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Stevia and Saccharin Preferences in Rats and Mice

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Abstract

Use of natural noncaloric sweeteners in commercial foods and beverages has expanded recently to include compounds from the plant *Stevia rebaudiana*. Little is known about the responses of rodents, the animal models for many studies of taste systems and food intake, to stevia sweeteners. In the present experiments, preferences of female Sprague–Dawley rats and C57BL/6J mice for different stevia products were compared with those for the artificial sweetener saccharin. The stevia component rebaudioside A has the most sweetness and least off-tastes to human raters. In ascending concentration tests (48-h sweetener vs. water), rats and mice preferred a high-rebaudioside, low-stevioside extract as strongly as saccharin, but the extract stimulated less overdrinking and was much less preferred to saccharin in direct choice tests. Relative to the extract, mice drank more pure rebaudioside A and showed stronger preferences but still less than those for saccharin. Mice also preferred a commercial mixture of rebaudioside A and erythritol (Truvia). Similar tests of sweet receptor T1R3 knockout mice and brief-access licking tests with normal mice suggested that the preferences were based on sweet taste rather than post-oral effects. The preference response of rodents to stevia sweeteners is notable in view of their minimal response to some other noncaloric sweeteners (aspartame and cyclamate).

Key words: C57BL/6J mice, noncaloric sweeteners, rebaudioside A, Sprague–Dawley rats, *Stevia rebaudiana*, sweet taste receptor, T1R3

Introduction

Stevia is a natural sweetener extract derived from the plant *Stevia rebaudiana* Bertoni (Geuns 2003; Singh and Rao 2005). It includes several compounds that have a sweet taste, with the major sweet components being stevioside and rebaudioside A (Prakash et al. 2008). Recently stevia products have been marketed as a natural, noncaloric tabletop sweetener and included in soft drinks (Carakostas et al. 2008; Prakash et al. 2008). Little is known about the sweetener potency of stevioside and rebaudioside A in laboratory rats and mice, which have been extensively studied for their behavioral and physiological response to caloric and noncaloric sweeteners. Rats and mice show preferences for some noncaloric sweeteners that humans prefer; for example, saccharin but not for others, for example, aspartame and cyclamate (Murray et al. 1953; Wagner 1971; Sclafani and Abrams 1986; Bachmanov et al. 2001). Although saccharin has been a useful tool in the study of sweet taste, it is a poor sugar substitute for rats: at its maximally preferred concentration it is only as attractive as dilute sucrose (Smith and Sclafani 2002). Limited information is available on the taste response of other species to stevia sweeteners. Gerbils appear to taste stevioside as sweet, that is, sucrose-like, based on chorda tympani nerve recordings and conditioned taste aversion data, but behavioral preference results were not reported (Jakinovich 1981; Jakinovich et al. 1990). Chorda tympani nerve and glossopharyngeal nerve recordings in sucrose-best fibers indicate some but not strong responses to stevioside in pigs; again behavioral preference results were not reported (Danilova et al. 1999).

In the present study, we evaluated stevia taste preferences in Sprague–Dawley rats and C57BL/6J (B6) mice, 2 commonly used rodent strains. Both species were tested because, although rats and mice show similar preference (or no-preference) responses to some artificial sweeteners (saccharin, aspartame, cyclamate, SC45647), they diverge in their response to another sweetener (sucralose) (Murray et al. 1953; Wagner 1971; Sclafani and Abrams 1986; Bachmanov et al. 2001; Sclafani and Clare 2004; Bello and Hajnal 2005; Dess et al. 2009). The stevia taste preference of rats is of particular interest in view of the recent discovery of sweet taste receptors in

the intestinal tract (Dyer et al. 2005; Margolskee et al. 2007), and a study investigating the endocrine response to gastric infusions of a stevia extract in rats (Fujita et al. 2009). Various stevia extract products are available on the market; we selected the extract (Stevia Max) used in the recent gastric infusion study to compare the taste preferences of rats and mice with the sweetener. In addition, mice were tested with a purified rebaudioside A (rebiana) used in some soft drink products and in a tabletop sweetener packet (Truvia). The stevia preferences of rats and mice were compared with their preference for saccharin, the most extensively studied noncaloric sweetener in rodents. In addition to evaluating preferences in 2-bottle sweetener versus water and stevia versus saccharin tests, sweetener acceptance was also evaluated by comparing the absolute intakes of the different sweetener solutions. The importance of the sweet receptor T1R3 in stevia preferences was demonstrated in tests of receptor knockout (KO) mice, and brief intake preference tests of normal mice showed that post-oral contributions to stevia preferences are unlikely. The discovery of a noncaloric sweetener that, unlike saccharin, is as attractive as concentrated sucrose solutions would be very useful to investigate the role of sweet taste in sugar appetite.

Experiment 1: stevia and saccharin preferences in rats

This experiment compared rats' preferences for a commercial stevia preparation and the standard noncaloric sweetener saccharin. Female rats were used because their responses to sweeteners are generally more pronounced than those of male rats (Valenstein 1967) and because they were used in our prior studies of aspartame and sucralose sweeteners (Sclafani and Abrams 1986; Sclafani and Clare 2004).

Materials and methods

Animals

Twenty female Sprague–Dawley rats were purchased from Charles River Laboratories and delivered to our laboratory 10 days prior to the start of the study. They were 10-weeks old at testing. The rats were singly housed in hanging stainless steel cages with ad libitum access to chow (5001, PMI Nutrition International) and deionized water in a room maintained at 20 °C with a 12:12 light:dark cycle. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Brooklyn College and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Test solutions

Solutions were prepared using saccharin (sodium saccharin, Sigma Chemical), a stevia extract (Stevia Max, JG Group), and deionized water. According to the company Stevia Max contains 61% rebaudioside A and 6–10% stevioside; other components are not described. Because the molecular weight of Stevia Max is unspecified, the stevia and saccharin solutions were formulated on a percent basis rather than a molar basis. A preliminary study indicated that a concentration range of 0.001–1% was appropriate to compare the 2 sweeteners. The solutions were prepared as wt/wt solutions because intakes were measured by weight.

