Cellular/Molecular

Gs\(\alpha\) Is Involved in Sugar Perception in

*Drosophila melanogaster*

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In *Drosophila melanogaster*, gustatory receptor genes (Grs) encode G-protein-coupled receptors (GPCRs) in gustatory receptor neurons (GRNs) and some olfactory receptor neurons. One of the Gr genes, *Gr5a*, encodes a sugar receptor that is expressed in a subset of GRNs and has been most extensively studied both molecularly and physiologically, but the G-protein \(\alpha\) subunit (Gs) that is coupled to this sugar receptor remains unknown. Here, we propose that Gs is the Gs that is responsible for Gr5a-mediated sugar-taste transduction, based on the following findings: First, immunoreactivities against Gs were detected in a subset of GRNs including all Gr5a-expressing neurons. Second, trehalose-intake is reduced in flies heterozygous for null mutations in DGs\(\alpha\), a homolog of mammalian Gs, and trehalose-induced electrical activities in sugar-sensitive GRNs were depressed in those flies. Furthermore, expression of wild-type DGs\(\alpha\) in sugar-sensitive GRNs in heterozygotic DGs\(\alpha\) mutant flies rescued those impairments. Third, expression of double-stranded RNA for DGs\(\alpha\) in sugar-sensitive GRNs depressed both behavioral and electrophysiological responses to trehalose. Together, these findings indicate that DGs\(\alpha\) is involved in trehalose perception. We suggest that sugar-taste signals are processed through the Gs\(\alpha\)-mediating signal transduction pathway in sugar-sensitive GRNs in *Drosophila*.

**Key words:** gustatory receptor; Gs; *Drosophila melanogaster*; sweet taste; trehalose; cAMP transduction pathway

Introduction

Sugar is a major source of energy for animals, and its taste is appealing, but the transduction pathway by which animals detect sugar in their environment and then process sugar-taste information into neuronal signals remains undetermined. In *Drosophila melanogaster*, gustatory receptor neurons (GRNs) express ~60 gustatory receptor genes (Grs), which are members of the G-protein-coupled receptor (GPCR) family (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003). The natural ligands recognized by the Grs are mostly unknown, except one that has been identified, namely trehalose (\(\alpha\)-\(\beta\)-glucopyranosyl-\(\alpha\)-\(\beta\)-glucopyranoside), the receptor of which is encoded by *Gr5a* (Dahanukar et al., 2001; Ueno et al., 2001; Chyb et al., 2003). Gr5a is activated by trehalose and coupled to the G-protein \(\alpha\) subunit (G\(\alpha\)), which potentially routes the signal to several distinct transduction pathways (Neves et al., 2002; Wong, 2003). In this study, we wished to identify which G\(\alpha\) is coupled to the sugar-taste transduction pathway.

In the *Drosophila* genome, 11 genes encode G\(\alpha\) (Ishimoto et al., 2005), and one of them, DGs\(\alpha\), is a homolog of mammalian Gs (Quan et al., 1989). The primary function of the Gs family is to elevate the concentration of cAMP via adenylyl cyclase (AC), and in vertebrates, this transduction pathway is involved in a variety of cellular functions (Tesmer and Sprang, 1998; Hurley, 1999; Simonds, 1999). Electrophysiological and biochemical studies have shown that cAMP is involved in sugar taste in vertebrates, although it is still unclear which isoform of Gs is coupled to the sugar receptor (Avenet and Lindemann, 1987; Striem et al., 1989; Naim et al., 1991). Gs is also involved in other sensory functions, for example, olfactory signaling in the rat is mediated by Golf, an isoform of Gs, and Golf is expressed in olfactory receptor neurons, localized at cilia and coupled to type III AC (Jones and Reed, 1989; Menco et al., 1992).

The *Drosophila* homolog DGs\(\alpha\) is likely to play an important role in various neuronal functions, because expression of a constitutively active form of DGs\(\alpha\) in the mushroom bodies impairs the learning abilities of flies (Connolly et al., 1996). Furthermore, DGs\(\alpha\) is required for the normal growth and function of synapses (Wolfgang et al., 2004), and synaptic transmission at the neuromuscular junction is compromised in DGs\(\alpha\)-null mutant embryos (Hou et al., 2003). Thus, DGs\(\alpha\) might be the G\(\alpha\) that is coupled to Grs.

To determine whether the G\(\alpha\)-mediating transduction pathway is involved in sugar-taste signaling in *Drosophila*, we first immunohistochemically demonstrated the localization of the DGs\(\alpha\) protein in GRNs. Next, we showed that gustatory responses were depressed in heterozygotic DGs\(\alpha\)-null mutant flies using behavioral and electrophysiological assays. These impaired
phenotypes were rescued by expression of wild-type DGr5a in GRNs in the mutants. We also found that reduced DGr5a expression induced by the RNAi technique depressed the trehalose responses. Together, we conclude that DGr5a is involved in sugar perception and suggest that Gso mediates sugar-taste signaling in *Drosophila*.

**Materials and Methods**

*Fly cultures.* All flies were reared on standard cornmeal medium at 25 ± 2°C, in 60% relative humidity and under a 12 h light/dark cycle. They were used for experiments on 2–5 d after eclosion.  

*Construction of a transgene* Gr5a-GALA. Gr5a-GALA was constructed by first generating a PCR product of 853 bp, corresponding to a sequence between that immediately upstream of the ATG first codon of Gr5a and the transcriptional starting site of an adjacent gene, CG3171, from *Drosophila* genomic DNA. This putative Gr5a upstream fragment and GAL4 sequence were subcloned into a pP[CaSpeR-4] vector. Insecting the Gr5a-GALA constructs, w Gr5a−; Gr5a-GALA flies were generated.

