Sweet Taste Responses of Mouse Chorda Tympani Neurons: Existence of Gurmarin-Sensitive and -Insensitive Receptor Components

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INTRODUCTION

Gurmarin (gur) is a peptide (mol wt, 4,209, isolated from the plant Gymnema sylvestre) that is known to specifically inhibit responses to sweeteners (substances taste sweet to humans) but not to other basic taste stimuli, such as NaCl, HCl, and quinine, in the rat (Imoto et al. 1991) and mouse chorda tympani nerve (Ninomiya and Imoto 1995; Ninomiya et al. 1997, 1998) and gerbil taste cells (Uchina and Sato 1997). In mice, it has been shown that the gur inhibition of sweetener responses is strain and nerve specific (Ninomiya and Imoto 1995; Ninomiya et al. 1997), that is, responses of the chorda tympani nerve innervating taste buds in the fungiform papillae to various sweeteners were suppressed by gur to ~50% of control in C57BL mice, whereas no such suppression was observed in BALB mice. Even in C57BL mice, responses of the glossopharyngeal nerve innervating taste buds in the circumvallate and foliate papillae were not suppressed by gur. These results suggest that there may exist two different receptor types for sweeteners in mice, one gur sensitive and the other gur insensitive. The C57BL strain possesses both types in the taste buds innervated by the chorda tympani nerve, whereas BALB mice almost exclusively have the gur-insensitive type. However, because this evidence is based on the analysis of relative magnitudes of whole nerve responses of peripheral taste nerves, more convincing evidence differentiating the gur-sensitive and -insensitive response components is required.

Similar mouse strain and nerve specificities have been reported in inhibition of salt responses by amiloride, a sodium channel blocker, which suppressed NaCl responses of the chorda tympani in C57BL but not BALB mice (Ninomiya et al. 1989), and no such suppression was observed in the glossopharyngeal nerve even in C57BL mice (Ninomiya et al. 1991). Furthermore, in single-fiber response analysis of the chorda tympani in C57BL mice (Ninomiya 1996, 1998), rats (Ninomiya and Funakoshi 1988), and hamsters (Hettinger and Frank 1990), it has also been shown that amiloride primarily inhibits NaCl responses of fibers that selectively respond to sodium salts (labeled N type), whereas it hardly affects NaCl responses of fibers that are broadly sensitive to electrolytes (labeled H or E type). This suggests the existence of selective synapse formation between particular classes of taste fibers and amiloride-sensitive and -insensitive taste cells in those animals. Therefore by analogy, it is possible that, if gur-sensitive and -insensitive receptor components for sweeteners in mice actually exist, they would differentially localize in taste cells and might be innervated by different groups of taste fibers. If this were the case, chorda tympani fibers innervating sweetener-responsive taste cells might be divided into two groups, according to their susceptibilities to gur.

To investigate this possibility, we examined single-fiber responses of the chorda tympani nerve to various taste stimuli including sweeteners before and after lingual treatment with gur in gur-sensitive C57BL mice (Ninomiya and Imoto 1995). The results suggested the existence of two groups of sucrose-responsive chorda tympani fibers that would selectively innervate taste cells possessing gur-sensitive and -insensitive receptor components for sweeteners.

METHODS

Subjects were adult male and female mice of C57BL/6CrSlc strain (age 8–25 wk, n = 24 males and 12 females), ranging in weight from 20 to 35 g. Each mouse was anesthetized with an injection of sodium pentobarbital (40–50 mg/kg ip) and maintained at a surgical level of
anesthesia with supplemental injections of sodium pentobarbital. The trachea was cannulated, and the mouse was then fixed in the supine position with a head holder to allow dissection of the chorda tympani nerve. The chorda tympani nerve was exposed at its exit from the lingual nerve and cut near its entrance to the bulla. Single or a few fibers of the nerve were dissected apart with a pair of needles and lifted on a silver wire electrode. An indifferent electrode was placed in nearby tissue. Impulse discharges resulting from chemical stimulations of the tongue were fed into an amplifier (Iyodenshikogaku K-1), monitored on an oscilloscope and audiometer, recorded on a recorder (Nihon-kohden, WS-641G), and stored on magnetic tape for future analysis.