Apparatus

The 2-bottle tests were conducted in the animals' home cages. Fluid was available through sipper spouts attached to glass bottles that were held on the front of the cage with springs. Fluid intakes were measured to the nearest 0.1 g by weighing the drinking bottles on an electronic balance interfaced to a laptop computer. Daily fluid spillage was estimated by recording the change in weight of 2 bottles that were placed on an empty cage. The estimated spill throughout the experiment was ~0.6 g, and intake measures were corrected by this amount.

Method

For 6 days, the rats were given access to 2 bottles of water. The animals were then divided into 2 groups of 10 animals each (Stevia and Saccharin) matched for mean body weight (253 vs. 254 g) and water intake (38.1 vs. 39.6 g/day). They were given a series of 48-h 2-bottle sweetener versus water tests at ascending concentrations of 0.001%, 0.003%, 0.01%, 0.03%, 0.1%, 0.3%, and 1% (Test 1A). The left–right position of the sweetener and water was alternated daily in this and subsequent tests.

The animals were then given only water for 6 days. During the next 8 days, they were given a series of stevia and saccharin tests. Test 1B was 2-bottle access to 0.1% sweetener versus water for 4 days. Half of the rats in each group were given their previous sweetener on the first 2 days and the opposite sweetener on the second 2 days; this was reversed for the other half of the animals. This was followed by Test 1C, 4 days of direct comparison of the sweeteners in 2-bottle tests. The animals were given 0.1% saccharin versus 0.1% stevia for 2 days and then the concentrations of both sweeteners were increased to 0.3% for the second 2 days.

Data analysis

Daily solution and water intakes were averaged over the 2 days at each solution concentration. Sweetener intakes were also expressed as percent intakes (sweetener intake/(sweetener + water intakes) × 100). Group differences in sweetener intakes and preferences were evaluated using separate mixed-model analyses of variance with group and sweetener concentration as between-group and within-group factors, respectively.

The significance of the 2-bottle sweetener preference at each concentration was evaluated within each group by comparing sweetener versus water intakes using paired *t*-tests

corrected for multiple comparisons using the Bonferroni procedure.

Results

In Test 1A (Figure 1), the Saccharin group consumed more sweetener than the Stevia group, $F_{1,18} = 10.44$, $P < 0.0001$. Both groups increased and then decreased solution intake as concentration increased ($F_{6,108} = 26.9$, $P < 0.0001$). There was a group × concentration interaction ($F_{6,108} = 11.78$, $P < 0.0001$) due to greater intakes ($P < 0.05$) of saccharin than stevia at 0.1% and 0.3% concentrations. The Saccharin rats at their peak saccharin intakes (0.3% concentration) consumed 2.7 times more sweetener than their water baseline (104.8 vs. 38.1 g/day), whereas the Stevia rats at their peak intake (0.1% concentration) consumed no more sweetener than their water baseline (39.9 vs. 39.6 g/day); this group difference in sweetener-stimulated intake was significant, $t_{18} = 3.66$, $P < 0.01$.

Percent sweetener intake did not differ between the groups but did differ as a function of concentration, $F_{6,108} = 26.39$, $P < 0.0001$; there was no group × concentration interaction. Numerically, peak preference was at the 0.1% concentration, although preferences for the midrange 0.03%, 0.1%, and 0.3% concentrations did not differ. Preferences for the lowest and highest concentrations tested, 0.001% and 1%, were just below 50%. In the sweetener versus water tests, the rats significantly preferred saccharin at 0.01–0.3% concentrations and stevia at 0.01–0.1% concentrations.

In Tests 1B and 1C, intakes of the original Saccharin and Stevia groups did not differ and therefore their data are combined in Figure 2. Intakes were greater in the 0.1% saccharin test than in the 0.1% stevia test ($F_{1,19} = 18.16$, $P < 0.001$), which was due to a much greater intake of saccharin than stevia with no difference in water intake (interaction $F_{1,19} = 12.88$, $P < 0.01$). The 93% preference for saccharin over water did not differ significantly from the 82% preference for stevia versus water. Overall, the rats strongly preferred saccharin over stevia ($F_{1,18} = 61.13$, $P < 0.0001$) with no effect of sweetener concentration. Percent preference for saccharin was not affected by original group membership or concentration.

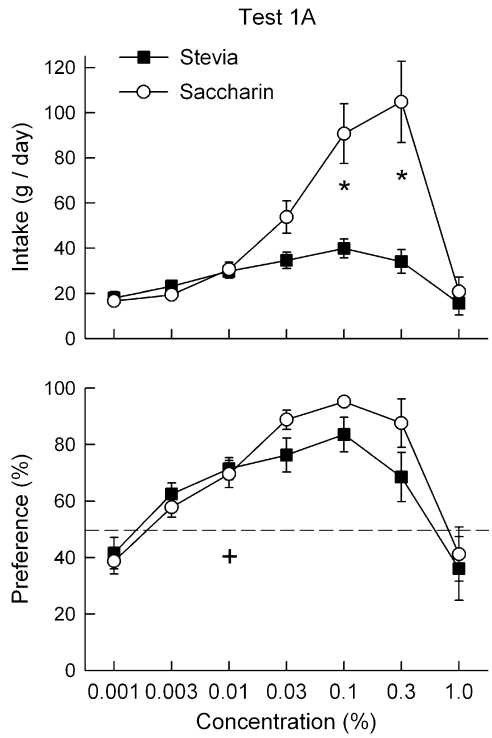

Figure 1 Experiment 1. Mean (±standard error of the mean) stevia and saccharin intakes (top) and preference (bottom) of female rats in 2-bottle tests with sweetener versus water. Significant ($P < 0.05$) between-group differences are indicated by an asterisk (*), and the plus sign (+) indicates the preference threshold (lowest concentration at which the rats consumed more sweetener than water).