*Immunohistochemistry.* Labela were dissected from heads and fixed in 4% paraformaldehyde/PBS for 30 min at 4°C. Primary antibodies used were rabbit anti-Gso peptide antiserum (catalogue#–383; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution and anti-GFP IgG2a (catalogue# A11120; Invitrogen, Carlsbad, CA) at a 1:100 dilution. The Gso peptide corresponds to the sequence of 18 C-terminal amino acid residues, which is a conserved sequence of the Gso family (Wolfgang et al., 1990). Secondary antibodies were goat anti-rabbit IgG coupled to Alexa-568 (#A-21069; Invitrogen) at a 1:200 dilution and goat anti-mouse IgG coupled to Alexa-488 (#A-11017; Invitrogen) at a 1:200 dilution.

*DGr5a mutants.* Two strains of DGr5a-null mutants were used: *cn bw DGr5a* R19/S6M and *cn bw DGr5a* R19/S6M. Because homozygous DGr5a mutations are lethal (Wolfgang et al., 2001), we generated heterozygous male flies (*cn bw DGr5a* R19/S6M) and used *cn bw DGr5a* R19/+ by crossing female flies of a wild-type strain to male flies of either of the *DGr5a* strains or *cn bw* and used *cn bw*/+ flies as a control for both strains of heterozygous DGr5a-null mutant flies (supplemental Table and Fig. 1, available at www.jneurosci.org as supplemental material).

*Measurements of sugar intake.* Procedures to measure the amount of trehalose intake have been described previously (Shimada et al., 1987). Briefly, after 9 h of starvation, ~30 flies were allowed to feed on sugar solutions containing 1% agar and a blue food dye (0.125 mg/ml brilliant blue FCF) in the dark for 1 h on a 60-well micro-test plate (Nunc, Roskilde, Denmark). After this feeding session, flies were killed at ~20°C and homogenized with 500 μl of PBS/ETOH. After centrifugation at 15,000 rpm for 10 min, absorbance of the supernatant was measured at 630 nm by a spectrophotometer (GE Healthcare Bio-Sciences, Piscataway, NJ). The absolute amount of intake was calculated from a calibration curve of the dye. Flies drink a certain amount of water regardless of sugar. To correct for this offset of intake, the mean amount of water intake was measured separately and subtracted from the mean amount of intake of sugar solutions for a given group of flies. In all experiments, the amount of water intake in each fly was not different among flies with various genetic backgrounds used in this study (*p > 0.05*).

*Two-choice test with bitter solutions.* Before the behavior test, flies were starved for 9 h in empty vials. Thirty to forty of those flies were introduced onto a 60-well micro-test plate and allowed to feed in the dark for 1 h. The wells in a micro-test plate were alternately filled with bitter and control solutions that were colored with red and blue food dyes, respectively. All solutions contained 1% agar. The concentration of food dye was 0.5 mg/ml for amaranth and 0.25 mg/ml for brilliant blue FCF. Because the quinine and denatonium benzoate solutions were acidic, we neutralized them with HEPES buffer (10 mM), pH 7.0. After feeding, flies were killed in a freezer and classified under a dissection microscope into four groups according to their abdominal color: blue (Nb), red (Nr), purple (Np), and no staining (Nn). The preference index (PI) of the control solution over a bitter solution was calculated as *(Nb − Nr)/(Nb + Nr + Np) × 100*. The percentage of Nn flies was smaller than 10% in all experiments. The PI close to 100 indicates that the flies avoid the bitter solution. All behavioral tests were performed at 25°C and in 60% relative humidity.

**GALA/upstream activator sequence analysis.** For the experiment shown in Figures 3, 4, and 5, we generated *cn bw DGr5a* R19/S6M; upstream activator sequence (UAS)-DGr5a and crossed it with Gr5a−; Gr5a-GALA or Gr5a+ (Canton-S). The UAS-DGr5a strain carried a wild-type DGr5a cDNA sequence linked to UAS (Wolfgang et al., 2001). Thus, we obtained Gr5a−; *cn bw DGr5a* R19/Gr5a-GALA; UAS-DGr5a+ and w Gr5a−; *cn bw DGr5a* R19/+; UAS-DGr5a−.

We crossed *cn bw DGr5a* R19/S6M and Gr5a−; Gr5a-GALA to generate Gr5a+; *cn bw DGr5a* R19/Gr5a-GALA; +/+.

For the experiment shown in Figure 6, we generated Gr5a−ΔEPI; P24 and Gr5a-GALA and crossed it with *cn bw DGr5a* R19/S6M; UAS-DG6a-or *cn bw DGr5a* R19/S6M to generate Gr5a−ΔEPI; *cn bw DGr5a* R19/Gr5a-GALA; UAS-DG6a− and Gr5a−ΔEPI; *cn bw DGr5a* R19/Gr5a-GALA; +++. We crossed Gr5a−ΔEPI with *cn bw DGr5a* R19/S6M; UAS-DG6a to generate Gr5a−ΔEPI; *cn bw DGr5a* R19/+; UAS-DG6a+ (supplemental Table and Fig. 2, available at www.jneurosci.org as supplemental material).

