1 cells.

It has been suggested that 5-HT inhibits GAS [5–9] and plays a key role in the regulation of gastric motility [10–12] *via* the 5-HT_3 receptor [11]. As 5-HT endocrine cells have been identified in the human stomach [71–73], the investigation of the 5-HT secretory abilities of HGT-1 cells, a well-established *in vitro* parietal cell model that displays the characteristic features of gastric parietal cells [22–24], was conducted in the second study (*(2) "Serotonin biosynthesis and release from human gastric tumour cells (HGT-1) and its functional role in cellular proton secretion"*). Studies performed at the transcriptional and functional levels in HGT-1 cells revealed mRNA expression of the enzymes involved in 5-HT synthesis (TPH1, TPH2, and AADC), as well as several 5-HT receptors (5-$HT_{3C/D}$ and 5-HT_7) and 5-HT re-uptake receptor (SERT). In addition, immunostaining experiments verified that 5-HT is present in HGT-1 cells. Overall, these experiments allowed us to conclude that HGT-1 cells are well-equipped to synthesize 5-HT. We also found that human tissue samples excised from the antrum section of stomach stained positive for 5-HT. Additionally the presence of 5-HT-positive cells in healthy stomach tissue samples has been reported [9]. Taken together, these results indicate that 5-HT is present in both healthy and diseased donors. Given that HGT-1 cells have the ability to secrete 5-HT, we chose to analyze 5-HT secretion upon stimulation with the amino acid L-arginine, an amino acid which regulates food intake [82] and gastric motility [83]. The performed experiments suggested that L-arginine-induced 5-HT secretion is mediated in a 5-HT_3-dependent manner, as the 5-HT_3 receptor is known to be involved in the regulation of GAS [9]. We then investigated the impact of 5-HT on proton release in HGT-1 cells and found that proton release was significantly reduced by 5-HT in HGT-1 cells, suggesting that there is an association between serotonergic pathways and proton secretion in HGT-1 cells, as Lai et al. [9] previously reported. In addition, the impact

of L-arginine on proton secretion was shown to be reduced by granisetron, indicating an involvement of 5-HT_3 receptors.

To summarize, the second investigation led to two major findings: 1) we discovered that the HGT-1 cells, having the capabilities of biosynthesizing 5-HT and secreting it upon stimulation, is a promising cell model system for studying peripheral serotonin in the stomach 2) the 5-HT_3 receptor is involved in both 5-HT and proton secretion.

After comprehensively identifying and describing the ability of HGT-1 cells to produce and release 5-HT, the impact of NCSs on 5-HT secretion were then investigated in the fourth study *((3) ""Non-caloric sweeteners induce peripheral serotonin secretion via a T1R3-dependent pathway in human gastric cells in culture"*). 5-HT secretion was significantly promoted in response to cyclamate, acesulfame K, saccharin, sucralose, and NHDC, and those effects on 5-HT release were reduced by T1R3 antagonist lactisole. Gene expression analysis also suggested that the T1R3 receptor is involved in 5-HT secretion when in the presence of NCSs. This finding was corroborated by a knockdown experiment performed in HGT-1 cells using siRNA targeted against the *TAS1R3* mRNA. Indeed, the impact of cyclamate and acesulfame K on 5-HT release was found to be reduced in *TAS1R3*-knocked down cells. In addition to tasting sweet, it has also been shown that saccharin binds to T2R31 and T2R43 [77]. Therefore, the effects of saccharin on the mRNA expression level of the relevant bitter taste receptor genes *TAS2R31* and *TAS2R43* were also addressed. However, no regulation was observed after 10 to 60 min treatments with 50 mM saccharin. To understand the sole role of T1R3 in 5-HT release induced by NCSs with bitter aftertaste, further studies are needed as downstream signaling pathways controlled by T1Rs and T2Rs are similar. Taken together, functional and transcriptional experiments suggest that T1R3, the key component of the sweet taste receptor in HGT-1 cells, is involved in the NCSs-induced 5-HT release in HGT-1 cells. As a next step, we then analyzed the downstream of T1R3. The impact of all tested NCSs on 5-HT release was reduced when the AC inhibitor NKY80 and IP_3 inhibitor neomycin [84] were present; pointing out to AC and Ca^{2+} being involved in the NCSs-evoked 5-HT release. Except for NHDC, the caloric sweetener glucose enhanced 5-HT release induced by four NCSs (cyclamate, acesulfame K, saccharin, sucralose), and this effect was