Discussion

Stevia, like saccharin, was attractive to rats, as measured by their preference for stevia over water in the 2-bottle tests. However, stevia did not promote overdrinking relative to the water baseline, whereas saccharin almost tripled daily fluid intake. In addition, saccharin was strongly preferred to stevia in direct 2-bottle tests. In contrast to these results, a recent paper (Figlewicz et al. 2009) reported substantial overdrinking in rats given a different stevia product (Stevia Now). However, it was subsequently revealed that this product was not a pure stevia extract but was mixed with rice maltodextrin as a bulking agent (Figlewicz et al. 2010). Maltodextrin has a very attractive taste to rats as well as post-oral effects that stimulate intake (Sclafani 1987; Sclafani and Nissenbaum 1988) and thus likely contributed to the overconsumption of the stevia solution. Note that rats also overconsume the sweetener sucralose when it is packaged with maltodextrin (Splenda) but show a much weaker and inconsistent preference response to solutions containing pure sucralose (Sclafani and Clare 2004; Bello and Hajnal 2005; Dess et al. 2009).

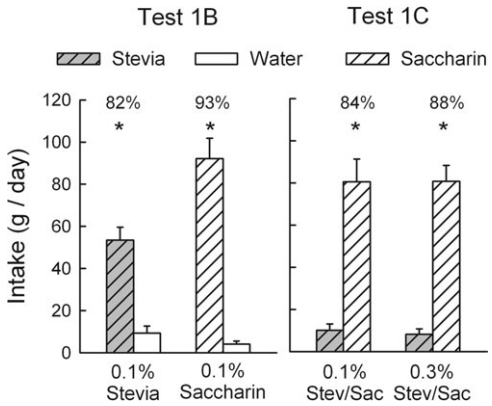

Figure 2 Experiment 1. Mean (+standard error of the mean) intakes of female rats in 2-bottle tests 1B (sweeteners vs. water) and 1C (stevia vs. saccharin). Significant ($P < 0.05$) differences between solutions are indicated by an asterisk (*).

The failure of rats to overdrink stevia solutions or prefer them to saccharin solutions indicates that stevia cannot replace saccharin as a sugar substitute for experimental purposes. This is of practical interest because saccharin is a relatively poor sugar substitute for rats. That is, the most preferred saccharin solutions (0.2–0.4%) are isopreferred to only 2–4% sucrose solutions and not nearly as potent as more concentrated sugar solutions in motivating behavior (Smith and Sclafani 2002). Earlier studies indicate that cyclamate, aspartame, and sucralose are even less effective than stevia as sweeteners for rats, although rats are attracted to the artificial sweetener SC45647 (Murray et al. 1953; Wagner 1971; Sclafani and Abrams 1986; Sclafani and Clare 2004; Bello and Hajnal 2005; Dess et al. 2009). Acesulfame K has been used as a sweetener in a few rat studies but there are no published acesulfame K versus water preference data (Hughes et al. 1987; Swithers et al. 2009). In our laboratory, 0.1% acesulfame K and 0.1% saccharin (~5 mM) were equally preferred and overconsumed relative to water by female rats (90.5 vs. 10.1 g, 88%; 80.3 vs. 6.5 g, 83%), and acesulfame K was equally preferred to saccharin at 0.1% and 0.3% concentrations in direct choice tests (53% and 56%; Sclafani A, Ackroff K, unpublished data). This equivalent response is interesting in light of the demonstration that the bitter quality of these 2 sulfonyl amides is detected in humans by the same receptors, T2R43 and T2R44 (Kuhn et al. 2004).

Experiment 2: stevia and rebiana tests in mice

We conducted the same series of stevia and saccharin preference tests in female B6 mice as in Experiment 1. In addition, we tested a source of relatively pure rebaudioside A (rebiana), which is the stevia component with the sweetest taste and least off-taste in human evaluation (Tanaka 1997; Prakash et al. 2008), and a consumer tabletop version of rebaudioside A (Truvia).

Materials and methods

Animals

Thirty female B6 mice, born in our laboratory from stock purchased from the Jackson Laboratories, were studied in 2 subsets as described below. The mice were 18-weeks old at testing. The mice were singly housed in plastic tub cages with ad libitum access to chow (5001) and deionized water in a room maintained at 22 °C with a 12:12 light:dark cycle.

Test solutions

Saccharin and the stevia extract (Stevia Max) were prepared in deionized water as in Experiment 1. Rebaudioside A solutions were prepared at 0.001–1% concentrations using rebiana, also known as reb A, which is 97.8% purified (Cargill, Inc.). Note that the molecular weights of sodium saccharin and pure rebaudioside A are 205.2 and 697.01, respectively. Thus, the percentage range of 0.001–1% corresponds to 0.049–48.73 mM saccharin and to 0.014–14.35 mM rebaudioside A.

In addition to stevia and rebiana, solutions containing a consumer version of rebaudioside A were prepared at concentrations of 1%, 2%, 4%, and 8% using the tabletop sweetener Truvia, which is a mixture of rebaudioside A, the sugar alcohol erythritol and unspecified natural flavors (Cargill). According to the company, erythritol is added to provide bulk and sweetening and to reduce the after-taste and off-flavors of intense sweeteners. The nutrition label lists erythritol as 3 g per 3.5 g serving, about 86% by weight. The exact rebaudioside A content is not specified but its maximal amount would be ~14% by weight. Because B6 mice display a preference for erythritol over water (Bachmanov et al. 2001), the Truvia preference was compared with the preference for erythritol (Honeyville Grain) solutions. The solution concentrations (1–8%) selected include the range previously studied in B6 mice using erythritol (Bachmanov et al. 2001).

Apparatus

The 2-bottle tests were conducted in the animal's home cage. Fluid was available through sipper spouts attached to 50-mL plastic tubes that were placed on top of the cage. The sipper spouts were inserted through holes positioned 3.7 cm apart in a stainless steel plate, and the drinking tubes were fixed in place with clips. Measurement of intakes followed the method of Experiment 1. The estimated spill throughout the experiment was ~0.4 g, and intake measures were corrected by this amount.