**Electrophysiological recording of taste responses from GRNs.** The response of GRNs to various substances was recorded from the L-I-L, L-V, and L-VII chemosensilla in a labelum (Hiroi et al., 2002) by the tip recording method (Hodgson et al., 1955). A reference glass electrode, containing the Ephrushi-Beadle Ringer’s solution (128 μM NaCl, 4.7 mM KCl), was inserted in the abdomen of an anesthetized male fly, and its tip was placed in the head. To prevent changes in the stimulant concentration by evaporation, the solution in the recording capillary tube was constantly flowed out from the tip by positive pressure. All stimulant solutions contained 7.5 mM KCl as an electrolyte. Signals were filtered with a low-pass filter (2.5 kHz), digitized by an A/D converter, and stored on a computer (Molecular Devices, Union City, CA). Spontaneous sugar spikes and/or salt spikes responded to 7.5 mM KCl. However, the number of spontaneous sugar spikes was very low (for example, 0.28/100 ms in *cn bw*+), and no significant difference was found among flies with various genetic backgrounds used in this study (*p > 0.05*). They were subtracted from all sugar responses to trehalose solutions in each chemosensillum. UAS-DG6a RNAi analysis. UAS-DG6a RNAi was constructed with double-stranded RNA representing nucleotides 42 to 1381 of the transcript encoding DGr5a and cloned into a pUAST vector at the EcoRI and KpnI sites. This RNAi construct suppresses the expression of two isoforms of DGr5a (Quan et al., 1989; Quan and Forte, 1990). The Gr66a-GALA fly was a gift from Dr. Hubert Amrein (Duke University, Durham, NC). We crossed Gr66a-GALA flies and Gr5a− (Canton-S) flies and generated Gr5a−; Gr66a-GALA. To express DG6a RNAi in DGr5a-GRNs, we crossed UAS-DG6a RNAi males with Gr5a−; Gr5a-GALA females to generate Gr5a−; Gr5a-GALA/UAS-DG6a-RNAi. To express DG6a RNAi in other neurons, we crossed UAS-DG6a RNAi males with Gr5a−; Gr5a-GALA females to generate Gr5a−; Gr66a-GALA/UAS-DG6a RNAi. (supplemental Table and Fig. 3, available at www.jneurosci.org as supplemental material).

**Western blotting.** To express DGr5a RNAi in the larval CNS, we crossed UAS-DG6a RNAi with 1407-GAL4 flies and generated 1407-GAL4/UAS-DG6a RNAi larvac. The 1407-GAL4 expresses UAS transgene in the larval CNS (Luo et al., 1994). The CNS from third-instar larvae were dissected in a saline (in mM: 130 NaCl, 36 sucrose, 5 KCl, 5 HEPES, 2 MgCl2, and 0.5 EGTA, pH 7.2) containing protease inhibitors. Subsequently, the saline was removed and replaced with 1X SDS gel sample buffer. After brief homogenization, the CNS tissue was incubated at 95°C for 4 min. The equivalent of one larval CNS was then loaded per lane, and proteins were separated by electrophoresis on a 10% polyacrylamide gel. Separated proteins were transferred to nitrocellulose, and resulting blots were probed with the rabbit anti-Gso peptide antiserum used in immunohistochemical studies described above. Blots were then probed with horse-radish peroxidase-conjugated anti-rabbit secondary antibodies, and labeled bands were detected by incubating with chemiluminescent substrates.

*norpA mutant.* The norpA P24 mutant was a gift from Dr. William L. Pak (Purdue University, West Lafayette, IN). Because Gr5a and norpA are located near one another on the X chromosome, we crossed *w*; Gr5a− and norpA P24 and generated *w*; Gr5a−; norpA P24 and *w*; norpA P24, *Gr5a*+ flies. cx is
Figure 1. Expression of the DGsα protein in labela. GFP is expressed in a subset of GRNs in a labelum (A) and tarsus (B) but not in nongustatory tissues in Gr5a-GAL4/UAS-mCD8::GFP flies. C, I. Merged images of immunofluorescence stained with anti-GFP antibody (arrows) and a transmission light image of a labelum of a Gr5a-GAL4/UAS-mCD8::GFP fly. D. An image of immunofluorescence stained with an anti-Gsα antiserum (red) of the same labelum as in C, E. A merged image of immunofluorescence stained with the anti-GFP antibody (C) and with the anti-Gsα antiserum (D). The GRNs that reacted to the anti-GFP antibody (arrows) also reacted to the anti-Gsα antiserum, resulting in yellow, but some GRNs reacted only to the anti-Gsα antiserum (red). F, L. A high-magnification image of a chemosensillum. Anti-GFP fluorescence (green) was diffuse. In contrast, anti-Gsα fluorescence (red) was in clusters indicated by arrowheads (G). H, Merged images of F and G. J, M. An image of immunofluorescence treated with preimmune rabbit serum of the same labelum as in I and J. N. A merged image of L and M. There was no red signal in the labelum or in the chemosensillum treated with the preimmune rabbit serum (J and M). The white dotted line indicates the outline of a chemosensillum. Scale bars: C–E, I–K, 50 μm; F–H, L–N, 5 μm.

Located close to Gr5a and tightly linked to the Gr5a+ allele (Ueno et al., 2001). Because norpA is essential for phototransduction (Bloomquist et al., 1988), we first recorded the electroretinogram in these flies and confirmed a lack of response to orange light (data not shown). In the behavioral and electrophysiological tests, we used w cx Gr5a+ as a control.

