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pH-Dependent Inhibition of the Human Bitter Taste Receptor hTAS2R16 by a Variety of Acidic Substances

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Some acidic peptides are known to reduce bitterness, but the detailed mechanism underlying this effect remains to be elucidated. In this study, we analyzed the effects of acidic dipeptides on the inhibition of the human bitter taste receptor hTAS2R16. Calcium imaging analysis of HEK293T cells expressing hTAS2R16 revealed that their response to the bitter tastant salicin was reduced in the presence of acidic dipeptides. A similar inhibitory effect was observed in a variety of other acids. The inhibition depended on the pH values resulting from the addition of acids but not on their concentrations. Our results suggest that the inhibition of the bitter taste receptors can be attributed to the bitterness-masking effect of the acidic dipeptides and that acidic pH may be one of the critical factors responsible for this sensory event.

KEYWORDS: Bitter taste receptor; hTAS2R16; salicin; bitter masking; acidic peptides; pH

INTRODUCTION

The idea that there are five basic tastes, namely, sweetness, bitterness, saltiness, sourness, and umami, has generally been accepted, and they are regarded as taste modalities that can be discriminated from one another (*1*). Toxic substances are often characterized by a bitter taste, and they induce aversive actions in animals. However, the proper and reasonable use of some bitter food ingredients can provide a unique and favorable character to foods, as in tea, coffee, and beer. It is thus important for the food industry to use bitter taste ingredients properly.

Certain food constituents mask bitterness. A good example of such activity is provided by Glu-Glu, which is present in the acidic fraction of a tryptic hydrolysate of a fish−protein concentrate; the addition of 0.1% Glu-Glu was found to induce a marked decrease in the bitterness scores of a number of bitter substances (*2*). The inhibitory effect of Glu-Glu on the IXth nerve response to bitter stimulation was also confirmed in rats (*3*), but the detailed mechanism underlying this effect remains to be elucidated.

Recent progress in taste molecular biology has revealed that taste receptors belonging to the G protein-coupled receptor (GPCR) family are expressed in some taste cells to recognize specific ligands. In humans, a GPCR of the TAS2R (T2R) family acts as the receptor for bitter taste recognition (*4*−*6*). Heterologous expression experiments have identified several cognate bitter ligands for human TAS2Rs (*7*−*15*). The hTAS2R16 receptor responds to salicin, an authentic bitter β-glucopyranoside extracted from willow bark (*9*). A clear dose−response relationship between hTAS2R16 and salicin was obtained by in vitro calcium imaging analysis of cultured cells, and this result closely resembled that obtained in a human sensory test (*9*).

Here, we investigated the bitterness-masking effects of acidic dipeptides and other acids by performing calcium imaging analysis of the hTAS2R16 receptors that were heterologously expressed in human embryonic kidney 293T (HEK293T) cells. This article reports pH-dependent inhibition on the human bitter taste receptor hTAS2R16 by a variety of acidic substances.

MATERIALS AND METHODS

Compounds. Salicin, L-glutamic acid, L-aspartic acid, DL-tartaric acid, *N*-phenylthiourea (PTC), and 6-propyl-2-thiouracil (PROP) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Citric acid and phosphoric acid were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). L-(+)-Ascorbic acid, glycine, sodium L-glutamate, sodium L-aspartate, and L-lysine monohydrochloride were products of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glu-Glu and Gly-Gly were synthesized by Peptide Institute, Inc. (Osaka, Japan). Asp-Asp was purchased from Bachem AG (Bubendorf, Switzerland) and isoproterenol from Sigma-Aldrich (Tokyo, Japan).

Construction of Expression Plasmids for hTAS2Rs and the Chimeric G Protein. To construct an expression plasmid for hTAS2R16,