Method

For the first week, the mice were given access to 2 bottles of water. The original 20 mice were then divided into 2 groups of 10 animals (Stevia and Saccharin) equated for body weight (22.0 vs. 22.3 g) and water intake (5.0 vs. 5.2 g/day). The later 10 mice were added as the Rebiana group and had similar baseline body weights (22.6 g) and water intakes (5.2 g/day) as the Stevia and Saccharin groups. Each group was given a series of 48-h 2-bottle sweetener versus water tests at ascending concentrations of 0.001%, 0.003%, 0.01%, 0.03%, 0.1%, 0.3%, and 1% (Test 2A).

Following the ascending series, the Saccharin and Stevia groups had 9 days of access to water only. During the next 8 days, they were given a series of stevia and saccharin tests. Test 2B was 4 days of 2-bottle access to 0.1% sweetener versus water. Half the mice in each group were given stevia in the first 2 days and saccharin in the second 2 days; the order was reversed for the other half of the animals. This was followed by Test 2C, 4 days of direct comparison of the sweeteners in 2-bottle tests. The animals were given 0.1% saccharin versus 0.1% stevia for 2 days and then the concentrations of both sweeteners were increased to 0.3% for the second 2 days.

The Rebiana group was treated similarly, except that they had 6 days of access to water only following Test 2A. For Test 2B, half the mice were given 0.1% rebiana versus water in the first 2 days and 0.1% saccharin versus water in the second 2 days; the order was reversed for the other animals. For Test 2C, the animals were given 0.1% saccharin versus 0.1% rebiana for 2 days and then the concentrations of both sweeteners were increased to 0.3% for the second 2 days.

Following Test 2C, the Saccharin and Stevia groups had 8 days of access to water only. Then they were distributed into 2 new groups of 10, with 5 mice each from the Saccharin and Stevia groups. In Test 2D, one group was given a series of 48-h 2-bottle Truvia versus water tests at ascending concentrations of 1%, 2%, 4%, and 8%. The second group was similarly tested but with 1–8% erythritol versus water.

The data were analyzed as in Experiment 1.

Results

In Test 2A (Figure 3), intakes of sweeteners differed among the groups, $F_{2,27} = 13.86$, $P < 0.0001$. All 3 groups increased and then decreased solution intake as concentration increased ($F_{6,162} = 145.04$, $P < 0.0001$) and the group × concentration interaction was significant, $F_{12,162} = 30.04$, $P < 0.0001$. There were no group differences at 0.001%, 0.003%, and 0.03% concentrations but at 0.1% the Saccharin group consumed more ($P < 0.05$%) sweetener than did the Stevia group. All 3 groups differed at the remaining concentrations; at 0.01%, intakes from highest to lowest were rebiana > stevia > saccharin, but at 0.3% and 1%, the order was saccharin > rebiana > stevia. The Saccharin mice at their peak saccharin intake (0.3% concentration) consumed 2.4 times more sweetener than their water baseline (11.7 vs. 5.0 g/day), whereas the Rebiana mice consumed 1.6 times more (8.3 vs. 5.2 g/day) and the Stevia mice consumed 1.4 times more (7.4 vs. 5.2 g/day) sweetener than water at their peak concentrations of 0.1%. The difference in sweetener-stimulated fluid intake was significantly greater ($P < 0.05$) for the Saccharin group than for the Rebiana and Stevia groups, $F_{2,27} = 20.84$, $P < 0.0001$.

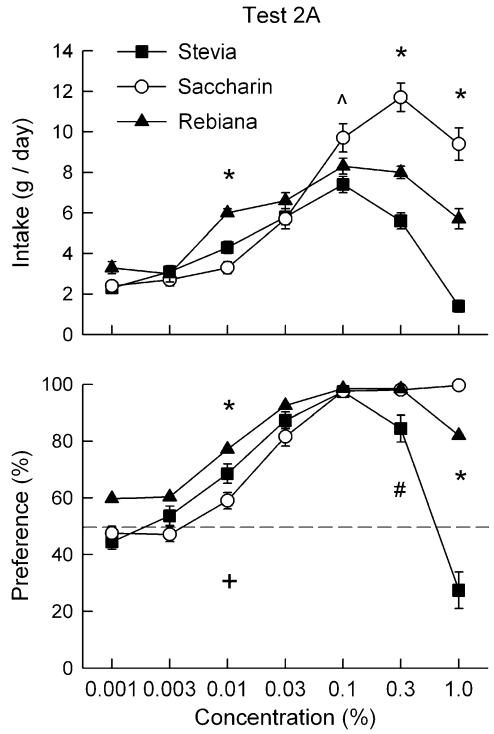

Figure 3 Experiment 2. Mean (±standard error of the mean) stevia, rebiana, and saccharin intakes (top) and preference (bottom) of female mice in 2-bottle tests with sweetener versus water. Significant ($P < 0.05$) between-group differences indicated by an asterisk (*; all groups differ), a carat (^; saccharin > stevia), or a pound sign (#; saccharin and rebiana > stevia). The plus sign (+) indicates the preference threshold (lowest concentration at which the mice consumed more sweetener than water).

Percent intakes of sweeteners differed among the groups, $F_{2,27} = 14.87$, $P < 0.0001$, as a function of concentration ($F_{6,162} = 100.55$, $P < 0.0001$), and the group × concentration interaction was also significant, $F_{12,162} = 20.27$, $P < 0.0001$. Rebiana preference exceeded that for the other sweeteners at 0.001% but was less than the preference for saccharin at 1%. Stevia preference was less than for the other sweeteners at 0.3% and 1% and intermediate to the others at 0.01%.

In Test 2A, the Saccharin and Rebiana groups drank more ($P < 0.05$) sweetener than water at 0.01–1% concentrations. The range was slightly smaller for the Stevia group, which drank more sweetener than water at 0.01–0.3% concentrations but less sweetener than water at the 1% concentration.

