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Primary Culture of Gustatory Receptor Neurons from the Blowfly, *Phormia regina*

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Abstract

Flies provide a powerful model system for exploring signaling systems in gustatory receptor neurons (GRNs). To elucidate the cellular and molecular bases of these signaling systems, we sought to develop techniques to dissociate GRNs. We developed a primary culture of GRNs isolated from the labella of the blowfly, *Phormia regina*, 4–5 days after pupation. Dissected labella were treated with papain in a low Ca^{2+} saline solution and shaken in Leibovitz's L-15 medium supplemented with 20-hydroxyecdysone, L-ascorbic acid, and trehalose with a test tube mixer. Released cells were plated and kept at 29°C in a medium containing fetal bovine serum. After a minimum of 2 days in culture, we observed survival or growth of bipolar cells with the characteristic morphology of GRNs. We also examined taste responsiveness by monitoring intracellular Ca^{2+} with a Ca^{2+}-sensitive fluorescent dye, fluo-3. For some bipolar cells, application of sucrose, NaCl, or LiCl for 5–20 s transiently increased the intracellular Ca^{2+} levels in cell bodies for 20–30 s. The primary cell culture described here is useful for functional analysis of GRNs.

Key words: calcium imaging, fly, intracellular calcium ion concentration, taste cells

Introduction

Gustatory receptor neurons (GRNs) in flies are located in hair-shaped structures called sensilla. A single sensillum generally houses one mechanoreceptor neuron and four functionally differentiated GRNs. Three of the four GRNs are termed sugar, salt, and water receptor neurons after their adequate stimulants. The other GRN is known to be sensitive to deterrent compounds such as bitter taste stimulants.

The nature of the signal transduction pathways in fly GRNs is controversial. Recent genomic and molecular approaches in the fruit fly, *Drosophila melanogaster*, have revealed the putative gustatory receptor (Gr) gene family (Clyne *et al.*, 2000; Dunipace *et al.*, 2001; Scott *et al.*, 2001; Robertson *et al.*, 2003) encoding seven transmembrane G protein–coupled receptors. To date, only a few have been functionally identified as Gr genes: Gr5a is used for the sugar compound trehalose (Dahanukar *et al.*, 2001; Ueno *et al.*, 2001), Gr66a, and some other Gr genes are used for bitter taste (deterrent) compounds (Thorne *et al.*, 2004; Wang *et al.*, 2004). The transduction pathway involving G proteins has been demonstrated in sugar receptor neurons of the flesh fly, *Boettcherisca peregrina* (Koganezawa and Shimada, 1997), and the blowfly, *Phormia regina* (Ahamed *et al.*, 2002), by electrophysiological and pharmacological analyses. However, the downstream processes are complicated. Previous reports using extracellular recordings indicated that cyclic guanosine 3′,5′-monophosphate (Amakawa *et al.*, 1990), inositol 1,4,5-triphosphate (Ozaki and Amakawa, 1992; Koganezawa and Shimada, 2002), Ca^{2+} (Ozaki and Amakawa, 1992; Liscia *et al.*, 2002), and/or nitric oxide (Murata *et al.*, 2004) function as second messengers in sugar receptor neurons. In addition, it has also been suggested by patch clamping recordings that there are ion channels directly gated by sugars in sugar receptor neurons of the flesh fly (Murakami and Kijima, 2000). Further cellular and molecular experiments are necessary to elucidate the transduction pathways in fly GRNs. To this end, it will be advantageous to establish a way to obtain dissociated live GRNs.

Dissociating fly GRNs has been hindered by the robustness of the sensillum cuticle around the dendrites and by the tight adhesion of the thecogen cell to the GRNs. In addition to a small body size, the limited number of receptor organs makes the isolation of the fly GRNs difficult. Therefore, to date, no preparations of dissociated receptor neurons have been reported. Studies have been limited to preparations of

regenerated dendrites from tip-cut sensilla in the flesh fly (Murakami and Kijima, 2000).