Chemicals. Trehalose (α(+)-trehalose dihydrate), sucrose, fructose (d(-)-fructose), glucose (α(+)-glucose), caffeine (caffeine anhydrous), quinine (quinine hydrochloride dihydrate), denatonium benzoate, and brilliant blue FCF were purchased from Wako Pure Chemical Industries (Osaka, Japan). Amaranth was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Statistical analysis. We used the Student’s t test for paired comparisons and the one-way ANOVA followed by the Sheffe’s test for multiple comparisons. We also used the Steel-Dwass test to compare the numbers of impulses that were not normally distributed.

Results

DGsα is expressed in gustatory receptor neurons

If DGsα were required for taste signaling, we would expect the DGsα protein to be expressed in GRNs. As expected, we detected mRNA of DGsα in labela by reverse transcription-PCR analysis (data not shown). We then immunohistochemically examined the localization of the DGsα protein in GRNs using an antiserum against a Gs peptide. To this end, we first examined the distribution of GFP expressed in GRNs in transgenic flies carrying Gr5a-GAL4 and UAS-GFP under a fluorescence stereomicroscope and found that GFP was expressed specifically in a subset of GRNs in labela and tarsi (Fig. 1A, B). This finding is in accord with previous reports (Chyb et al., 2003; Thorne et al., 2004; Wang et al., 2004).

Under a confocal microscope, a set of GFP-expressing GRNs was located near the proximal end of the chemosensillum (Fig. 1C, I, green cells, indicated by arrows). The immunofluorescence against Gs was observed in GFP-expressing GRNs (Gr5α-GRNs, indicated by arrows) as well as in nonexpressing GRNs (non-Gr5α GRNs) (Fig. 1D, E). All Gr5α-GRNs had anti-Gs immunoreactivities. We counted the numbers of Gr5α-GRNs and other GRNs that showed anti-Gs immunoreactivities in a labelum. They were 35 ± 3 and 77 ± 6 (n = 5), respectively. The number of Gr5α-GRNs that we found is close to that in previous reports (~30 in a labelum (Chyb et al., 2003) and 71 ± 11 in a palp that contains two labela (Thorne et al., 2004)). From the morphological and electrophysiological experiments, the total...
number of GRNs in a labelum is estimated to be ~150 (Amrein and Thorne, 2005). Hence, our results indicate that approximately one-half of GRNs are expressing DGsa, although the intensity of immunofluorescence against Gs in Gr5a-GRNs was higher than that in non-Gr5a GRNs (Fig. 1D). In the chemosensillum, GRNs extended their dendrites (Fig. 1F-I), and anti-Gs fluorescence clusters were found at the dendrite, revealing punctuated localization of Gs (Fig. 1G,H, arrowheads). Immunoreactivities in GRNs were not detected in negative controls, in which preimmune rabbit serum was used (Fig. 1f,k,m,n). These results indicate that DGsa is expressed in GRNs, including all Gr5a-GRNs, in a labelum.

Sugar intake is depressed in heterozygous DGsa-null mutants

To examine the behavioral response to sugars in heterozygous DGsa-null mutant flies, we measured the amount of intake of 20 mM trehalose, 5 mM sucrose, 20 mM fructose, and 20 mM glucose in DGsa^{R19/+} and in cn bw/+ , a control. The amount of intake of each sugar solution in DGsa^{R19/+} flies was significantly lower than that in control flies (Fig. 2A) (p < 0.05).

It is known that a variety of gustatory receptors, Gr22b, Gr22c, Gr22e, Gr22f, Gr28b, Gr32a, Gr59b, and Gr66a, are expressed in non-Gr5a GRNs and that those GRNs are required for bitter taste perception (Thorne et al., 2004; Wang et al., 2004). It is then possible that bitter-taste perception is also mediated by Gsa and that the DGsa-expressing GRNs other than Gr5a (Fig. 1D,E) are bitter-sensitive GRNs. To test this possibility, we examined the behavioral response to bitter substances in DGsa^{R19/+} flies using the two-choice test. We tested three bitter substances, caffeine (at 1, 5, and 10 mM), quinine (at 0.1, 0.25, 0.5, 1, and 2.5 mM), and denatonium benzoate (at 0.025, 0.1, 0.25, 0.5, and 1 mM). These bitter substances have been shown to induce the behavioral and electrophysiological bitter responses in Drosophila (Meunier et al., 2003; Hiroi et al., 2004). In contrast to sugars, no significant difference was found in the preference index between DGsa^{R19/+} and control flies with any bitter substances tested and at any concentrations (Fig. 2B). This finding indicates that the behavioral response to bitter substances was not impaired in the heterozygous DGsa-null mutant.

We next examined the dose–response relationship between the amount of intake and the trehalose concentration in DGsa^{R19/+}. Although the trehalose intake in the mutant increased with the trehalose concentration, the amount of intake at 10, 20, 40, and 80 mM trehalose in DGsa^{R19/+} flies was significantly lower than that at the corresponding concentration in control flies (Fig. 2C, open columns, p < 0.05, asterisks). We also measured the amount of intake in another heterozygous DGsa-null mutant, DGsa^{R600/+} (Wolfgang et al., 2001), and found that those at 40 and 80 mM trehalose in DGsa^{R600/+} flies were significantly lower than those at the corresponding concentrations in control flies (Fig. 2C, shaded columns, p < 0.05, asterisks). The depressed trehalose intake in DGsa^{R19/+} flies was rescued by the Gs27 construct that contains the entire DGsa gene (Wolfgang et al., 2001) (Fig. 2D, shaded columns). These results indicate that the behavioral responses to trehalose in heterozygous DGsa-null mutants were impaired.