In Test 2B, the intakes of the Stevia and Saccharin groups did not differ and their data are combined in Figure 4 (top). The mice strongly preferred 0.1% stevia and saccharin to water ($F_{1,19} = 957.2$, $P < 0.0001$). The Rebiana group strongly preferred both 0.1% rebiana and saccharin to water, $F_{1,9} = 931.9$, $P < 0.0001$ (Figure 4, bottom). However, the animals consumed less stevia or rebiana than saccharin when each was offered versus water, as indicated by fluid × test interactions ($F_{1,19} = 36.47$, $P < 0.0001$, $F_{1,9} = 26.02$, $P = 0.001$) and simple main effects tests.

In the direct sweetener comparison of Test 2C (Figure 4), the Saccharin and Stevia mice drank much more saccharin than stevia ($F_{1,18} = 349.2$, $P < 0.0001$). The mice drank more in the 0.3% test than the 0.1% test ($F_{1,18} = 10.07$, $P < 0.001$); the greater intake reflected a larger intake of 0.3% than 0.1% saccharin, whereas the small stevia intakes did not differ (interaction $F_{1,18} = 16.59$, $P < 0.0001$ and simple main effects). The percent intake of saccharin versus stevia was greater at 0.3% than at 0.1% ($t_{19} = 3.16$, $P < 0.01$). The Rebiana mice drank much more saccharin than rebiana ($F_{1,9} = 115.9$, $P < 0.0001$). They drank more 0.3% saccharin than 0.1% saccharin, whereas the small rebiana intakes did not differ (interaction $F_{1,9} = 7.67$, $P < 0.05$ and simple main effects). The percent intake of saccharin versus rebiana was greater at 0.3% than at 0.1% ($t_9 = 2.59$, $P < 0.05$).

In Test series 2D (Figure 5), the Truvia group drank more sweetener than the Erythritol group, $F_{1,18} = 10.26$, $P < 0.01$.

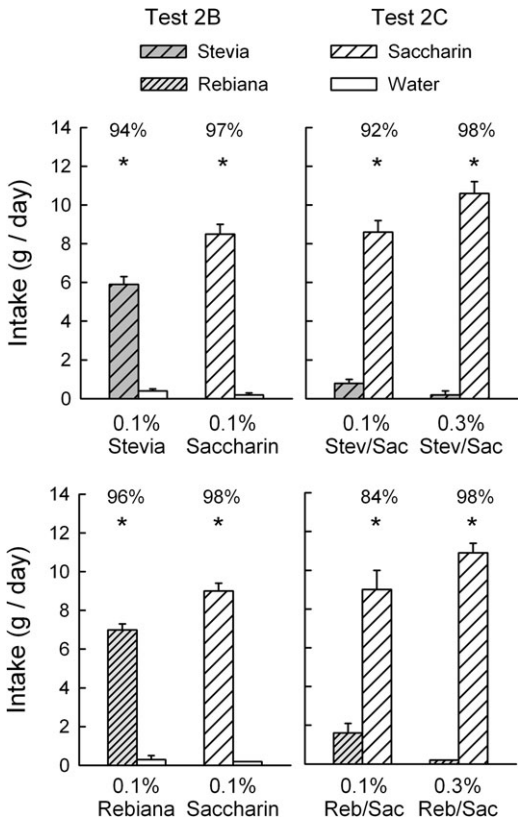

Figure 4 Experiment 2. Mean (+standard error of the mean) intakes of female mice in 2-bottle tests 2B (sweeteners vs. water) and 2C (sweetener vs. sweetener). The top panel shows the averaged intakes of the stevia and saccharin groups, which did not differ. The bottom panel shows the intakes of the Rebiana group. Significant ($P < 0.05$) differences between solutions are indicated by an asterisk (*).

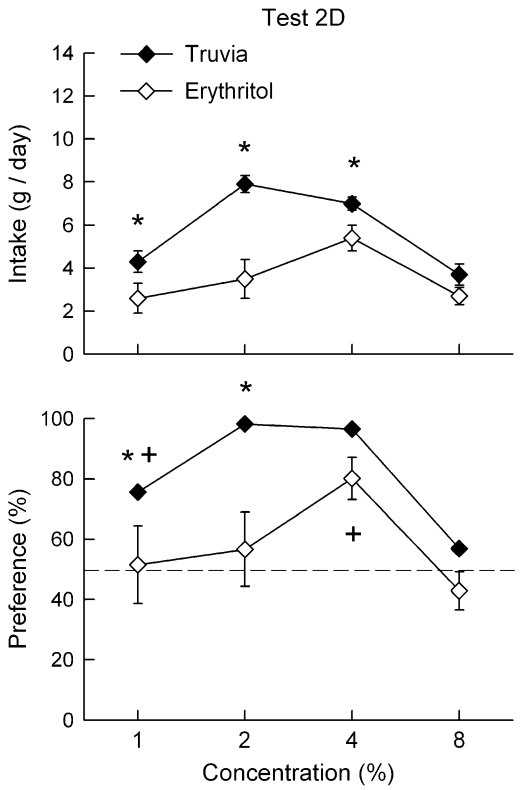

Figure 5 Experiment 2. Mean (±standard error of the mean) Truvia and erythritol intakes (top) and preference (bottom) of female mice in 2-bottle tests with sweetener versus water. Significant ($P < 0.05$) between-group differences are indicated by an asterisk (*), and the plus sign (+) indicates the preference threshold (lowest concentration at which the mice consumed more sweetener than water).

Both groups increased and then decreased solution intake as concentration increased ($F_{3,54} = 45.54$, $P < 0.0001$), and the group × concentration interaction was significant, $F_{3,54} = 10.64$, $P < 0.0001$. The Truvia group drank more sweetener at 1–4% concentrations than did the Erythritol group. The Truvia mice at their peak intake (2% concentration) consumed 1.5 times more sweetener than water baseline (7.9 vs. 5.1 g/day), whereas the Erythritol mice at their peak intake (4% concentration) consumed no more sweetener than their water baseline (5.4 vs. 5.6 g/day); this difference in sweetener-stimulated intake was significant, $t_{18} = 3.98$, $P < 0.001$.