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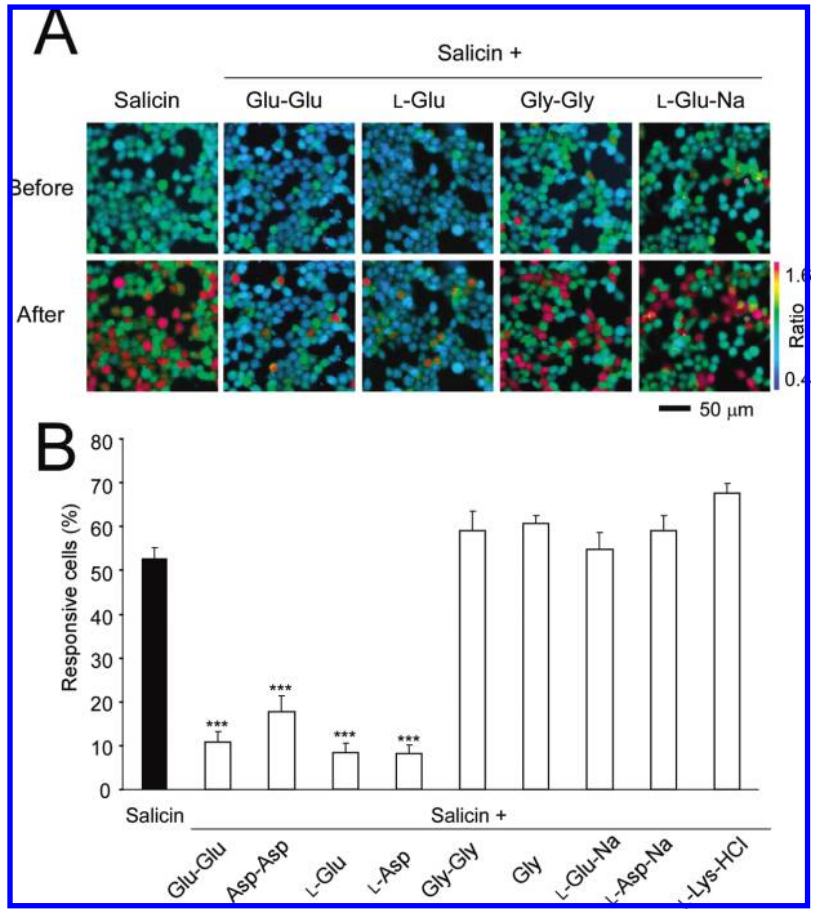

Figure 1. Response of hTAS2R16 to salicin in the presence of peptides, amino acids, or amino acid salts. (**A**) Representative ratiometric images of fura-2 loaded HEK293T cells coexpressing hTAS2R16 and Gα16gust44 in response to 1 mM salicin in the absence or presence of 10 mM peptides, amino acids, or amino acid salts. The top and bottom columns show the representative cell images obtained before and after ligand application, respectively. The color scale indicates the F340/F380 ratio as a pseudocolor. (**B**) Responses of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to 1 mM salicin in the absence and presence of 10 mM peptides, amino acids, or amino acid salts. Each of the columns represents the percentage of the responsive cells in randomly selected DsRed-positive cells (mean ± SE from at least 3 independent measurements). The significance of the differences between the control (1 mM salicin) and test values were tested by using one-way ANOVA followed by the Dunnett's test. ***, $p < 0.001$ vs salicin.

DNA fragments encoding hTAS2R16 (NCBI refseq number: NM_016945) were obtained from human genomic DNA (BD Clontech, Mountain View, CA, USA) by polymerase chain reaction (PCR). The coding region of hTAS2R16 (1−876) was tagged at the N-terminal with the bovine rhodopsin amino acid 1−39 site (*16*) and subcloned into the *Eco*R I-*Not* I site of the pEAK10 expression vector (Edge Biosystems, Gaithersburg, MD, USA).

An expression plasmid for hTAS2R38 was constructed by using an hTAS2R38-PAV variant with the polymorphism most responsive to PTC and PROP (*15*). DNA fragments encoding hTAS2R38 (NCBI refseq number: NM_176817) were also obtained from human genomic DNA by PCR amplification. The obtained sequence encoding the hTAS2R38-AVI mutant was used to generate constructs corresponding to the PAV haplotypes through the subsequent introduction of point mutations by PCR using mutation introducing primer sets. The constructs of hTAS2R38-PAV were verified by sequencing analysis. The coding sequence of hTAS2R38-PAV(1−1002) was tagged with the first 45 amino acids of rat somatostatin receptor type 3 (ssr) at the N-terminal (*17*) and was subcloned into the *Eco*R I-*Not* I site of the pEAK10 expression vector.

The expression plasmids for the chimeric G-protein subunit Gα16gust44 subcloned into the pcDNA3.1 expression vector (Invitrogen, San Diego, CA, USA) were kindly gifted by Dr. Ueda and Dr. Shimada (Nagoya City Univ.) (*16*).

Cell Culture and Transfection. Human embryonic kidney 293T (HEK293T) cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen). For the transfection, the cells were seeded onto 35 mm dishes and transiently transfected with the plasmid expressing the hTAS2R16 or hTAS2R38 along with both Gα16gust44 and red fluorescent protein (DsRed2) (pDsRed2-N1; Takara Bio, Shiga, Japan) in the ratio 40:10:0.8 using Lipofectamine 2000 (Invitrogen). The response to isoproterenol was detected by transfecting the cells with an empty vector instead of using the hTAS2Rs-expression vector.

Ca^{2+} Imaging Analysis. For ordinary experiments, transfected cells were transferred into a 96 CytoWell plate (Nalge Nunc International, Rochester, NY, USA) 6 h after transfection. The cells were incubated for an additional 18−20 h and rinsed and loaded with 5 μM of the calcium indicator dye, fura-2 AM (Invitrogen), that was diluted with the assay buffer (130 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM HEPES, pH 7.4). The incubation was continued for another 30−40 min at room temperature. The cells were rinsed and incubated in 100 μL of the assay buffer for more than 10 min, prior to adding 100 μL each of the 2× ligand solution by pipetting. Each of the final solutions obtained after ligand application was characterized on the basis of the ligand concentration and the resulting pH value. The pH was adjusted with 1 N NaOH if necessary.