Transgene DGsa expressed in Gr5a-GRNs rescues impairment of trehalose intake

To determine whether the depressed trehalose intake in heterozygous DGsa-null mutants is attributable to a defect of trehalose response in the Gr5a-GRNs, we expressed the wild-type DGsa
DGα is functionally involved in trehalose-induced electrical responses in sugar-sensitive GRNs

To further confirm the involvement of DGα in the trehalose response in Gr5a-GRNs, we studied their electrical responses to trehalose in heterozygous DGα-null mutants. It is known that sugar, water, low concentrations of salt and bitter/high concentrations of salt stimuli induce the responses in corresponding four types of GRNs in an L-type chemosensillum (Fujishiro et al., 1984; Hiroi et al., 2004). When an L-type chemosensillum was stimulated with water, a single train of monophasic spikes was observed (Fig. 4A2, expanded trace on the left). In Fig. 4A1, monophasic spikes are marked with asterisks. In contrast, when the chemosensillum was stimulated with 20 mM trehalose, two kinds of spikes, monophasic and biphasic ones, were observed (Fig. 4A2, an expanded trace on the right is biphasic. In Fig. 4A1, biphasic spikes are marked with dots). The biphasic spikes are most likely to be generated in sugar-sensitive GRNs, whereas monophasic spikes originate from water-sensitive GRNs because only monophasic spikes were observed when the chemosensillum was stimulated with water and the frequency of monophasic spikes were slightly decreased (15.0 spikes/200 ms at 0 mM trehalose and 11.2 spikes/200 ms at 320 mM trehalose in cn bw/+ flies), whereas that of biphasic spikes increased with the trehalose concentration (Fig. 4A1). When the chemosensillum in Gr5a-null mutant flies [Gr5aΔEP19 (Ueno et al., 2001)] was stimulated with trehalose, the number of biphasic spikes was very low (data not shown), in accord with a previous finding that the number of sugar-sensitive GRNs in Gr5aΔEP19 is extremely low (Dahanukar et al., 2001). Therefore, we considered that the biphasic spikes that we recorded in these experiments were generated in sugar-sensitive Gr5a-GRNs.

It is possible that the monophasic spikes are not only generated by water-sensitive GRNs but also by GRNs sensitive to low concentrations of salt because we always had 7.5 mM KCl in the recording solution as an electrical conductor. However, we observed two types of spikes, monophasic and biphasic spikes, in 20 mM NaCl solution. When the NaCl concentration was increased to 100 mM, the frequency of monophasic spikes decreased slightly, whereas that of biphasic spikes dramatically increased (data not shown). Thus, we believe that the monophasic spikes are generated by water-sensitive GRNs.

All transformants responded to trehalose (Fig. 4A1). However, the response in DGα+ flies carrying both Gr5a-GAL4 and UAS-DGα was significantly stronger than the responses in the other genotypes at 5, 20, and 80 mM trehalose (Fig. 4B, asterisks, p < 0.05). This result indicates that the electrical responses to trehalose in Gr5a-GRNs in DGα+ flies are rescued by the expression of wild-type DGα exclusively in Gr5a-GRNs.

The amount of trehalose intake in DGα+ flies carrying both Gr5a-GAL4 and UAS-DGα was lower at 80 mM trehalose and higher at 5 mM trehalose than that in cn bw/+ flies (Fig. 5A). In contrast, the electrical response in DGα+ flies carrying both Gr5a-GAL4 and UAS-DGα was not significantly different from the responses in cn bw/+ flies at 5, 20, 80, and 320 mM trehalose (Fig. 5B). To account for this discrepancy, we suggest that DGα in non-Gr5a cells, for example, CNS neurons, is also involved in the behavioral response.

Neuronal electrical responses to water were not different between DGα+ flies carrying both Gr5a-GAL4 and UAS-DGα and cn bw/+ flies (Fig. 5B). This result indicates that the response to water in water-sensitive GRNs is not impaired in DGα+ flies.

Trehalose response in double mutants of DGαR19/+ and Gr5a-null was not rescued by exogenous wild-type DGα

The depressed electrophysiological trehalose response in DGαR19/+ flies was rescued when exogenous DGα was expressed in Gr5a-GRNs (Figs. 4B, 5B). However, it is still possible that expressing exogenous DGα nonspecifically enhanced the activity of sugar-sensitive Gr5a-GRNs. Furthermore, it is also possible that the increase of biphasic spike frequency in DGαR19/+ flies carrying both Gr5a-GAL4 and UAS-DGα, compared with DGαR19/+ flies, is attributable to recruitment of non-sugar-sensitive Gr5a-GRNs, because it is known that L-type chemosensilla contain multiple Gr5a-GRNs (Thorne et al., 2004), and another report suggests that some of Gr5a-GRNs serve as salt-sensitive GRNs (Wang et al., 2004). To rule out these possibilities, we generated double mutants of DGαR19 and Gr5aΔEP19. Gr5aΔEP19 is a Gr5a-null allele and has an extremely low sensitivity to trehalose, as mentioned above (Dahanukar et al., 2001; Ueno et al., 2001). If exogenous DGα nonspecifically enhanced the activity of sugar-sensitive Gr5a-GRNs or enhanced the activity of salt-sensitive Gr5a-GRNs, exogenous DGα should also have increased the number of biphasic spikes even in the Gr5a-null allele.