Overall, percent intake of sweetener was greater in the Truvia than the Erythritol group, $F_{1,18} = 6.51$, $P < 0.05$. Percentages differed as a function of concentration ($F_{3,54} = 27.28$, $P < 0.0001$), and the group × concentration interaction was also significant, $F_{3,54} = 3.78$, $P < 0.05$. Truvia preference exceeded erythritol preference at 1% and 2%, and the group preferences did not differ significantly at higher concentrations. The Truvia group drank more sweetener than water at 1–4% concentrations, whereas the Erythritol group only drank more sweetener than water at the 4% concentration.

Discussion

During the ascending series, when each animal was exposed to only one sweetener, intakes of B6 mice differed little at the lower concentrations, and at higher concentrations, the rank order of sweetener intakes was saccharin > rebiana > stevia. In direct comparisons among the 3 sweeteners, the mice consumed substantially more saccharin than rebiana and stevia. Thus, saccharin was most preferred and most effective in stimulating intake among the 3 sweeteners. Although rebiana and stevia were not directly compared, in the sweetener versus water tests, rebiana produced stronger preferences and stimulated greater intakes than did stevia. This can be attributed to the higher rebaudioside A concentration of rebiana compared with the Stevia Max product (97.8% vs. 61%). Note that although rebiana was less effective than saccharin in stimulating fluid intake, it was more potent on a molar basis. That is, rebiana intake peaked at a 1 mM (0.1%) concentration, whereas saccharin intake peaked at a 14.6 mM (0.3%) concentration.

In the comparison of the sugar alcohol erythritol versus Truvia, which is primarily erythritol by weight but contains as much as 14% rebiana, the Truvia solution was more

effective at stimulating intake and was preferred across more concentrations. This indicates that the addition of rebiana to erythritol enhanced the attractiveness of the mixture, although the role of the unspecified natural flavors in the product is not known. The lower intakes of both solutions at the lowest and highest concentrations suggest that we tested an adequate range to evaluate preferences in these animals. A preference for erythritol over water was apparent only at 4%, which is consistent with the previous report that B6 mice preferred erythritol at 3% and 6% concentrations but not at lower or higher concentrations (Bachmanov et al. 2001). The most preferred Truvia solution (2%) stimulated fluid intake to the same degree as the most preferred rebiana solutions (0.1–0.3%; 7.9 vs. 8.0–8.3 g/day). The maximal rebaudioside A concentration of the 2% Truvia solution (~0.28%) is close to that of the most preferred rebiana solutions (0.1% and 0.3%).

Experiment 3: rebiana and saccharin preferences in T1R3 KO and B6 mice

The strong preference rats and mice displayed for saccharin over stevia or rebiana suggests that saccharin is more effective in stimulating the rodent sweet taste receptor than are stevioside and rebaudioside A compounds. Alternatively, saccharin may have less of an off-taste to rodents than the stevia compounds. We evaluated this second possibility using KO mice missing the T1R3 sweet taste receptor. Prior studies indicate that T1R3 KO mice avoid rather than prefer saccharin solutions, which is attributed to insensitivity to the sweet taste but sensitivity to the bitter taste of saccharin (Damak et al. 2003; Blednov et al. 2008; Zukerman et al. 2009). Differential avoidance of saccharin and rebaudioside A by T1R3 KO mice would suggest that the 2 sweeteners differ in their off-taste to mice.

Materials and methods

Animals

Female T1R3 KO mice ($n = 11$) were derived from mice produced by homologous recombination in C57BL/6J embryonic stem cells and maintained on this background (Damak et al. 2003). C57BL/6J wild-type (B6 WT) mice ($n = 10$) were derived from mice obtained from the Jackson Laboratories. They were 36-weeks old at testing and were housed as in Experiment 2.

Method

Solutions of 0.3% saccharin and 0.3% rebiana were prepared as in Experiment 2. For the first 2 days, the mice were given access to 2 bottles of water. During the next 6 days, they were given a series of rebiana and saccharin tests. Test 3A was 4 days of 2-bottle access to 0.3% sweetener versus water. Half the mice were given 0.3% rebiana versus water in the first 2 days and 0.3% saccharin versus water in the second 2 days; the order was reversed for the other animals. For Test 3B, the animals were given 0.3% saccharin versus 0.3% rebiana for 2 days. The significance of the 2-bottle sweetener preference at each concentration was evaluated within each group by comparing sweetener versus water intakes using paired *t*-tests corrected for multiple comparisons using the Bonferroni procedure.

Results and discussion

The KO mice consumed significantly more water than either sweetener in Test 3A ($P < 0.01$); they avoided rebiana and saccharin to the same degree (percent intakes of 17% and 19%, respectively; Figure 6). The KO mice did not differ in their rebiana versus saccharin intakes in Test 3B, displaying a nonsignificant 60% preference for saccharin. In contrast, the B6 WT mice consumed significantly ($P < 0.01$) more rebiana and saccharin than water in Test 3A (85% and 99%, respectively; Figure 6) and more saccharin than rebiana in Test 3B (97%; $P < 0.01$). In addition, the B6 mice