The anterior one-half of the mouse's tongue was enclosed in a flow chamber. Solutions were delivered into the flow chamber by gravity flow and flowed over the tongue for a controlled period. Solutions used were (in M) 0.1 NaCl, 0.01 HCl, 0.02 quinine HCl, 0.03–1.0 sucrose, 0.5 fructose, 0.5 glucose, 0.02 saccharin Na, 0.03 d-tryptophan, and 0.1 d-phenylalanine. These chemicals were dissolved in distilled water at ~23°C. Methods for chemical stimulation were the same as those described in our previous reports (Ninomiya and Imoto 1995; Ninomiya et al. 1997). The order of chemical stimulation during the first survey to find fibers responding to sweeteners was used as (in M) 0.5 sucrose, 0.1 M NaCl, 0.01 HCl, and 0.02 quinine HCl. If the fiber clearly responded to sucrose, we continued and further applied other sweet substances, such as (in M) 0.02 saccharin Na; 0.03, 0.1, 0.3, and 1.0 M sucrose; 0.03 d-tryptophan, and 0.1 d-phenylalanine. Then 0.5 M sucrose was applied once more to check the reproducibility of the response before the lingual treatment with gur. In some fibers, we did not try 0.03, 0.1, 0.3, and 1.0 M sucrose. During chemical stimulation of the tongue, the test solution flowed at the same flow rate as the distilled water used for rinsing the tongue (~0.5 ml/s). The tongue was rinsed for >1 min during the intervals between successive stimulations. To examine gur inhibition of chorda tympani responses, the tongue was treated with 4.8 μM (≈20 μM/ml) gur dissolved in 5 mM phosphate buffer (pH 6.8) for 5 min in the similar manner as that described by our previous study (Ninomiya and Imoto 1995). Gur is reported to be very stable against selective cleavage by proteases, even under conditions such as high temperature, low pH, and the presence of urea (Araki et al. 1995). Inhibitory effects of gur on rat chorda tympani responses to sucrose were hardly changed when gur solution was heated to 90°C, and stored for ≥2 mo (Imoto et al. 1991). To obtain similar potencies of gur throughout the experiments, we used aliquots of the same stock solution of gur that were stored at ~20 °C and warmed to the room temperature immediately before application to the tongue. We chose a concentration of gur (4.8 μM) that exhibited the maximum suppressive effect on chorda tympani responses in C57BL strain (Ninomiya and Imoto 1995). The time for the gur treatment (5 min) was shorter than that (10 min) used in our previous studies. However, our pilot experiments showed that the lingual treatment with 4.8 μM gur for 5 min produced the maximum suppressive effect of gur on sucrose responses (~50% of control). In the fibers whose sucrose responses were suppressed by gur, to facilitate the recovery of the suppressed sweetener responses, animal's tongue was rinsed for 10 min with 15 mM β-cyclodextrin (β-CD), which could remove the effect of gur (Ninomiya et al. 1998). In most animals, gur was applied to the tongue only once, and thereby data from only one preparation were obtained from each animal. In some animals, we tested the second gur treatment for the second preparation. However, we did not use the data from the second trial for the current analysis except in those cases when the first preparation of the animal was sensitive to gur and its suppressed response frequencies to sucrose after gur recovered to >85% of control (not stored by a spike counter) after rinsing the tongue with β-CD. In some fibers, 2% pronase E (dissolved in 50 mM phosphate buffer at pH 6.8), a specific inhibitor for sweetener responses in rats (Hiji 1975) and mice (Ninomiya et al. 1997), was further applied for 10 min to the tongue after gur and β-CD experiments to check its inhibitory effects on sweetener responses.

Single fibers were identified by uniform spike height, singular wave form, and examination of latencies between contiguous spikes. In total, 30 single fibers responding to sucrose were obtained from 26 mice (1 each from 22 mice and 2 each from 4 mice). Frequency-time histograms of impulse discharges before, during, and after chemical stimulation of the tongue were made by means of a spike-analysis system (Iyodenshikogaku, SAS-1). For data analysis, we used the net average frequency for the first 5 s after the stimulus onset obtained by subtracting the spontaneous frequency for a 5-s period before stimulation.

**RESULTS**

Thirty chorda tympani fibers responding to sucrose were classified into two groups according to gur sensitivity of sucrose responses. In 16 of 30 fibers, responses to 0.5 M sucrose were suppressed by gur to <50% of control (Figs. 1, A and B, and 2, Gur-sensitive). Percent control responses of this group to 0.5 M sucrose after gur ranged from 4.3 to 48.3% with a mean of 20.3 ± 13.1% (SD; Fig. 1A, Gur-sensitive). Mean number of impulses/5 s of 16 fibers to 0.5 M sucrose after gur (12.5 ± 8.2) was significantly smaller than that before gurmrin (66.8 ± 30.3, t-test, P < 0.001, Fig. 1B, Gur-sensitive, A–P).