attenuated by a GLUT1 inhibitor cytochalasin B [62], suggesting that GLUT1 is involved in 5-HT release stimulated by the four aforementioned NCSs. The total amount of ERK1/2 was increased in response to all tested NCSs, and this effect was attenuated when using a MEK inhibitor PD98059 [85]. In addition, 5-HT secretion induced by all tested NCSs was significantly diminished by PD98059. Overall, these results indicate that ERK play a role in the NCSs-induced 5-HT release in HGT-1 cells. To summarize the third study, four tested NCSs (cyclamate, acesulfame K, saccharin and sucralose) showed distinct patterns of intracellular signals in 5-HT-secreting HGT-1 cells. All tested sweet tastants acted as T1R3 agonists while lactisole acted as a T1R3 antagonist. 5-HT release induced by NCSs is dependent on the activation of the T1R3 which is accompanied by the activation of cAMP/ERK/Ca^{2+} pathway. In order to assess whether the effects obtained from HGT-1 cells can also be observed *in vivo*, further studied in human or animals are warranted. In addition, studies in T1R3 knock-out models need to be performed in order to elucidate the mechanisms of GAS and 5-HT secretion regulated by the T1R3 receptor. Finally, 5-HT_3 knock-out models may serve as models to study the association between GAS and 5-HT secretion.

Worldwide, total food energy consumption has significantly increased in half a century, and the excess energy intake, particularly sweet energy-based, is considered to be one of the major contributors to the rising rates in overweight and obesity [1]. Reports in the literature demonstrate that sweet taste signaling mechanisms elucidated in the oral cavity also function in the GI system and have an impact on the progress of satiety. Therefore, the sweet taste signaling system is essential in understanding the concepts for the development of satiety [1]. The heterodimer complex T1R2/T1R3 is believed to be a central component of one of the sweet taste detection systems [33]. There is, however, evidence indicating that that T1R3 homodimers have been found to recognize sugars [43–45]. Overall, our results and data illustrate the importance and the level of involvement of the sweet taste receptor system in diverse cellular processes in HGT-1 cells. Finally, this cumulative thesis may be beneficial in guiding the design and the format of future *in vitro* and *in vivo* studies dedicated to comprehensively investigate the biological processes affected by artificial NCSs in the GI tract, and especially in the stomach.

V. References

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VI. Abstract

Non-caloric sweeteners (NCSs) are used as sugar substitutes in foods and beverages to increase the sweetness without providing dietary energy. Although their physiological roles in the gastrointestinal tract (GI) have been explored, the impact of NCSs on mechanisms regulating gastric acid secretion (GAS) has not been elucidated *in vitro*. Gastric acid has a remarkable role in the stomach by providing a chemical barrier against ingested pathogens, serving in the digestion of foods, and regulating mechanisms of satiety. GAS can be mediated via hormonal, neuronal, and paracrine pathways. Therefore, the present thesis aimed to identify the impact of NCSs on mechanisms of GAS, and serotonin (5-HT)-one of the regulatory factors of GAS as well as satiation. In addition, the involvement of sweet taste receptors (T1R2/T1R3) and its downstream mechanisms on the modulation of GAS and 5-HT was studied in the HGT-1 cell system (human gastric parietal adenocarcinoma cells). In HGT-1 cells, cyclamate stimulated and acesulfame K inhibited proton secretion, as a key mechanism of GAS. Analysis by means of RT-qPCR and immunocytochemistry revealed that T1R3 is the major unit of T1R2/T1R3 in HGT-1 cells, and mRNA expression of which was regulated by tested NCSs. Moreover, T1R3 receptor antagonist lactisole attenuated the effect of cyclamate, and acesulfame K on proton release. Experiments revealed that HGT-1 cells constitute essential components to produce 5-HT. Regarding this, selected NCSs cyclamate, acesulfame K, saccharin, sucralose and neohesperidin dihydrochalcone (NHDC) promoted 5-HT secretion in HGT-1 cells, and lactisole diminished the NCSs-induced 5-HT secretion. A siRNA knockdown approach targeting *TAS1R3* demonstrated the involvement of T1R3 receptor in both proton and 5-HT secretion induced by cyclamate and acesulfame K. Co-treatment of HGT-1 cells with glucose enhanced the effect of selected NCSs on both proton and 5-HT secretion. As a result, this study exposes a new insight of physiological potentials of NCSs in the field of food substituents in order to enlighten the mechanisms regulating GAS.