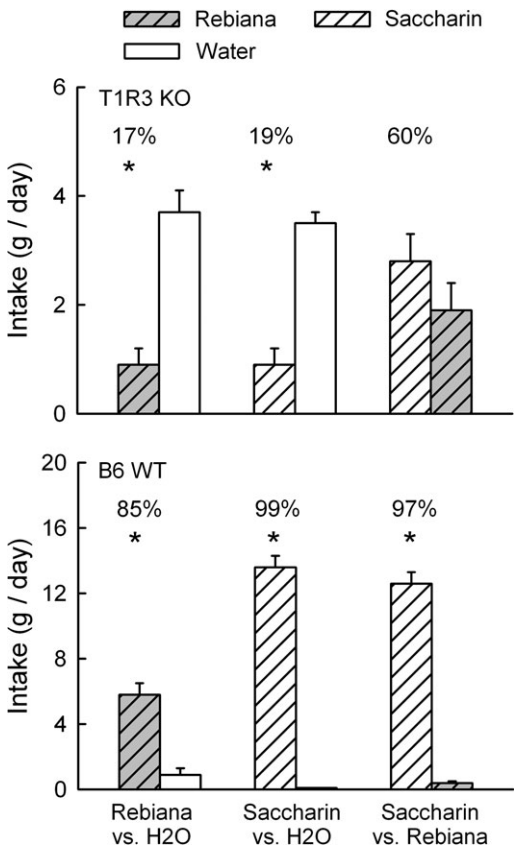

Figure 6 Experiment 3. Mean (+standard error of the mean) intakes of female T1R3 KO mice (top panel) and B6 WT mice (bottom panel) in 2-bottle tests 3A (sweeteners vs. water) and 3B (sweetener vs. sweetener). Significant ($P < 0.01$) differences between solutions are indicated by an asterisk (*).

consumed more saccharin than rebiana in the sweetener versus sweetener tests ($P < 0.01$), replicating the preference for saccharin over rebiana in Test 2C. Thus, like saccharin, stevia is unattractive to mice without a functioning sweet taste receptor. The responses of T1R3 KO mice suggest that saccharin and rebiana do not differ in their aversive off-tastes; compared with rebiana, the stronger attraction to saccharin in normal mice likely represents superior stimulation of rodent sweet receptors.

Experiment 4: rebiana and saccharin brief licking tests in mice

The recent discovery of sweet taste receptors in the gut and pancreas raise the possibility that post-oral factors may contribute to the long-term intake of and preference for caloric and noncaloric sweeteners (Jang et al. 2007; Mace et al. 2007; Margolskee et al. 2007; Nakagawa et al. 2009). It is also possible that compounds in stevia extracts may have metabolic effects independent of sweet taste receptors (Chatsudthipong and Muanprasat 2009). To determine if oral stimuli alone can account for the strong preference B6 mice displayed for saccharin over rebiana in Experiments 2 and 3, we measured sweetener preference in naïve B6 mice using 60-s 2-bottle tests that greatly reduce or eliminate post-oral effects.

Materials and methods

Animals

Female B6 mice ($n = 10$) were derived from mice obtained from the Jackson Laboratories. They were 26-weeks old at testing and were housed as in Experiment 2.

Apparatus

Tests were conducted in clear plastic cages ($15 \times 15 \times 32$ cm) with a stainless steel perforated floor. Fluid was available from 1 or 2 stainless steel sipper spouts through slots (5×20 mm, 32-mm apart) in a stainless steel plate at the front of the cage. The sipper spouts were attached to motorized bottle holders (ENV-252M; Med Associates) that positioned the spouts 1 mm in front of the cage at the start of a trial and retracted them at the end of the trial. Licking behavior was monitored with electronic lickometers (ENV-250B, Med Associates) interfaced to a microcomputer.

Solutions

Solutions of 0.1% and 0.3% saccharin and rebiana were prepared as in Experiment 2. In addition, saccharin was prepared at a 0.06% concentration, which is isomolar to 0.3% rebiana.

Method

The mice were initially water deprived overnight and trained to drink water in the test cages during a 5-min session. The next day, while still water deprived, the animals were given 60-s 2-bottle choice tests between 0.1% rebiana versus 0.1% saccharin. (We have found this to be a rapid technique for training mice to drink in the cages, which transfers well when deprivation is shifted from water to food.) The mice were then given ad libitum access to water but food restricted overnight. The next day, while food deprived, a second set of 60-s 2-bottle choice tests between 0.1% sweeteners was conducted. This was followed on the next days by 2-bottle tests with 0.3% sweeteners and 3.1 mM sweeteners. On all sweetener test days, the mice were first given 5-s access to one sipper tube and then the other sipper tube to allow them to sample the contents of the left and right bottles; this was followed immediately by 60-s access to both sipper tubes. The animals were then returned to their home cages for 1 h and then given a second 70-s test. The left–right positions of the 2 sweeteners were switched from the first to second daily test sessions. Sweetener preference was measured by recording licks on the left and right sipper tubes. The timing of each session duration for each mouse began with the 10th lick. The sipper tubes were automatically retracted away from individual cages 5 or 60 s after the 10th lick according to the session requirement. Food rations (2.5 g) were given to the animals 1 h after the second test of each day.

Data analysis

The 60-s lick data were averaged for the 2 sessions of each sweetener comparison in the food-restriction test series. Saccharin preference was expressed as the percent of licks on the saccharin tube (saccharin licks/(saccharin + rebiana licks) $\times$ 100). The significance of the saccharin preference in each comparison was evaluated by comparing saccharin and rebiana licks using paired *t*-tests corrected for multiple comparisons using the Bonferroni procedure.

Results and discussion

The mice licked more for saccharin than rebiana in all 3 comparisons (Figure 7). When offered 0.1% or 0.3% saccharin versus rebiana solutions, the mice displayed significant ($P < 0.01$) saccharin preferences (74% and 85%, respectively). In tests with isomolar (3.1 mM) solutions, they preferred saccharin (0.06%) to rebiana (0.3%) by 81%. In addition, they licked more for 0.3% saccharin than 0.1% saccharin or 3.1 mM (0.06%) saccharin ($P < 0.01$).

Preferences for saccharin over rebiana in the 60-s tests approached those observed in Tests 2C (84%, 98%) and 3B (98%). Because these animals were given only a limited number of brief tests, their responses should reflect only their orosensory reactions to the solutions. Thus, oral factors alone can explain the relative saccharin preference displayed by mice (and presumably rats). The 60-s data, however, do not exclude possible post-oral metabolic effects on the intake of the sweeteners in 48-h tests.