Dissociated olfactory receptor neuron preparations have been available for various species of insects: the sphinx moth, *Manduca sexta* (Stengl and Hildebrand, 1990), the locust, *Locusta migratoria* (Wegener *et al.*, 1992), the honeybee, *Apis mellifera mellifera* (Gascuel *et al.*, 1994; Laurent *et al.*, 2002), the blowfly, *P. regina* (Nakagawa and Iwama, 1995), and the cabbage moth, *Mamestra brassicae* (Lucas and Shimahara, 2002). With the exception of the locust, one of the key points for successful preparation of dissociated cells is the use of pupal stage tissue because pupae provide soft cuticles and loose adhesion of thecogen cells. Another point to consider is the establishment of primary cultures of the dissociated cells, which seems to be essential for developing olfactory receptor neuron characteristics after dissociation.

In the present paper, we overcame the robustness of the cuticle and tight adhesion of thecogen cells to the GRNs by using pupae. Because the blowfly, *P. regina*, is a comparatively large fly species, it was easier to dissect its taste organ. Cell suspensions from the labellar tissues included both bipolar and vesicular cells. The bipolar cells showed morphological characteristics of the fly GRNs. After 2–6 days in culture, a subset of bipolar cells exhibited a transient increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), responded to some different qualities of taste stimulants, which corresponded to electrophysiological responses of GRNs *in vivo* reported in previous works. Thus, we describe, for the first time in insects, primary cultures of isolated and dissociated GRNs from the pupal blowfly, *P. regina*.

Materials and methods

Experimental animals

Blowflies, *P. regina*, were raised in an environmental chamber with 12:12 h light:dark cycle in the laboratory at 24 ± 1°C and fed on chicken liver and yeast bait at the larval stage. Pupae were selected 4–5 days after pupation when all labella structures were almost complete (see Results).

Cell isolation and culture

Dissected labella of 20 pupal blowflies were incubated with 0.4 mg/ml papain (Calbiochem, Darmstadt, Germany) plus 0.2 mg/ml bovine serum albumin (Sigma, St Louis, MO) in nominally Ca^{2+}-free Waterhouse's saline (Buck, 1953) with some modifications (in mM: NaCl 121, KCl 5.5, $MgCl_2$ 0.8, $NaHCO_3$ 1.2, NaH_2PO_4 0.06, glucose 10, and mannitol 100) at 30°C for 40 min. After rinsing in Leibovitz's L-15 medium (Gibco, Grand Island, NY) supplemented with 50 mM trehalose (Sigma), 100 μM L-ascorbic acid (Vargas and Lucero, 1999; Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 1 μg/ml 20-hydroxyecdysone (0.1% ethanol, Sigma), labella were disrupted by shaking in 100 μl L-15 medium with a test tube mixer for 30 s. The suspension (50 μl), including the dispersed cells, was plated on a concanavalin A (Sigma)–coated coverslip in a 35-mm plastic dish and left for 20 min, which allowed the cells to adhere to the surface of the coverslip. Cell culture was carried out following the method by Nakagawa and Iwama (1995) with some modifications. Briefly, the medium was replaced with 2 ml of L-15 medium further supplemented with 10% fetal bovine serum (Sigma); the osmotic pressure of the medium was 380 mOsm. Cultures were maintained at 29°C under normal air with high humidity for ≥2 days. Cells were observed on a modified Hoffman contrast inverted microscope (CKX41; Olympus, Tokyo, Japan). Images were captured with either a digital camera (COOLPIX 885; Nikon, Tokyo, Japan) or a cooled CCD camera (Penguin 600CL; Pixera, Los Gatos, CA) with data acquisition software (InStudio; Pixera) on a personal computer.

Fluorescence imaging of intracellular Ca^{2+}

Cells on a coverslip were held in an experimental chamber under continuous perfusion at a rate of ~1 ml/min with a physiological saline solution (in mM: NaCl 112, KCl 5, $CaCl_2$ 0.9, $MgCl_2$ 1.7, $NaHCO_3$ 1.2, 4-2-hydroxyethyl-1-piperazine ethanesulfonic acid 5, and mannitol 100; pH 7.6, 340 mOsm). Stimulating solutions were prepared by replacing mannitol with one of the taste stimulants (in mM: sucrose 100/200, NaCl 500, or LiCl 500) in the physiological saline solution. The stimulating solutions were pressure ejected from multibarrel glass pipettes placed within 10–20 μm downstream from the cell soma.