For the perfusion assay, the cells were transferred onto a glass bottom dish (AGC techno glass Co., Chiba, Japan) 6 h after transfection. After 18−32 h, these cells were loaded with 5 μM fura-2 AM and incubated for 30 min at room temperature. The cells were washed with the assay buffer perfusing under gravity at a flow rate of 8 mL/min. We prepared two different assay buffers with pH values 7.4 and 4.0. The buffer of pH 7.4 had the same composition as the buffer described above, whereas the buffer of pH 4.0 also contained 10 mM L-Glu. The pH was adjusted with 1 N NaOH. The ligand solutions (3 mM salicin) were prepared

by using these buffers. The salicin solutions were applied to the cells for a period of 20 s.

The fura-2 fluorescence intensities resulting from excitation at 340 and 380 nm were measured at 510 nm by using a computer-controlled filter changer (Lambda 10−2 or 10−3; Sutter Instrument, San Rafael, CA, USA), a MicroMax CCD camera (Princeton Instruments, Trenton, NJ, USA), or CoolSNAP HQ2 (Photometrics, Tucson, AZ, USA), and an inverted fluorescence microscope (IX-70 or IX-81; Olympus, Tokyo, Japan). The images were recorded at 4 s intervals and analyzed using the MetaFluor software (Molecular Devices, Sunnyvale, CA, USA). The resultant changes in the intracellular calcium ion concentration ($[Ca^{2+}]_i$) were measured by randomly selected DsRed2-positive cells that were regarded as the transfected cells. The changes were represented as a ratio of the fluorescence intensities at the two excitation wavelengths (F340/F380). The cells were regarded as responsive when the increase in the F340/F380 ratio was larger than 0.2. We confirmed that a minor response was detected when the ligand was applied in the absence of the receptors or the chimeric G-protein (data not shown).

RESULTS

Response of hTAS2R16 to Salicin in the Presence of Added Acidic Peptides or Amino Acids. We used calcium imaging analysis to investigate the stimulatory responses of the HEK293T cells transiently expressing the hTAS2R16 receptors along with Gα16gust44 and DsRed2 to its cognate ligand, salicin, in the presence or absence of various peptides, amino acids, or amino acid salts. The application of salicin at 1 mM, a concentration that was close to the EC_{50} value (*9*), induced a response in approximately 50% of the DsRed-positive cells (**Figure 1**). However, when 10 mM each of Glu-Glu, Asp-Asp, L-Glu, or L-Asp was applied to the cells along with 1 mM salicin, the number of responsive cells reduced significantly ($p < 0.001$) (**Figure 1**). In contrast, the application of 1 mM salicin along with 10 mM of substances such as neutral Gly-Gly, Gly, sodium salts of L-Glu, and L-Asp or Lys-HCl did not induce a significant reduction in the response of hTAS2R16 to salicin (**Figure 1**). These results suggest that the acidic dipeptides and amino acids examined inhibit the hTAS2R16 response.

Response of hTAS2R16 to Salicin in the Presence of a Variety of Added Acidic Substances. We used a variety of acidic substances to compare their ability to reduce the hTAS2R16 response to salicin (**Figure 2**). We selected Glu-Glu and Asp-Asp as acidic dipeptides, L-Glu and L-Asp as acidic amino acids, citric acid, L-(+)-ascorbic acid, and DL-tartaric acid as organic acids, and phosphoric acid as an inorganic acid. We found that a few of the HEK293T cells transiently transfected with the hTAS2R16 receptor and Gα16gust44 responded to individual application of 10 mM of each of the acidic substances (data not shown). Then, 1 mM salicin along with each of the acidic substances at different concentrations (1−10 mM) was applied to the cells. The response to 1 mM salicin was significantly inhibited at higher concentrations of each acidic substance ($p < 0.001$) (**Figure 2**). Moreover, all of the tested acidic substances showed a similar effect. These results suggest that the inhibition of hTAS2R16 response to 1 mM salicin is not specific to acidic dipeptides and acidic amino acids, but common to acidic substances. Acidic solutions are characterized by their concentrations and pH values; therefore, we investigated the influence of these two factors on the inhibition of the hTAS2R16 response to salicin.

pH-Dependent Change in hTAS2R16 Function. Initially, we compared the influence of acidic substances with the same concentrations and different pH values on the hTAS2R16 response to salicin. We used ligand solutions with constant concentrations of the acidic substances to prepare a series of 2× ligand solutions containing 2 mM salicin and 20 mM each