In the remaining 14 fibers, sucrose responses were only slightly if at all inhibited by gur (Figs. 1, A and B, and 2, Gur-insensitive). Their percent control responses ranged from 76.9 to 105.6% with a mean of 91.2 ± 8.1% (SD; Fig. 1B, Gur-insensitive). The mean number of impulses/5 s of the 14 fibers before gur (62.6 ± 28.6) was not significantly different from that after gur (57.2 ± 26.7, t-test, P > 0.05, Fig. 1B, Gur-insensitive, a–n).

Figure 2 shows sample records of responses of gur-sensitive and -insensitive fibers to four taste stimuli (0.1 M NaCl, 0.01 M HCl, 0.02 M saccharin Na, and 0.03 M D-tryptophan, and 0.1 M D-phenylalanine).

**FIG. 1.** A: distribution of mouse chorda tympani fibers according to their percent control responses to 0.5 M sucrose after 4.8 μM gurmarin (gur) (control, before gur = 100%). Fibers were classified into gur-sensitive and -insensitive groups. B: responses of gur-sensitive (n = 16, A–P) and gur-insensitive types (n = 14, a–n) of mouse chorda tympani fibers to 0.5 M sucrose before (□) and after gur (■).
M NaCl, 0.01 M HCl, 0.02 M quinine HCl, and 0.5 M sucrose) before and after gur. Both fibers strongly responded to 0.5 M sucrose but only slightly if at all to other stimuli, indicating typical sucrose-best fibers (Frank 1973). Responses to sucrose in the gur-sensitive fiber was almost abolished by gur, whereas no such suppression was observed in the gur-insensitive fiber. The suppressed sucrose response after gur in the gur-sensitive fiber recovered to some extent after rinsing the tongue with 15 mM β-CD for 10 min, which by itself has no effect on sucrose response of the gur-insensitive fiber. The more or less complete recovery of sucrose responses after rinsing the tongue with 15 mM β-CD was observed in all 11 gur-sensitive fibers tested. Percent sucrose responses after β-CD among the 11 fibers were ranging from 40.0 to 88.1% of control (mean = 66.3 ± 16.0%). This mean percent sucrose response (~66%) of the gur-sensitive fibers after β-CD was comparable with that presumed on a basis of possible interaction between 4.8 μM gur and 15 mM β-CD (~20% of free gur (~0.96 μM) remained) (Ninomiya et al. 1998). The mean response (impulses/5 s) of the 11 gur-sensitive fibers tested with β-CD, which were 69.9 ± 23.9 before gur and 12.1 ± 9.0 after gur, were recovered to 45.9 ± 17.2 after β-CD. As shown in Fig. 2, further treatment with 2% pronase for 10 min almost abolished sucrose responses in both types of fibers. The mean response (impulses/5 s) of six gur-sensitive and six gur-insensitive fibers tested, which were 69.0 ± 18.3 and 60.7 ± 16.5 before gur, reduced to 3.2 ± 1.7 and 3.3 ± 4.1 after pronase, respectively. Concentration-response relationships for sucrose before and after for two groups are shown in Fig. 3A. Responses of gur-sensitive fibers to 0.03–1.0 M sucrose decreased to ~15% of control (10.4–22.2%) after gur [ANOVA, F(1,8) = 23.0, P < 0.01], whereas those of gur-insensitive fibers were not significantly changed after gur [ANOVA, F(1,8) = 0.1, P > 0.05]. Response profiles of the two groups of sucrose-best fibers to eight stimuli are shown in Fig. 3B. In gur-sensitive fibers, responses to not only sucrose but other sweeteners were significantly suppressed by gur (t-test, P < 0.05–0.001), whereas no such inhibition was observed in responses to any stimulus in gur-insensitive fibers (t-test, P > 0.05). Comparing responses to six sweeteners before gur between two groups, a significant difference was observed in response to 0.1 M D-phenylalanine, which was larger in gur-sensitive than -insensitive fibers (t-test, P < 0.05).