Intracellular Ca^{2+} was monitored with a fluorescent dye, fluo-3 (Molecular Probes, Eugene, OR). To load the dye, cells were incubated in a saline solution containing 2 μM fluo-3/AM and 0.1% dimethylsulfoxide at room temperature for 1 h. Cells were perfused with dye-free physiological saline for 10 min. The fluo-3–loaded cells were imaged using a real-time confocal laser scanning system (CSU10, Yokogawa, Tokyo, Japan) attached to a Carl Zeiss Axioscop upright microscope (Göttingen, Germany) with a water immersion objective (40×, NA 0.95, Carl Zeiss). The excitation light (488 nm) was supplied from an argon ion laser (Melles Griot, Carlsbad, CA), the emitted light was passed through a long-pass filter (≥515 nm), and fluorescent images were captured at a rate of 30 per second with an intensified CCD camera (ICCD-350F, Videoscope International, Dulles, VA). Time series 8-bit confocal images (of a maximum of 640 × 480 pixels) were stored on a Macintosh PowerMac G4 microcomputer and analyzed with IPLab 3.6.4 software (Scanalytics, Fairfax, VA). Time series fluorescent intensities in different cell compartments were measured by reading out the average pixel values in the regions of interest on the stored images. The fluorescence changes were normalized to baseline fluorescence intensity ($\Delta F/F$) and were plotted as a time function.

Results

Appropriate developmental stage for obtaining discrete cells from pupae

First, we determined the developing stage of the blowfly appropriate for dissociation of GRNs in view of the feasibility of dissecting labella and obtaining an adequate number of discrete cells. We succeeded in the dissection of the labella from ≥4-day-old pupae, whose morphology was indistinguishable from their adults. From the labella of 4- to 5-day-old pupae, many discrete cells were dissociated. The number of discrete cells obtained from the labellar of adults and ≥5-day-old pupae decreased in an age-dependent manner. Therefore, the 4- to 5-day-old pupae were selected for the following experiments.

Morphological characteristics of discrete cells

In cell suspensions derived from the pupal labella, two distinguishable types of discrete cells (with and without processes) were observed. Each cell type was divided into subgroups based on their size, shape, and surface appearance of somata.

Figure 1 shows representative cells with processes. About 10% of all discrete cells in a culture dish belonged to this type. Somata of these cells were round or oval with diameters of 4–12 μm in the short axis (Figure 1A–C). Some of these cells possessed a rough surface (Figure 1D). Trypan blue staining demonstrated that almost all the cells with processes survived the isolation procedures (data not shown). After 2–6 days in culture, these cells extended their processes over several hundred micrometers, sometimes with elaborate branches (Figure 1E,F). The length of the processes of each bipolar cell measured 45.0 ± 6.3 μm on day 0, 128.1 ± 27.0 μm on day 2, and 279.4 ± 51.9 μm on day 6 (mean ± SEM, n = 20, Figure 2). Absence of 20-hydroxyecdysone in the medium inhibited the prolongation of the processes. Depending on plating density, 10–20 of these cells were found in a single dish after 2–6 days in culture. Almost all the cells that had processes after 2–6 days in culture excluded trypan blue (Figure 1F). Cell survival seemed to be optimized by using L-15 medium supplemented with 10% fetal bovine serum. As far as we observed, some bipolar cells survived up to 17 days and elongated their processes to about 1 mm.

Figure 3 shows representative round (A–C) and oval (D,E; arrowhead) cells without processes. The round cells measured 2–30 μm in diameter. Most of the round cells with a diameter of 10–30 μm possessed granules on the surface. Oval cells measured 6–30 μm in the short axis. More than 60% of

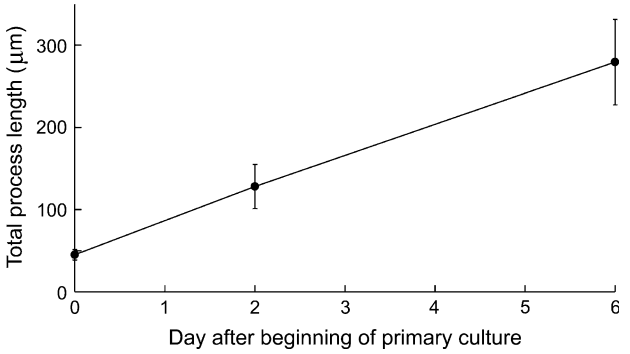

Figure 2 Growth of the bipolar cell processes in primary culture. Total length of the processes for each cell in Figure 1A,B was calculated as the sum of the process length on both sides including the branches (mean ± SEM, n = 20).