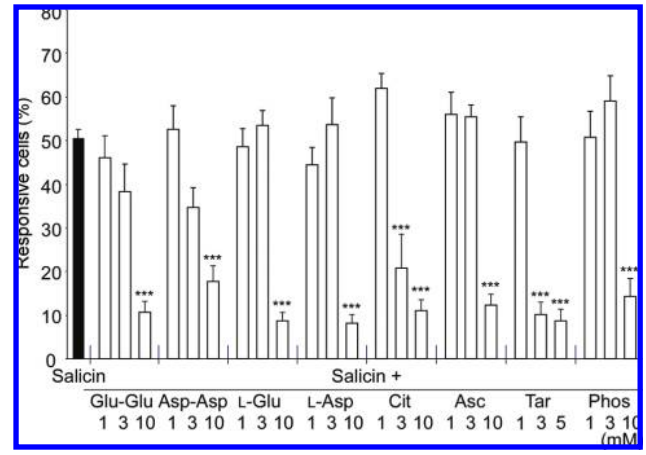

Figure 2. Response of hTAS2R16 to salicin in the presence of a variety of acidic substances. Responses of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to 1 mM salicin in the absence and presence of acidic substances. Each of the columns and points represents the mean ± SE from at least 3 independent measurements. The significance of differences between control (1 mM salicin) and test values was tested by one-way ANOVA followed by Dunnett's test. ***, $p < 0.001$ vs salicin. Cit, citric acid; Asc, L-(+)-ascorbic acid; Tar, DL-tartaric acid; and Phos, phosphoric acid.

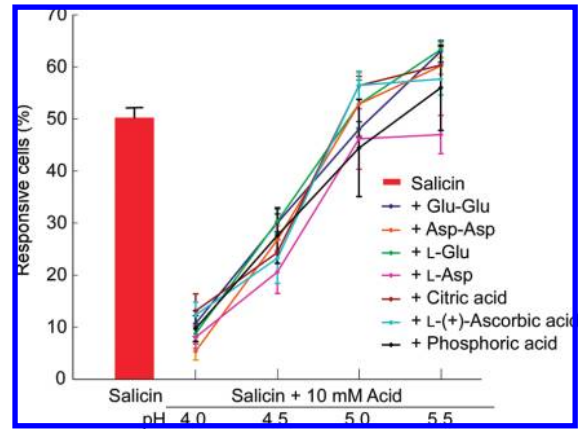

Figure 3. Response of hTAS2R16 to salicin in the presence of a variety of acidic substances under different pH conditions. Responses of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to 1 mM salicin in the absence and presence of 10 mM acidic substances. The pH values of the ligand solution were adjusted with 1 N NaOH such that the pH values after adding the ligands to the cells were equivalent to the indicated pH values (pH 4.0−5.5). Each of the columns and points represents the mean ± SE from at least 3 independent determinations.

of Glu-Glu, Asp-Asp, L-Glu, L-Asp, citric acid, L-(+)-ascorbic acid, and phosphoric acid (**Figure 3**), and their pH values were adjusted using 1 N NaOH such that the resultant pH values were equivalent to the indicated ones (pH 4.0−5.5) after addition of the ligand to the external solution of the cells. In these cases, the final concentrations of salicin and the acidic substances were set at 1 mM and 10 mM, respectively. The inhibitory effects were not observed above pH 5.0, whereas the ratio of the responsive cells decreased significantly when the pH value was below 4.5 ($p < 0.05$), and there was no appreciable difference between the inhibitory effects of the acidic substances (**Figure 3**). Furthermore, we tested the effect of a decrease in pH on the hTAS2R16 receptor response to other agonists: phenyl-β-D-glucopyranoside, helicin, and arbutin, all of which were reported

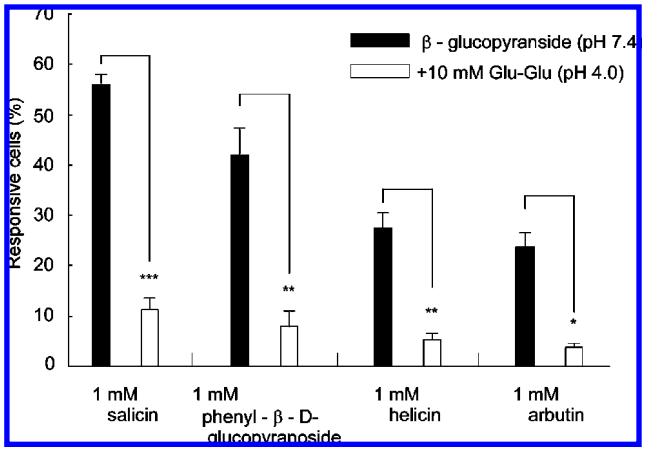

Figure 4. Response of hTAS2R16 to various β-glucopyranosides in the presence of Glu-Glu. Responses of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to 1 mM of various β-glucopyranosides in the presence and absence of 10 mM acidic substances. The pH values of the ligand solution were adjusted with 1 N NaOH such that the pH values after adding the ligands to the cells were equal to the indicated pH values (pH 4.0). Each of the columns and points represents the mean ± SE from at least 3 independent determinations. The significance of differences between the control (1 mM each of phenyl-β-glucopyranoside, pH 7.4) and test values was tested by Student's *t*-test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ vs control.