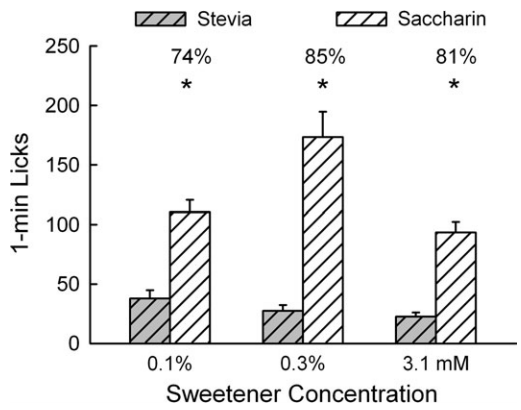

Figure 7 Experiment 4. Mean (+standard error of the mean) 1-min lick totals of female mice in 2-bottle preference tests with 0.1% and 0.3% rebiana versus saccharin and with 3.1 mM solutions of rebiana (0.3%) versus saccharin (0.06%). Significant ($P < 0.01$) differences between solutions are indicated by an asterisk (*).

General discussion

These experiments provided new information on the responses of common laboratory strains of rats and mice to stevia compounds. Both species displayed preferences for a stevia extract over water at a range of concentrations (0.01% to 0.1% or 0.3%). Mice also preferred solutions prepared with pure rebaudioside A (0.01–1%) to water and rebaudioside A plus erythritol mixture (2–4% Truvia) to water. However, stevia and rebaudioside A were less effective than saccharin in stimulating fluid intake and were much less preferred to saccharin in direct 2-bottle tests. When the influence of sweet taste was removed, the remaining taste qualities of saccharin and stevia were similar. When the influence of post-oral effects was removed by offering the solutions in brief tests, mice showed similar preferences to those with more prolonged access.

The species showed some similarities and some differences in their responsiveness to the tested sweeteners. Rats showed similar preference curves for saccharin and stevia (vs. water) across the tested range of concentrations, but they consumed much more saccharin than stevia at their preferred concentrations. In fact, unlike saccharin, stevia did not stimulate rats to overdrink relative to their water baseline. In contrast, stevia did stimulate mice to overdrink but less so than did saccharin. Saccharin stimulated fluid intake to about the same degree in rats and mice at 0.1–0.3% concentrations, but rats lost their saccharin preference at 1%, whereas mice continued to prefer the sweetener. This divergence at high saccharin concentrations has been observed previously (Collier and Novell 1967; Bachmanov et al. 2001). The difference between sweetener preferences of rats and mice may be due to species differences in the sensitivity of the sweet taste receptor. Note, however, that this study only examined one strain each of rats and mice, and both species are known to differ considerably in sweet taste responsiveness across strains (Goodwin and Amit 2000; Bachmanov et al. 2001; Reed et al. 2004; Lu et al. 2005; Sclafani 2006; Tordoff et al. 2008). Furthermore, animals, like humans, have different sensitivities to bitter tastes (Prutkin et al. 2000; Blizard 2007; Tepper 2008), which are components of these intense sweetener tastes, at least by human evaluation. Saccharin activates specific human T2R bitter receptors (Kuhn et al. 2004); the details of bitter sensing of saccharin and other intense sweeteners by rodents have not yet been deduced.

In spite of the well-known off-taste of saccharin, it was superior in attractiveness to rodents when the animals chose between it and the stevia or rebiana solutions at concentrations that elicited the most drinking. Thus even pure rebaudioside A, which is said to have less of an off-taste than stevioside extracts, was less attractive than saccharin to rats. Human tasters evaluate the sweetness of 0.03% rebiana (in the middle of our tested range) as equivalent to 6% sucrose. Above this level, they report off-tastes of bitterness and a licorice flavor (Schiffman et al. 1995; Prakash et al. 2008). The rats and mice displayed significant stevia and rebiana preferences even at 10 times this concentration, although they did reduce preference and/or intake as concentrations were raised to 1%. The similar avoidance of saccharin and rebiana by T1R3 KO mice insensitive to sweet taste shows that the difference in sweetener intake and preference is unlikely to reflect large differences in off-tastes. Human tasters rate the sweetness of stevioside and rebaudioside A similarly as concentration increases, but the bitter rating for stevioside is more prominent than that of rebaudioside at higher concentrations (Schiffman et al. 1995). If rodent responses are similar, at high concentrations the stevia extract, due to its stevioside content, might be more avoided than pure rebaudioside by KO mice.

A notable aspect of the present data is the differences in the various measures of sweetener avidity: sweetener versus water preference, sweetener versus sweetener preference, and sweetener acceptance (total daily intake). For example, the B6 mice showed identical preferences (98%) for 0.3% saccharin and rebiana over water, yet they drank significantly more saccharin than rebiana (11.7 vs. 8.0 g/day) and showed a 98% preference for 0.3% saccharin over rebiana in a direct choice test. These findings indicate that strong sweetener versus water preferences do not provide an accurate measure of the relative preference and acceptability of different sweeteners. This is presumably due to a ceiling effect (preferences near 100%) that obscures how attractive the sweetener is to the animal. At lower concentrations (0.01–0.03%) that did not produce near-total preferences (vs. water), the preference scores and daily intake measures of saccharin, rebiana, and stevia were correlated. These results indicate that the stimulatory actions of the different sweeteners on taste receptors can largely account for the differential daily intakes and relative preference in sweetener versus sweetener tests. The possibility that post-oral actions of saccharin or stevia compounds may influence sweetener intake has not been investigated.

Stevia-derived products are being marketed as "natural" sweeteners because of their plant origin but whether they are superior to other noncaloric sweeteners for human use remains to be determined. The present findings demonstrate that stevia and rebiana, unlike some other noncaloric sweeteners, are preferred by rodents, although they are much less preferred and stimulate less overdrinking compared with saccharin.

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