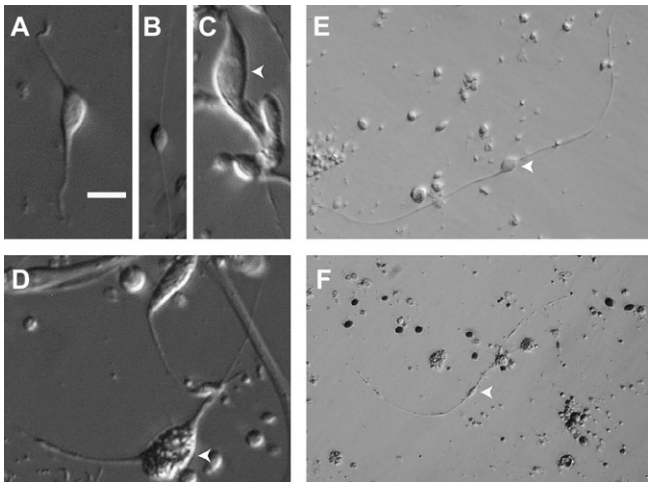

Figure 1 Morphology and survival of the cells with processes dissociated from labella of *Phormia regina*. Modified Hoffman contrast images of typical cell preparations just after isolation and plating (**A**, **B**; **C**, **D**, arrowhead), cultured for 2 days (**E**, arrowhead) and 6 days (**F**, arrowhead; trypan blue exclusion after incubation of 0.5% trypan blue for 5 min). Scale bar in (A) is 10 μm for (A–D), 20 μm for (E), and 40 μm for (F).

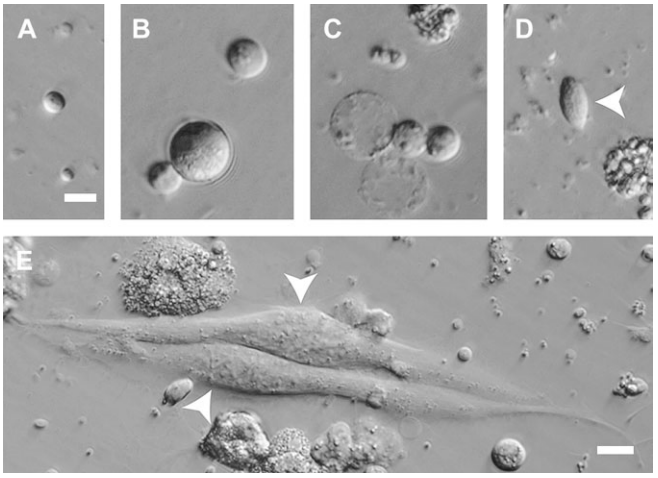

Figure 3 Morphology of the cells without processes dissociated from labella of *Phormia regina*. Modified Hoffman contrast images of typical cell preparations just after isolation and plating. Scale bars in **(A)** and **(E)** are 5 μm for **(A–D)** and 20 μm, respectively.

the cells without processes in primary culture after 6 days stained with trypan blue, although almost all of them excluded trypan blue just after isolation.

In the cells described above, cells showing processes could be the taste or mechanoreceptor neurons from labella of flies. The number of GRNs is larger than mechanoreceptor neurons in labella of flies because a labellar chemosensillum is a sensory unit containing four GRNs and one mechanoreceptor neuron. A taste receptor cell *in situ* has a long dendritic process (up to 300 μm), but the mechanoreceptor neuron has a tubular body instead. The bipolar cells in our culture tended to have two long processes on both sides. Thus, the cells having processes in our culture system are very likely to be GRNs.

The L-15 medium selected in our study supported the growth of the bipolar cells while tending to inhibit the growth of the nonbipolar cells. In contrast, Grace's or Schneider's medium, which we also tested, supported the growth of nonbipolar cells, not the bipolar cells. No medium was found suitable for both cell types.