VII. Zusammenfassung

Nicht-kalorische Süßstoffen (NKSs) werden als Zuckerersatz in Nahrungsmitteln und Getränken verwendet, um die Süßkraft zu erhöhen, ohne Nahrungsenergie bereitzustellen. Obwohl ihre physiologischen Funktionen im Gastrointestinaltrakt (GIT) erforscht wurden, wurde der Einfluss von NKSs auf Mechanismen zur Regulierung der Magensäuresekretion (MS) *in vitro* nicht aufgeklärt. Magensäure hat eine bemerkenswerte Rolle im Magen, indem sie eine chemische Barriere gegen aufgenommene Pathogene bereitstellt, die der Verdauung von Nahrungsmitteln dient und Sättigungsmechanismen reguliert. MS kann über hormonelle, neuronale und parakrine Wege vermittelt werden. Ziel der vorliegenden Arbeit war es daher, den Einfluss von NKSs auf Mechanismen der MS und auf Serotonin (5-HT), einer der regulatorischen Faktoren der MS sowie Sättigung, zu identifizieren. Darüber hinaus wurde die Beteiligung der Süßrezeptoren (T1R2/T1R3) und deren modulierende Wirkung auf die MS und auf 5-HT in HGT-1 Zellen (humane parietale Magenkarzinomzellen) *in vitro* untersucht. Es konnte gezeigt werden, dass in HGT-1 Zellen die Protonensekretion als Schlüsselmechanismus der MS von Cyclamat stimuliert und von Acesulfam K inhibiert wurde. RT-qPCR Analysen und immuncytochemische Darstellungen demonstrierten, dass T1R3 die Haupteinheit von T1R2/T1R3 in HGT-1 Zellen darstellt und deren mRNA Expression durch die getesteten NKSs reguliert wurde. Darüber hinaus hat Lactisol, ein T1R3 Antagonist, die Wirkung von Cyclamat und Acesulfam K auf die Protonenfreisetzung abgeschwächt. Experimente zeigten, dass HGT-1 Zellen essentielle Bestandteile zur Produktion von 5-HT darstellen. In Anbetracht dessen förderten Cyclamat, Acesulfam K, Saccharin und Sucralose, und Neohesperidin-dihydrochalkon (NHDC) die Sekretion von 5-HT in HGT-1 Zellen, während Lactisol die NKSs-induzierte 5-HT-Sekretion verringerte. Ein *TAS1R3* knockdown mittels siRNA zeigte eine Beteiligung des T1R3-Rezeptors sowohl bei der Protonen- als auch bei der 5-HT-Sekretion, was durch Cyclamat und Acesulfam K induziert wurde. Eine gleichzeitige Behandlung von HGT-1 Zellen mit Glucose und ausgewählten NKSs verstärkte deren Effekt auf die Protonen- und 5-HT-Sekretion. Diese Ergebnisse geben neue Einblicke in die physiologischen Möglichkeiten von NKSs und deren Mechanismen hinsichtlich Regulierung der Magensäuresekretion.