The behavioral responses to trehalose in three strains of double mutants were very low and not significantly different among them (p > 0.05) (Fig. 6A), nor were electrical responses robust. The response to 20 mM trehalose was not detected in sugar-sensitive GRNs, and the response to 320 mM trehalose was at a low level in all strains of flies carrying Gr5aΔEP19 (Fig. 6B). No
significant differences were detected at any concentrations (5 ~ 320 mmoles/l) of trehalose among three transformants (p > 0.05) (Fig. 6B). These results indicate that the expression of exogenous wild-type Dgsa enhances neither the activity of sugar-sensitive Gr5a-GRNs nonspecifically nor the activity of salt-sensitive Gr5a-GRNs and suggest that Dgsa involved in the downstream transduction pathway of Gr5a in sugar-sensitive-GRNs.

RNAi for Dgsa depresses the expression of Dgsa in Gr5a-GRNs and both behavioral and electrophysiological trehalose responses

To confirm that Dgsa is involved in the trehalose response in sugar-sensitive Gr5a-GRNs, we used the RNAi technique. With the Western blotting analysis, we confirmed that the expression level of Dgsa is depressed in the brain of larvae by the UAS-Dgsa RNAi construct (Fig. 7A). Next, we immunohistochemically examined the effect of expressing the Dgsa RNAi construct in GRNs. In flies in which GFP was expressed in Gr5a-GRNs (Gr5a-GAL4/UA-mCD8::GFP), Gr5a-GRNs were stained as yellow by simultaneous staining with anti-GFP antibody (green) and anti-Gsa peptide antiserum (red) (Fig. 7B, top panel). In contrast, in flies in which Dgsa RNAi was coexpressed (Gr5a-GAL4/UA-mCD8::GFP/UAS-Dgsa RNAi), the majority of Gr5a-GRNs showed green fluorescence and weak yellow fluorescence (Fig. 7B, bottom panel). In either type of flies, non-Gr5a GRNs showed red immunofluorescence (Fig. 7B, top and bottom panels). Thus, we confirmed that introducing the Dgsa RNAi construct suppressed Dgsa expression in Gr5a-GRNs.

The behavioral responses to trehalose in two strains of transgenic flies carrying both Gr5a-GAL4 and UAS-Dgsa RNAi constructs (#32-1 and #20-1) were significantly lower than in flies carrying either the Gr5a-GAL4 or the UAS-Dgsa RNAi construct [p < 0.05 (Fig. 7C) in Gr5a-GAL4/UA-mCD8::GFP RNAi (#32-1) at 10, 20, and 80 mM and in Gr5a-GAL4/UAS-Dgsa RNAi (#20-1) at 20 and 40 mM].

Gr66a is expressed in bitter-sensitive GRNs and is not coexpressed with Gr5a, although natural ligands for Gr66a are not known (Thorne et al., 2004; Wang et al., 2004). The trehalose responses in transgenic flies carrying Gr66a-GAL4 and UAS-Dgsa RNAi were not impaired (Fig. 7C). Hence, we conclude that the reduction of trehalose responses in two strains of transgenic flies described above is the result of the inhibitory effects of Dgsa RNAi specifically in Gr5a-GRNs.

The electrophysiological responses in sugar-sensitive Gr5a-GRNs at 5, 20, and 80 mM trehalose in transgenic flies carrying both Gr5a-GAL4 and UAS-Dgsa RNAi constructs were significantly lower than those in transgenic flies carrying either Gr5a-GAL4 or UAS-Dgsa RNAi alone (Fig. 7D, p < 0.05). These findings indicate that the reduced expression level of Dgsa in the transformant carrying both Gr5a-GAL4 and UAS-Dgsa RNAi constructs depressed trehalose responses in sugar-sensitive Gr5a-GRNs.

**Figure 4.** Electrical responses in GRNs to trehalose in heterozygous Dgsc-null mutants. **A1,** Typical responses in L-type chemosensilla to water (7.5 mM KCl), 20 and 320 mM trehalose solutions in Dgsc[R19]/+ flies carrying both Gr5a-GAL4 and UAS-Dgsc (left three traces) and Dgsc[R19]+ carrying either Gr5a-GAL4 (middle three traces) or UAS-Dgsc (right three traces) alone. Spikes marked with asterisks and dots were counted separately as a measure of magnitude of the response in a water-sensitive GRN (asterisks) and in a sugar-sensitive GRN (dots) during a 200 ms period starting from the onset of stimulus (period between two vertical dotted lines). Arrowheads indicate the onset of stimulation. **A2,** Two expanded traces represent typical spikes from a water-sensitive GRN (asterisk) and a sugar-sensitive GRN. Each column represents the number of spikes in a sugar-sensitive GRN during a 200 ms period starting from the onset of stimulus. A significant difference was found between Dgsc[R19]+/ flies carrying both Gr5a-GAL4 and UAS-Dgsc (filled columns) and Dgsc[R19]+/ flies carrying either Gr5a-GAL4 (open columns) or UAS-Dgsc (shaded columns) alone at 5, 20, and 80 mM trehalose (asterisks, p < 0.05). Columns marked “Water” represent the response to water of a water-sensitive GRN. Each column represents the mean ± SEM of 15 samples. NS, Not significant.
Figure 5. The dose–response relations between behavioral responses and trehalose concentration (A), and electrical responses and trehalose concentration (B) in DGs (A) and Gr5a-GAL4 (B) double mutant. Wild-type DGs was expressed in Gr5a-GRNs in DGs (B) and in the Gr5a-null background using the GAL4/UAS system (Gr5a (A) and Gr5a-GAL4 (B)). Each column represents mean ± SEM of 10 experiments. No significant difference was found among three double mutants at any concentrations (0.8, 4, 20, 100, and 400 mM) (Fig. 8B, p > 0.05). These findings indicate that the behavioral and electrophysiological responses to trehalose in norpA flies were not impaired, although the expression of PLC encoded by norpA is eliminated in norpA flies (Pearn et al., 1996). Thus, we conclude that norpA does not mediate trehalose-taste signaling.