**DISCUSSION**

In our previous study measuring whole-nerve responses of the mouse chorda tympani (Ninomiya and Imoto 1995), we found that in C57BL mice gur at the most effective range of

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#GURMARIN-SENSITIVE AND -INSENSITIVE SWEET RECEPTORS

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FIG. 2. Sample recordings of 2 types (Gur-sensitive and Gur-insensitive) of single chorda tympani fibers to 0.1 M NaCl, 0.01 M HCl, 0.02 M quinine HCl, and 0.5 M sucrose before and after gur. Both fibers strongly responded to 0.5 M sucrose but only slightly if at all to other stimuli, indicating typical sucrose-best fibers (Frank 1973). Responses to sucrose in the gur-sensitive fiber was almost abolished by gur, whereas no such suppression was observed in the gur-insensitive fiber. The suppressed sucrose response after gur in the gur-sensitive fiber recovered to some extent after rinsing the tongue with 15 mM β-CD for 10 min, which by itself has no effect on sucrose response of the gur-insensitive fiber. The more or less complete recovery of sucrose responses after rinsing the tongue with 15 mM β-CD was observed in all 11 gur-sensitive fibers tested.

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FIG. 3. A: concentration-response relationships for sucrose before (○) and after 4.8 μM gur (●) in 2 types of mouse chorda tympani fibers [Gur-sensitive (n = 6) and Gur-insensitive (n = 5)]. Vertical bars indicate SDs. B: responses profiles to 0.1 M NaCl (Na), 0.01 M HCl (H), 0.02 M quinine HCl (Q), 0.5 M sucrose (Suc), 0.5 M fructose (Fru), 0.5 M glucose (Glc), 0.02 M saccharin Na (Sac), 0.03 M L-tryptophan (L-Try), and 0.1 M D-phenylalanine (D-Phe) before (○) and after 4.8 μM gur (●) in 2 types of mouse chorda tympani fibers [Gur-sensitive (n = 9–16) and Gur-insensitive type (n = 9–14)]. Vertical bars indicate SDs.
concentration suppressed chorda tympani responses to various sweeteners to \( \sim \)50% of control. Further, we hypothesized that this strain possesses two different response components for sweeteners, one gur sensitive and the other gur insensitive. The current single fiber study revealed the existence of both gur-sensitive and -insensitive types of sucrose-responsive and pronase-sensitive chorda tympani fibers in this strain and thereby clearly supported the previously mentioned hypothesis. Responses to 0.03–1.0 M sucrose in gur-sensitive fibers were suppressed to \( \sim \)20% of control by gur (Figs. 1–3), whereas no such suppression was evident in gur-insensitive fibers (\( \sim \)90% of control after gur) (Figs. 1–3). In total of both gur-sensitive and -insensitive fibers, the mean response (impulses/5 s) to 0.5 M sucrose (64.9 \( \pm \) 29.6, \( n = 30 \)) was reduced to 51.5% (impulses/5 s: 33.4 \( \pm \) 29.4, \( n = 30 \)) by gur, which was comparable with that found in whole-nerve experiments. In single-fiber studies on selective inhibitors, especially whose effects are long lasting such as gur, 2–3 h in rats and mice (Imoto et al. 1991; Ninomiya and Imoto 1995), and gymnemic acid, 1 h in humans (Kurihara 1969), there might generally be difficulty in determining if the reduction of response after the inhibitor was caused by the inhibitor or by inevitable gradual decrease in basic activity of fibers during a long-duration recording. To overcome this difficulty, we used \( \beta \)-CD, which reduced the effect of gur (Ninomiya et al. 1998), and found that in all fibers tested the suppression of sucrose responses by gur was to some extent (mean: \( \sim \)70% of control) reversed by rinsing the tongue with \( \beta \)-CD. This suggests that reduction of impulses of the fibers after gur observed in this study mostly was due to the effect of gur. \( \beta \)-CD is known to contain seven \( \alpha \)-1,4--linked \( \beta \)-glucopyranose units and have a hydrophobic cavity leading to specific binding of guest molecules, such as amino acids with phenyl rings (e.g., tyrosine and phenylalanine) (Li and Purdy 1992). Our recent studies (Ninomiya et al. 1998) have demonstrated that the inhibitory effect of \( \beta \)-CD on gur suppression of sweetener responses is due to formation of inclusion complexes between \( \beta \)-CD and tyrosine residues of gur. Therefore the observed effect of gur may be due to its action on extracellular domains of sweetener receptors of taste cells, and \( \beta \)-CD may prevent gur from binding with receptor sites by forming the inclusion complexes with gur on the taste cell membrane.