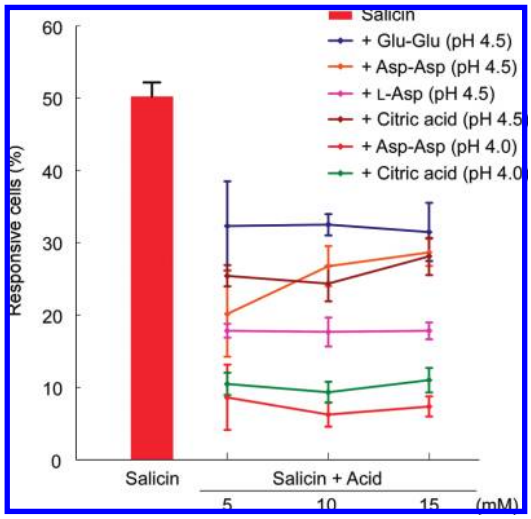

Figure 5. Effect of the concentrations of the acidic substances on the hTAS2R16 response to salicin. Responses of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to 1 mM salicin in the absence and presence of acidic substances (5, 10, or 15 mM). The pH values of the ligand solution were adjusted with 1 N NaOH such that the pH values after adding the ligands to the cells were equivalent to the indicated pH values (pH 4.0 or 4.5). Each of the columns and points represents the mean ± SE from at least 3 independent measurements.

as cognate ligands for hTAS2R16 (*9*). As a result, we were able to confirm the inhibition of hTAS2R16 responses to tested 1 mM β-D-glucopyranosides at pH 4.0 (**Figure 4**).

Ligand Concentration-Independent Inhibition of hTAS2R16. To examine the relationship between the inhibitory effects and the concentrations of acidic substances, we carried out an experiment by using different concentrations of ligand solutions at the same pH values of 4.5 or 4.0 (**Figure 5**). The hTAS2R16-transfected cells were stimulated with 1 mM salicin in the presence of various concentrations (5, 10, and 15 mM) of the acidic substances Glu-Glu, Asp-Asp, L-Asp, and citric acid at

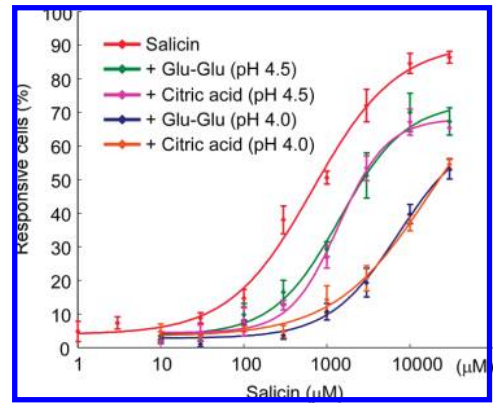

Figure 6. Dose−response relationship between hTAS2R16 and salicin in the absence or presence of acidic substances under different pH conditions. Dose−response relationship of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to salicin in the absence and presence of 10 mM Glu-Glu or citric acid. The pH values of the ligand solution were adjusted with 1 N NaOH such that the pH values after adding the ligands to the cells were equivalent to the indicated pH values (pH 4.0 or 4.5). Each of the points represents the mean ± SE from at least 3 independent measurements.

a pH value of 4.5 or 4.0. The inhibitory effect on the hTAS2R16 response was observed, as in the case of **Figure 3**. Irrespective of the acidic substance, there was little correlation between the inhibitory effect and the concentrations of the acidic substances under the same pH conditions (**Figure 5**). These results suggest that the inhibition of the hTAS2R16 response is independent of the concentration of the acidic substances, and the inhibitory effect was controlled just by the resultant pH values.

Dose−Response Relationship between hTAS2R16 and Salicin under Different pH Conditions. We confirmed that the hTAS2R16 and Gα16gust44-expressing cells responded to salicin in a dose-dependent manner at a neutral pH as reported (*9*), and the EC_{50} value was 683 ± 111 μM under our experimental conditions (**Figure 6**). We examined the dose−response relationship between hTAS2R16 and salicin in the presence of 10 mM Glu-Glu or citric acid (pH 4.5 or 4.0). The addition of these acidic substances reduced the response of hTAS2R16 to salicin stimulation, resulting in a right shift of the EC_{50} value. At pH 4.5, the EC_{50} value of hTAS2R16 for salicin slightly shifted toward 1.43 ± 0.24 mM in the presence of 10 mM Glu-Glu and toward 1.39 ± 0.13 mM in the presence of 10 mM citric acid (**Figure 6**). When the pH was set at 4.0, the EC_{50} shift was clearly observed at 7.26 ± 2.33 mM (10 mM Glu-Glu) or 17.4 ± 11.5 mM (10 mM citric acid). Furthermore, there was no appreciable difference between the inhibitory effects of Glu-Glu and citric acid, and it appeared that the response is exclusively dependent on the pH value (**Figure 6**).