Physiological properties of bipolar cells monitored by calcium imaging

Focusing on the putative GRNs with bipolar processes (Figure 1), we attempted to examine whether these cells responded to any taste stimulants such as sucrose, NaCl, or LiCl by monitoring intracellular calcium with a Ca^{2+}-sensitive dye, fluo-3. Cultured cells loaded with fluo-3 were stimulated under superfusion with a physiological saline solution and $[Ca^{2+}]_i$ images were continuously recorded.

First, it was examined whether bipolar cells exhibited an increase in $[Ca^{2+}]_i$ after being depolarized by a 2-s perfusion with 117.5 mM K^+ (Figure 4A). Depolarization caused an increase in fluo-3 fluorescence intensity in the somata of most noncultured and cultured bipolar cells in the presence of 2.5 mM Ca^{2+} in the bath. The increase in fluorescence intensity was repeatedly induced by repetitive depolarization. These results indicate that depolarization increases $[Ca^{2+}]_i$ in bipolar cells and confirm that changes in $[Ca^{2+}]_i$ are available for monitoring the excitation of the bipolar cells. Considering that the high K^+ solution can affect the viability of the cultured cells, it was applied at the end of each stimulation sequence.

Fly GRNs are functionally differentiated to respond to sugars, salts, water, and deterrent compounds. It was examined if these taste stimulants induced calcium responses in these bipolar cells in primary culture. However, the noncultured bipolar cells and cultured bipolar cells less than 2 days in culture did not respond to any stimulant applications (n = 93). In some bipolar cells (18 of 69 cells) cultured for 2–6 days, as seen in Figure 1E,F, $[Ca^{2+}]_i$ was increased by application of 200 mM sucrose for 5 s (Figure 4B). Bipolar cells responding to 200 mM sucrose did not respond to 500 mM NaCl (n = 5), corresponding to the electrophysiological results that one of the four GRNs in a labellar chemosensillum responds selectively to sugar. This suggests that these bipolar cells were not salt receptor neurons but sugar receptor neurons. In *P. regina*, glycyrrhizin is the only artificial (nonsugar) sweetener for humans that also stimulates sugar receptor neurons (Ahamed et al., 2001): glycyrrhizin induced the maximum response at 3 mM, which was much lower than that of sucrose. However, glycyrrhizin did not dissolve readily in the bath solution that included 100 mM mannitol, which was added to increase the osmotic pressure to bring it closer to that of the medium for primary cultures (see Materials and Methods). To determine if the increase in $[Ca^{2+}]_i$ depended on Ca^{2+} influx, we monitored the changes in $[Ca^{2+}]_i$ in response to sucrose under extracellular Ca^{2+}-free conditions. The bipolar cells responded up to twice to repeated stimulation of sucrose, and the magnitude of the response to the second stimulation was much smaller than that to the first one (data not shown). To obtain a result in between the two stimulations, the extracellular Ca^{2+}-free conditions were examined first followed by the normal conditions. Increase in $[Ca^{2+}]_i$ by application of 100 mM sucrose for 20 s was almost suppressed under the Ca^{2+}-free conditions; this increase was recovered after washing with the normal physiological saline solution (n = 3, Figure 4C). The results showed that the $[Ca^{2+}]_i$ increase in response to sucrose mostly consisted of Ca^{2+} influx.

In some other bipolar cells that did not show an increase in $[Ca^{2+}]_i$ after application of sucrose (five of 32 cells), $[Ca^{2+}]_i$ was increased by application of 500 mM NaCl for 5 s (Figure 4D). This type of bipolar cells appeared to be salt receptor neurons. A considerable transient decrease in fluorescent intensity was unavoidable at the onset of 100 mM sucrose stimulation in all the recordings of bipolar cells that responded to 500 mM NaCl. A transient decrease in fluorescent intensity was sometimes observed even when bath solution was applied. Additionally, a time course of study of this transient decrease in fluorescence roughly traced the stimulating puff. These are characteristic of artifacts that frequently appear in optical recordings.

No water receptor neurons were identified in the bipolar cells because there was no stimulating solution available for GRNs responsive to water under superfusion with saline.