Existence of more than one type of the sweet taste receptor in mammals has been suggested by many studies, including human psychophysical (Faurion et al. 1985; Schiffman et al. 1981) and neurophysiological studies recording whole chorda tympani responses in chimpanzees (Hellekant et al. 1985), dogs (Anderson et al. 1962), gerbils (Vlahopoulos and Jaki-novich 1986), rats (Yamada and Imoto 1987; Yamamoto and Kawamura 1971), hamsters (Yamada and Imoto 1987), and mice (Iwasaki and Sato 1986). In gerbils, previous studies with sweetener inhibitors (Vlahopoulos and Jaki-novich 1986) sug-gested the existence of at least two different receptor sites for sucrose because the gerbil chorda tympani responses to sucrose were inhibited by three different competitive inhibitors with different structural characteristics (methyl 4,6-dichloro-4,6-dideoxy-\( \alpha \)-d-galacto-pyranoside, \( \alpha \)-nitrophenyl \( \alpha \)-d-glucopyranoside, and chloramphenicol). In chimpanzees, gymnemic acid did not completely abolish the chorda tympani responses to sucrose, and \( \sim \)20–30% of the sucrose response remained after gymnemic acid, suggesting the existence of two different su-
crose sites, one gymnemic acid sensitive and the other gymnemic acid insensitive (Hellekant et al. 1985). Incomplete suppression of whole chorda tympani responses to sucrose by inhibitors has also been reported in cases of zizyphin in rats and hamsters (Yamada and Imoto 1987), Zn and/or Cu ions in rats (Yamamoto and Kawamura 1971), hamsters (Myers et al. 1993), and mice (Iwasaki and Sato 1986), and gur in rats (Imoto et al. 1991) and mice (Ninomiya and Imoto 1995). However, because the magnitude of suppression of responses might be depend on the potency of inhibitors or many other possible factors (i.e., concentration dependencies, competitive or noncompetitive), the existence of the residual responses to sucrose after treatment with such inhibitors may not always indicate the existence of two different sucrose receptor sites. For example, recent studies analyzing single-fiber responses in chimpanzee chorda tympani (Hellekant et al. 1998) demonstrated that gymnemic acid exclusively inhibited responses to sweeteners in S-type (sucrose-best) fibers that were classified by a hierarchial cluster analysis but not those in other types of fibers. Therefore the residual responses after gymnemic acid might be derived from those of fibers other than S-type cluster, which may mainly relate to recognition for taste substances other than sweeteners. The current results showing that two groups of sucrose-best chorda tympani fibers are classified by their gur sensitivities may thus be the first clear-cut evidence suggesting the existence of two different sucrose and/or sweetener sites in mice.

This study revealed that two groups of sucrose-responsive chorda tympani fibers selectively innervate taste cells whose sweetener responses were inhibited or unaffected by gur. This possible matching between taste cells possessing particular receptor mechanisms and their corresponding types of fibers has previously been suggested in the cases between amiloride-sensitive and -insensitive taste cells and axons in rats (Ninomiya and Funakoshi 1988), hamsters (Hettinger and Frank 1990), and mice (Ninomiya 1998) and two different types of amiloride-sensitive fibers with different temperature dependencies in mice (Ninomiya 1996). A recent study demonstrated that such selective synaptic formation between taste cells and axons with different amiloride sensitivities was not altered even after cross-regeneration between the chorda tympani and glossopharyngeal nerves in mice (Ninomiya 1998). This finding suggests that regenerated taste axons selectively recouple with the appropriate type of receptor cell whether they inner-vate the front or the back of the tongue. Existence of selective synapse formation between the amiloride-sensitive cell types was also suggested in the relay from the peripheral taste axons to the second-order neurons in the nucleus tractus solitarius (Scott and Giza 1990). Olfactory receptor neurons are known to express a particular type of receptor protein (Chess et al. 1994) and send their axons to any one or a few of some 2,000 modules, called glomeruli, in the olfactory bulb (Ressler et al. 1994; Vassar et al. 1994). For this highly specific mechanism of axon guidance, existence of guidance molecules (i.e., receptor proteins) (Singer et al. 1995) has been postulated. It is therefore possible that guidance molecules may exist also in taste cells and/or axons to effect the matching. Future extensive studies including molecular genetic analysis may lead to anwer to this question on taste axon–cell matching specificity.
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