Immediate Recovery of hTAS2R16 Response under Neutral Conditions. We sequentially changed the pH values of the external solutions from neutral to acidic conditions and then from acidic to neutral conditions, and investigated the influence of these changes in pH values on the hTAS2R16 response to salicin (**Figure 7**). The hTAS2R16- and Gα16gust44-transfected HEK293T cells were treated with 3 mM salicin in triplicate, at pH values of 7.4, 4.0, and 7.4. We continuously monitored the response of one hundred transfected cells and confirmed that the reduced response at pH 4.0 showed an immediate recovery at pH 7.4 (**Figure 7**).

Response of hTAS2R38-PAV to PTC and PROP in the Presence of Glu-Glu. Using the hTAS2R receptor in addition

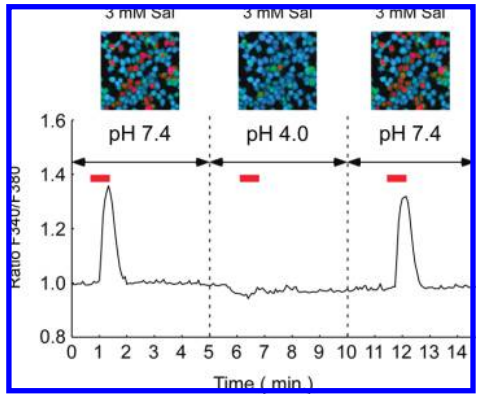

Figure 7. The reduced response of hTAS2R16 to salicin in the presence of L-Glu showed an immediate recovery at pH 7.4. Responses of the HEK293T cells coexpressing hTAS2R16 and Gα16gust44 to 3 mM salicin under sequentially changing pH values; a perfusion system was used to change the pH values from 7.4 to 4.0 and then to 7.4. Ligand solutions containing 3 mM salicin were perfused for 20 s, and the duration of each stimulation was indicated by red bars. The trace describes the average ratio of fluorescence intensities at the 2 excitation wavelengths (F340/F380) of the randomly selected 100 DsRed2-positive cells.

to hTAS2R16, we carried out an analysis with hTAS2R38, a receptor for 6-propyl-2-thiouracil (PROP) and *N*-phenylthiourea (PTC). To obtain a strong response to agonists in our heterologous expression system, we used a cDNA as the highest sensitive mutant of hTAS2R38 (P49/A262/V296) with an amino terminal tag of 45 amino acids of rat somatostatin receptor type 3 (ssr-hTAS2R38PAV) (*15*). At neutral pH, the hTAS2R38PAV and Gα16gust44-transfected HEK293T cells responded to PTC and PROP in a dose-dependent manner (data not shown), and the proportion of cells responding to 3 μM PTC or 3 μM PROP was 38.4 ± 4.4% or 58.3 ± 2.6%, respectively. However, the responses of hTAS2R38PAV to PTC and PROP were significantly reduced in the presence of 10 mM Glu-Glu (at pH 4.0) (**Figure 8**).

Gα16gust44-Mediated PLC-IP$_3$ Pathway within HEK293T Cells Was Not Always Affected at Low pH. To verify which molecules in a signaling cascade are involved in the inhibitory effects observed in the presence of acidic substances, we next investigated the effects of pH on the Gα16gust44-mediated Ca^{2+} response of the β-adrenergic receptor that is endogenously expressed in HEK293T cells. HEK293T cells were transfected with Gα16gust44 and examined for responsiveness to isoproterenol, a ligand for the β-adrenergic receptor, by using Ca^{2+} imaging. We observed that the HEK293T cells without Gα16gust44 transfection hardly responded to isoproterenol, indicating that Gα16gust44 could mediate the intracellular calcium mobilization (data not shown). Under neutral pH, the Gα16gust44-transfected HEK293T cells responded to isoproterenol in a dose-dependent manner, with an EC_{50} value of 23.6 ± 4.7 nM (**Figure 9A**), and the proportion of cells responding to 15 nM isoproterenol was 33.9 ± 5.5% of DsRed positive cells (**Figure 9B**). We found that the response of the Gα16gust44-transfected cells to isoproterenol at a concentration of 15 nM was not inhibited in the presence of 10 mM L-Glu even when the pH values after ligand addition were adjusted to 4.0 or 4.5 (**Figure 9B**).