Finally, LiCl was applied to bipolar cells as a stimulant for deterrent compound-sensitive GRNs ("deterrent" GRNs). LiCl is electrophysiologically known to stimulate deterrent GRNs as well as salt receptor neurons (Gillary, 1966). In three out of the 21 bipolar cells that did not respond to sucrose or NaCl, an increase in $[Ca^{2+}]_i$ was detected when 500 mM LiCl was applied for 10 s (Figure 4E). This responsiveness reflects the stimulatory property of LiCl on deterrent GRNs. It has also been reported that the deterrent GRNs are responsive to bitter compounds (e.g., Liscia et al., 2000; *Protophormia terranovae*) or food containing bitter compounds (e.g., Dethier, 1976; *P. regina*). To seek a bitter compound suitable as a standard stimulant to deterrent

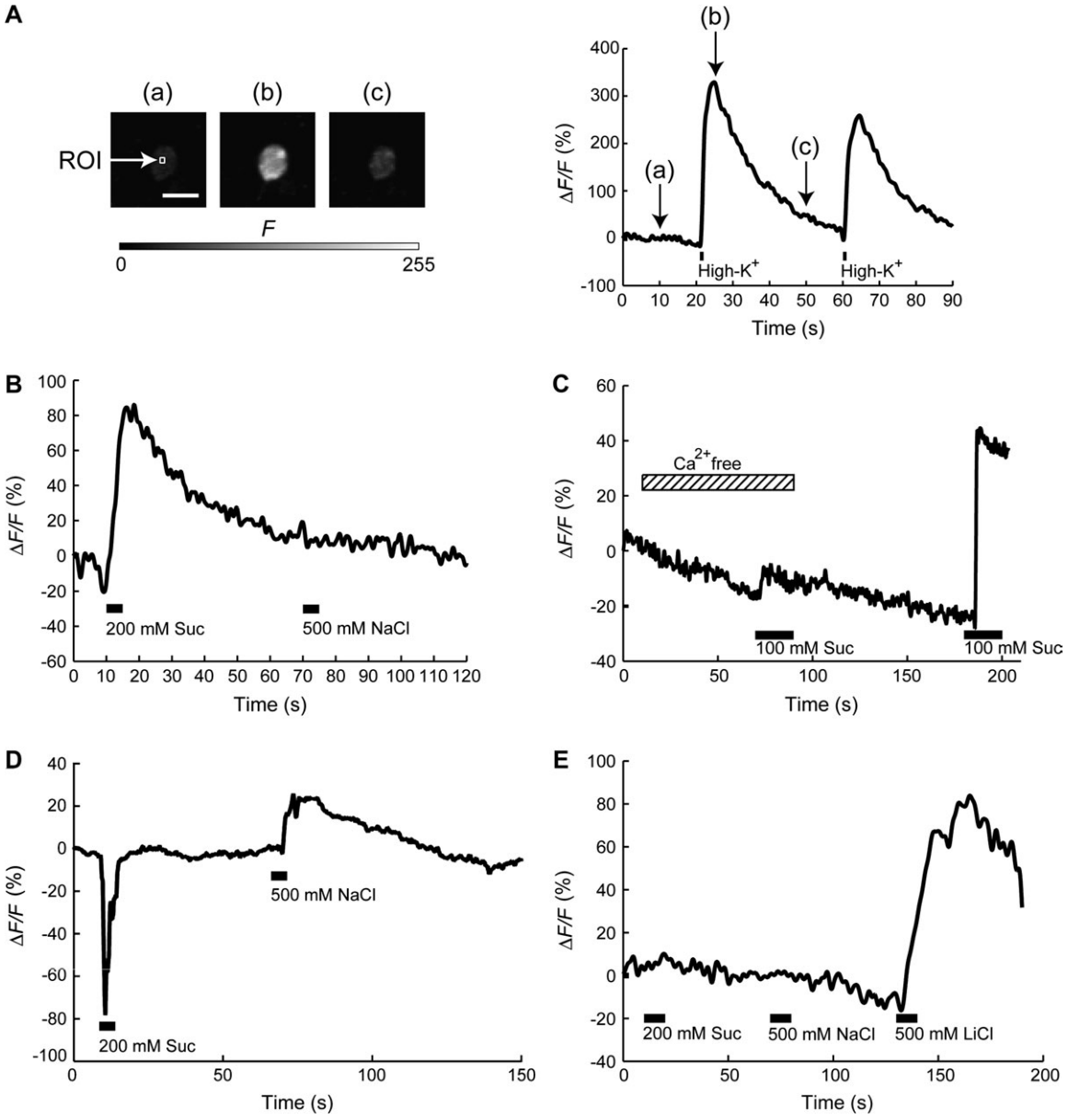

Figure 4 Ca^{2+} responses of putative cultured GRNs containing fluo-3 to **(A)** high K^+ solution, **(B)** 200 mM sucrose and 500 mM NaCl, **(C)** 100 mM sucrose under extracellular Ca^{2+}-free (shaded bar) and normal conditions, **(D)** 500 mM NaCl, and **(E)** 500 mM LiCl. Stimulants were applied during the periods indicated with solid bars. In (A), fluorescent images (a–c) of the same cell body are shown in gray scale: 10 s before high K^+ stimulation (a), 5 s (b), and 30 s (c) after the beginning of high K^+ stimulation. Each image was obtained by stacking 10 images recorded following the times shown. Scale bar is 10 μm. Fluorescence intensity in a region of interest set on the cell image was measured and plotted as a time function of fluorescence changes normalized to baseline fluorescence intensity ($\Delta F/F$).

GRNs of *P. regina*, general bitter compounds for humans were tested such as caffeine, denatonium, strychnine, and quinine by extracellular recording (tip recording) on the labellar chemosensilla in *P. regina* 5–7 days after emergence. However, only a small portion of the labellar chemosensilla was responsive to these bitter compounds in *P. regina*: less than 10% of the LL-type labellar chemosensilla showed a response to these compounds. These results indicate that only a few of the deterrent GRNs in the labella of *P. regina* respond to bitter compounds. In contrast, it is easy to find the labellar chemosensilla including the deterrent GRNs responsive to LiCl (Gillary, 1966). This precluded the use of the bitter compounds as stimulants for the deterrent GRNs.