Discussion

In this study, we conclude that a specific Gα, Gsα, which is encoded by DGsα, is involved in the sugar response in Gr5a-GRNs, and we suggest that the DGsα-mediated transduction pathway is coupled to Gr5a and processes sugar signaling in Drosophila.

After identifying Drosophila gustatory receptors as GPCRs, Clyne et al. (2000) postulated that the transduction pathway of taste in Drosophila is mediated by G-proteins. Recently, a γ subunit of the G-protein encoded by Gty1 was reported to be required for sugar perception (Ishimoto et al., 2005). Although this finding confirms the hypothesis that the sugar-gustatory receptors, including Gr5a, are coupled to G-proteins, it does not specify the downstream pathway for sugar-taste signaling in Drosophila. Our finding that Gsα is involved in sugar perception in Drosophila strongly suggests that the cAMP transduction pathway is involved in sugar-taste signaling. Although in vertebrates it has not been established which Gα mediates sugar-taste signaling, this is the first demonstration in Drosophila that Gsα is involved in sugar-taste signaling in vivo.

The responses to sugars, i.e., trehalose, sucrose, fructose, and glucose, were impaired in the heterozygous DGsα-null mutants (Fig. 2A), although it is known that Gr5a is narrowly tuned to trehalose (Chyb et al., 2003). To test this possibility, we used norpA, which encodes PLC, and measured the amount of trehalose intake in a norpA-null allele, norpA (Pearn et al., 1996). The amount of trehalose intake was not different between norpA and control flies at any concentrations, except at 0.8 mM, at which level trehalose intake was significantly higher in norpA than in the control (Fig. 8A, asterisk).

The electrophysiological responses in sugar-sensitive Gr5a-GRNs in norpA and control flies were not significantly different at any concentrations of trehalose (0.8, 4, 20, 100, and 400 mM) (Fig. 8B, p > 0.05). These findings indicate that the behavioral and electrophysiological responses to trehalose in norpA flies were not impaired, although the expression of PLC encoded by norpA is eliminated in norpA flies (Pearn et al., 1996). Thus, we conclude that norpA does not mediate trehalose-taste signaling.

Trehalose response in a norpA-null mutant

In addition to the involvement of DGsα in the Gr5a-mediated trehalose-signaling pathway, phospholipase C (PLC) might also play a role in this pathway (Koganezawa and Shimada, 2002;
erozygotes of two DGsa-null alleles [DGsaR19 and DGsaR60 (Wolfgang et al., 2001)] were lower than in the control (Fig. 2C). The trehalose intake in DGsaR19/+ was more severely depressed than in DGsaR60/+ . This difference could arise from an additional mutation in DGsaR19 (Wolfgang et al., 2001). However, the depression of trehalose intake in DGsaR19/+ was completely rescued by expressing exogenous DGsa in Gs27 (Fig. 2D) or in Gr5a-GAL4/UAS-DGsa (Fig. 3). These results indicate that the depression of trehalose intake in DGsaR19/+ flies is attributable to the DGsaR19 mutation. It is unclear at this moment what is causing the difference between DGsaR19/+ and DGsaR60/+.

It is possible that DGsa is not required for trehalose-taste signaling but is necessary for development of sugar-sensitive GRNs, and the low trehalose responses in transgenic flies expressing DGsa RNAi (Gr5a-GAL4/UAS-DGsa RNAi) were caused by developmental defects of the neurons. For example, DGsa might be required for expression of Gr5a or ion channels. However, the response induced by 320 mM trehalose in those flies was not different from that in the control (Fig. 7D). This result indicates that the sugar-sensitive GRNs in the transgenic flies are fully equipped with the molecular machinery for the maximal trehalose response. Thus, we suggest that the developmental effect of DGsa on sugar-sensitive GRNs is not a major contributor to the observed RNAi effect.

In the RNAi analysis, the responses to trehalose in the transgenic DGsa RNAi-expressing flies were reduced but not eliminated (Fig. 7C,D). The residual responses in the transgenic flies might be attributable to the residual DGsa protein, because the DGsa expression was not completely eliminated in the transgenic flies (Fig. 7A,B). However, it is possible that a non-DGsa transduction pathway is also involved in sugar perception. A previous report suggested that the Gq/PLC-mediated pathway is involved in the Gr5a-initiated signaling pathway in the S2 cell line (Chyb et al., 2003), and another report showed that dGq, a homolog of mammalian Gq, and norpA are expressed in the labelum (Talluri et al., 1995; Koganawaza and Shimada, 2002). It is, therefore, possible that the PLC system is synergistically contributing to sugar-taste perception together with the Gr5a/DGsa signaling pathway. Using a norpA-null allele, norpA P24 , we tested this possibility. We found that the amount of trehalose intake was not significantly different between norpA P24 and control flies at most concentrations tested (Fig. 8A). Furthermore, the electrophysiological response to trehalose was not significantly different at any concentrations between norpA P24 and control flies (Fig. 8B). It is unlikely that PLC encoded by genes other than norpA is involved in this pathway, because PLC activities are completely eliminated in adult heads of norpA P24 flies (Zhu et al., 1993). Therefore, we suggest that the PLC transduction pathway is not significantly contributing to the sugar response in Drosophila.