DISCUSSION

To evaluate the taste of foods, the sensory test is a very practical tool (*18*), and Noguchi et al. (*2*) found that some acidic dipeptides had a bitterness-masking effect. The addition of 0.1%

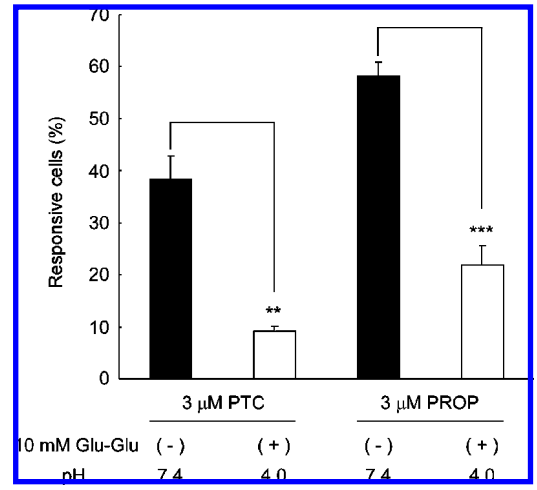

Figure 8. Response of hTAS2R38-PAV to PTC and PROP in the presence of Glu-Glu. Responses of the HEK293T cells coexpressing hTAS2R38PAV and Gα16gust44 to 3.0 μM each of PTC or PROP in the presence and absence of 10 mM Glu-Glu. The pH values of the ligand solution were adjusted with 1 N NaOH such that the resultant pH values after the ligand addition to the cells were equal to the indicated pH values (pH 4.0). Each of the columns and points represents the mean ± SE from at least 3 independent determinations. The significance of differences between the control (3.0 μM each of PTC or PROP, pH 7.4) and test values was tested by Student's *t*-test. ***, $p < 0.001$; **, $p < 0.01$.

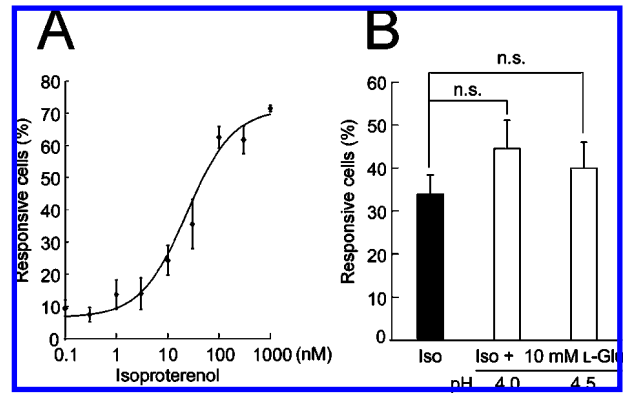

Figure 9. Response of Gα16gust44-expressing HEK293T cells to isoproterenol. (**A**) Dose−response relationship between Gα16gust44-expressing HEK293T cells and isoproterenol. (**B**) Responses of Gα16gust44-expressing HEK293T cells to 15 nM isoproterenol in the absence and presence of 10 mM L-Glu. The pH values of the ligand solution were adjusted with 1 N NaOH such that the resultant pH values after ligand addition to the cells were equivalent to the indicated pH values (pH 4.0 or 4.5). Each of the columns and points represents the mean ± SE from at least 3 independent measurements. n.s., not significant; Iso, isoproterenol.

Glu-Glu, which occurs in the acidic oligopeptide fraction of a fish-protein hydrolysate, to L-Ile, Gly-Leu, and other well-known bitter substances was found to induce a marked decrease in bitterness. While a sensory test is available as a standard method for taste evaluation, electrophysiological recordings from the glossopharyngeal or chorda tympani nerves have provided insights into taste reception and the responses of the nerves to tastant stimulation. Iwasaki et al. (*3*) examined the effect of Glu-Glu on the IXth nerve responses of rats to quinine, nicotine, Gly-Ile, and $MgCl_2$. They found that the magnitude of the response to the bitter substances mixed with Glu-Glu was smaller than the sum of the individual responses to the bitter solution and Glu-Glu. From these results, they concluded that

Glu-Glu decreases the taste intensity produced by various bitter compounds. The inhibitory effect, however, is so small that the data are not equivalent to those observed in the sensory test.

With recent progress in molecular biological studies of the taste receptor, the importance of evaluating taste by in vitro assay methods has been realized (*19*, *20*). In humans, 25 members of the TAS2R family have been identified, and heterologous expression experiments have identified their cognate bitter ligands (*7*−*15*). In the present study, we tried to investigate the bitterness-masking effect of Glu-Glu using calcium imaging analysis of the HEK293T cells heterologously expressing hTAS2Rs.

It is important to employ bitter ligands that are stable under acidic conditions because we observed the response of hTAS2Rs at both neutral and acidic pH. For example, denatonium benzoate, which is a representative bitter tastant and also a ligand for some of the bitter taste receptors, did not adequately perform as an agonist under our experimental conditions because it evoked increases in intracellular calcium concentrations in nearly all Gα16gust44-expressing HEK293T cells without expressing TAS2Rs (data not shown). Therefore, we selected salicin as a representative, acid-stable, bitter ligand. Furthermore, the threshold and EC_{50} values for salicin determined in the in vitro study (**Figure 6**) closely resembled those reported in the human sensory tests (*9*). Therefore, we selected salicin as a model bitter ligand for investigating the bitterness-masking effects of Glu-Glu in vitro.