It is unlikely that the Ca^{2+} responses to stimulating solutions in bipolar cells merely reflect changes in osmotic pressure produced by the stimulating solutions. First, we used a hyperosmotic solution containing 100 mM mannitol for the basic perfusion, which is the same as in the culture

medium. When the cell was stimulated with 100 mM sucrose, as shown in Figure 4C, the sucrose solution contained no mannitol and had nearly the same osmotic pressure as the perfusing solution. Second, the observed Ca^{2+} responses were independent of the changes in osmotic pressure that occurred when the stimulating solution was applied. As shown in Figure 4B, cells that responded to 200 mM sucrose did not respond to 500 mM NaCl, despite a larger osmotic pressure in the NaCl solution. The bipolar cells sensitive to 500 mM LiCl did not respond to 500 mM NaCl (Figure 4E). Taken together, these observations indicate that the Ca^{2+} responses of the GRNs were mediated by taste stimulants as chemical ligands.

In cells isolated from the labella of the blowfly, we found several kinds of cells that did not respond to taste stimulants. Especially, the silent bipolar cells with somata of ~12 μm in diameter with thick processes (Figure 1C,D) did not show responsiveness to any taste stimulants used in this study. Such silent bipolar cells could be water receptor neurons, although we could not examine their responsiveness to water. Another possibility is that, based on their morphology (Dethier, 1976), these cells are mechanoreceptor neurons that are located at the base of each sensillum. However, we did not obtain any evidence to support this possibility. The bipolar cells with somata of ~12 μm in diameter with thick processes did not exhibit any response when a puff of the simple bath solution was applied (data not shown). The cells without processes (see Figure 3) also exhibited no taste response.

Discussion

To date, there was no primary culture method for insect GRNs, although cell culture systems have been widely utilized for other organisms. We have developed culture conditions in which some of the bipolar cells with somata of 4–7 μm in diameter dissociated from labella of the blowfly, *P. regina*, exhibited a taste response to sucrose, NaCl, or LiCl. Therefore, we concluded that these bipolar cells were most likely GRNs, although we do not completely exclude the possibility that some of them, especially the "silent" cells, could be mechanoreceptor neurons.