Figure 7. The effects of DGsa RNAi on immunoreactivities in larval CNS and GRNs (A, B) and on behavioral (C) and electrophysiological responses (D) to trehalose. A, Immunoblot of the DGsa protein obtained from larval CNS. Lane 1, DGsa RNAi expressed in larval CNS (1407-GAL4/UAS-DGsa RNAi). Lane 2, Control (+ UAS-DGsa RNAi). The antisemur recognized short and long forms of DGsa (Quan and Forte, 1990). B, Effects of DGsa RNAi expressed in Gr5a-GRNs. Control images stained with anti-GFP antibody (top left panel) and with anti-Gs peptide antiserum (top middle panel), and merged (top right panel) in a transgenic fly expressing GFP in Gr5a-GRNs. The bottom panels are corresponding images in a transgenic fly expressing GFP and DGsa RNAi in Gs25-GRNs. Arrows indicate Gr5a-GRNs. In the top panels, two Gr5a-GRNs (arrows) show immunofluorescence against GFP (left panel) as well as against GsC (middle panel) resulting in yellow (right panel). In the bottom panels, in which DGsa RNAi was expressed, anti-GsC fluorescence was very dim in a Gr5a-GRN (arrow in middle panel), resulting in green in a merged image (arrow in right panel). Immunoreactivities against GsC in non-Gr5a GRNs (arrowheads) of both transgenic flies are visible. Scale bars, 10 μm. C, The amount of trehalose intake in DGsa RNAi-expressing flies (Gr5a-GAL4/UAS-DGsa RNAi (#32–1), open columns) was lower than in controls (black and red columns) at 10, 20, 40, and 80 mM trehalose (asterisks, p < 0.05). The amount of trehalose intake in another DGsa RNAi-expressing strain (Gr5a-GAL4/UAS-DGsa RNAi (#20–1), gray columns) was also lower than in control at 20 and 40 mM (asterisks, p < 0.05). Each column represents the mean ± SEM of 10 experiments. No significant difference was found among flies expressing DGsa RNAi in bitter-sensitive GRNs (Gr5a-GAL4/UAS-DGsa RNAi, blue columns) and controls (black and red columns) at 5, 20, and 80 mM trehalose (asterisks, p < 0.05). Each column represents the mean ± SEM of 15 samples. D, Electrical responses to trehalose in flies expressing DGsa RNAi (Gr5a-GAL4/UAS-DGsa RNAi), x- and y-axes are the same as Figure 4B. A significant difference was found among flies expressing DGsa RNAi (Gr5a-GAL4/UAS-DGsa RNAi (#32–1), open columns) and controls (black and red columns) at 5, 20, and 80 mM trehalose (asterisks, p < 0.05). Each column represents the mean ± SEM of 15 samples.
We found that the behavioral response to 0.8 mM trehalose in norpA 0.24 was higher than control flies (Fig. 8A), although the electrophysiological responses to 0.8 mM trehalose were not different between norpA 0.24 and control flies (Fig. 8B). These results suggest that norpA is involved in the higher processing of gustatory information, for example, in the CNS. It is known that norpA is expressed in the brain (Zhu et al., 1993).

We found that DGsα is localized not only in Gr5α-GRNs but also in non-Gr5α GRNs (~40 GRNs in a labelum) (Fig. 1D, E). In labela, there are at least four types of GRNs sensitive to sugar, low concentrations of salt, bitter-substances/high concentrations of salt, water, and mechanosensory neurons (Falk et al., 1976; Fujishiro et al., 1984; Meunier et al., 2003; Hiroi et al., 2004). Then, two questions arise: (1) which GRN, other than Gr5α-GRNs, contains DGsα? (2) Is DGsα in unknown GRNs involved in the taste signaling of GRNs? The behavioral responses to bitter solutions were not different between heterozygous DGsα-null mutant and control flies (Fig. 2B), and the behavioral and electrophysiological responses to water were not different among all DGsα strains examined in this study. It is known that salt responses in larvae require amiloride-sensitive channels encoded by ppk11 and ppk19 (Liu et al., 2003), and the low and high concentrations of salt responses do not require Gyr1 in adult flies (Ishimoto et al., 2005). These findings together with our results suggest that DGsα in non-Gr5α GRNs serve for other signaling than taste or that the non-Gr5α GRNs containing DGsα are mechanosensory neurons. However, because we did not examine the bitter and water responses in the homozygous DGsα R15 mutant, we cannot rigorously exclude the possibility of whether DGsα is involved in bitter and/or water tastes.

We suggest that, in Drosophila, the Gs-mediated cAMP transduction pathway is the main signaling route in sugar-sensitive GRNs. In contrast, the PLC/IP3, mediating pathway is involved in sugar-tasting in the fleshfly (Boettcherisca peregrina) (Koganezawa and Shimada, 2002) and the guanosine-3',5'-cyclic monophosphate/nitric oxide pathway in the blowfly (Phormia regina) (Amakawa et al., 1990; Murata et al., 2004). The cAMP pathway may be involved in sugar-taste perception in the frog, rat, and pig (Avenet and Lindemann, 1987; Striem et al., 1989; Naim et al., 1991), whereas a recent study on T1R2/T1R3 gustatory sugar receptors of the mouse supports involvement of the PLC pathway (Zhang et al., 2003). Additional comparative studies are necessary to elucidate the diversity of molecular mechanisms of sugar-taste signaling in various animals.

References


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