In our experiments, we found that some acidic dipeptides and acidic amino acids significantly reduced the response of hTAS2R16 to salicin, while no such effects were observed with Gly-Gly, Gly, and amino acid salts (**Figure 1**). This is apparently the first report on the inhibition of the bitter taste-receptor response by acidic substances. Interestingly, not only acidic dipeptides and acidic amino acids but also a variety of acidic substances such as organic and inorganic acids had a similar inhibitory effect on the response of hTAS2R16 (**Figure 2**). Furthermore, we investigated which acid concentration and the indicated resultant pH was critical for the inhibition of the hTAS2R16 response. The results indicated that this effect depended on the pH (**Figure 3**) and not on the concentration of the acidic substances (**Figure 5**). The dose−response relationship of hTAS2R16 to salicin showed that the inhibition was induced by a right-shift in the EC_{50} values at lower pH (**Figure 6**). However, because of the solubility limitation of salicin, its highest concentration was 30 mM under our experimental conditions (**Figure 6**). We therefore could not clearly indicate whether or not the dose−response curves are compressed at pH 4.5 or 4.0.

Another question was about whether or not the inhibitory effect at low pH was specific to hTAS2R16 and whether or not the pH dependent inhibition correlated with the bitterness-masking effects of Glu-Glu. Noguchi et al. (*2*) reported that Glu-Glu reduced the bitterness of PTC in the sensory test. On the other hand, PTC and PROP were reported as the ligands for hTAS2R38 (*15*). Examining the response of ssr-hTAS2R38PAV transfected HEK293T cells to PTC and PROP in the presence or absence of 10 mM Glu-Glu, we found that the inhibitory effects on hTAS2R38PAV activation by PTC and PROP at pH 4.0 were similar to that observed in the case of hTAS2R16 (**Figure 8**). Our results with hTAS2R38 may provide the link between the previously reported bitterness-masking effects of Glu-Glu and our experimental data from calcium-imaging analysis of the hTAS2R receptors heterologously expressed in HEK293T cells.

The influence of saliva having an enormous buffering capacity cannot be neglected. Therefore the pH-dependent inhibition of hTAS2R16, which was found in the cell assays, might not be translated directly to in vivo situations. However, our experimental data showed that at least two human TAS2R receptors, hTAS2R16 and hTAS2R38, were inhibited at low pH. Further experiments should be carried out to elucidate whether this inhibitory effect is generally observed in TAS2Rs.

It has been reported that some of the GPCRs, such as OGR1, GPR4, and calcium-sensing receptor, can sense the extracellular proton and are coupled to G-proteins to stimulate intracellular signaling pathways (*21*, *22*). However, we observed that the response of the Gα16gust44-transfected cells to isoproterenol was not inhibited in the presence of 10 mM L-Glu, irrespective of the pH values after ligand addition (**Figure 9B**). On the basis of this experimental result, it can be considered that the GPCR-mediated intracellular signaling pathways are not always affected even if the pH value of the extracellular solution is lowered. These results indicate that the Gα16gust44-mediated PLC-IP_3 pathway in the HEK293T cells is not affected at low pH and that the intracellular signaling transduction molecules downstream of the bitter taste receptors are probably independent of the inhibitory effect of acidic substances on hTAS2R16 activation.

We were able to demonstrate the pH-dependent inhibition of hTAS2Rs. However, a more accurate interpretation of the underlying mechanism may be obtained by further experimentation to investigate the possibility of a reduction in binding affinity between the receptor and the ligand in the presence of acidic substances or at a low pH. To clarify this point, one may need to measure the biding affinity by biochemical methods, which, however, was strictly difficult because no isotope labeled salicin was commercially available. Therefore, we were unable to demonstrate the experimental evidence of this aspect. However, we were able to successfully elucidate the reversibility of the inhibitory effect. As a result, we found that the reduction in the response at pH 4.0 showed an immediate recovery at pH 7.4 (**Figure 7**). This result suggests that the hTAS2R16 receptor protein is seldom irreversibly inactivated, even when the pH of the external solution is set at 4.0, and the inhibitory effects are probably induced by a transient structural change in the hTAS2R16 protein at low pH. Further experiments using point mutants of hTAS2Rs can reveal the detailed mechanism of the inhibition of bitterness at acidic pH values.

ABBREVIATIONS USED

GPCR, G protein-coupled receptor; HEK293T, human embryonic kidney 293T; PTC, *N*-phenylthiourea; PROP, 6-propyl-2-thiouracil; PCR, polymerase chain reaction.

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