Necessity of primary culture and critical culture period for taste responsiveness of bipolar cells

Bipolar cells isolated from the labella of *P. regina* pupae did not respond to any taste stimulants on the day of isolation. One of the reasons may be because the isolated bipolar cells lost the major portions of dendritic processes during the dissociation procedures: the processes of those cells were shorter than 50 μm immediately after isolation, while the dendritic processes of labellar GRNs *in vivo* (adult blowfly) measure up to 300 μm in length (Dethier, 1976). We suppose that the bipolar cells did not respond to taste stimulants because of a lack of dendritic processes, which is where the signal transduction is believed to occur. Another reason could be because the GRNs of the pupae 4–5 days after pupation have not fully expressed some functional proteins such as taste receptors or ion channels. This hypothesis is indirectly supported by the fact that labellar sensilla in pupal *P. regina* barely show a full response to sugars (T. Amakawa, personal communication) and that the dendrites of GRNs continue growing throughout the entire pupal period of about 7 days (Hansen and Hansen-Delkeskamp, 1983). In our experiments, the culture period of ≥2 days was critical for obtaining the bipolar cells responsive to taste stimulants. Interestingly, this period of 2 days almost corresponds to the remainder of the pupal stage before their effected emergence. We speculate that the culture of ≥2 days allows the bipolar cells to construct their taste transduction machinery.

Variation in GRN soma sizes

Taste-responsive cultured bipolar cells were classified into two groups according to their soma size: one group had somata of ~7 μm in diameter (Figure 1A,E), and the other group had somata of ~4 μm (Figure 1B,F). According to electron microscopic observations of taste organ slices, the size of GRNs seem to depend on taste sensilla size of the labella of *P. regina* (M. Ozaki, unpublished data). Recently, it was also reported in *Drosophila* that one of the four GRNs in a small type of labellar chemosensilla is comparatively large (Thorne *et al.*, 2004). In our experiments, response to sucrose was observed in five large bipolar cells and in 13 small bipolar cells, while response to NaCl or LiCl was seen only in small bipolar cells. These results imply that the soma size of bipolar cells cannot be used to predict the cell's specific taste modality.

Taste modality selectivity of single GRNs

In flies, a gustatory sensillum contains four functionally differentiated GRNs: sugar, salt, water receptor neurons, and deterrent GRNs. The response of a taste receptor cell in a sensillum is taste modality selective when observed by extracellular recordings. In our preparations, cultured putative GRNs also exhibited the same modality-selective responsiveness when observed by calcium imaging. Our preparations included GRNs responding to sucrose (Figure 4B), NaCl (Figure 4D), or LiCl (deterrent GRNs; Figure 4E), although water receptor neurons were difficult to identify under perfusing conditions. Sucrose-sensitive cells did not respond to NaCl, which was consistent with the responsiveness of sugar receptor neurons characterized by the tip recordings (Figure 4B). NaCl-sensitive cells did not respond to sucrose (Figure 4D), and LiCl-sensitive cells did not respond to either sugar or NaCl (Figure 4E). These response features corresponded to those of salt receptor neurons and deterrent GRNs, respectively. Our results demonstrated that a single GRN exhibits taste modality-selective responsiveness.

Possible involvement of Ca^{2+} in responses of fly GRNs

A transient increase of $[Ca^{2+}]_i$ induced by a taste stimulant indicates the possibility that Ca^{2+} is involved in transduction pathways in sugar and salt receptor neurons and deterrent GRNs. Especially in sugar receptor neurons, a transient increase in $[Ca^{2+}]_i$ mainly depended on Ca^{2+} influx because it was hardly seen under extracellular Ca^{2+}-free conditions (Figure 4C). Ca^{2+} influx may take place through ionotropic sugar receptors, cyclic nucleotide–gated cation channels, and/or voltage-gated cation channels. On the other hand, the increase in $[Ca^{2+}]_i$ in salt receptor neurons (19.1 ± 3.5%, mean ± SEM, $n = 5$) was lower than in sugar receptor neurons (108.9 ± 12.9%, mean ± SEM, $n = 18$; also see Figure 4D), although both cells were stimulated with high concentrations of chemicals that give maximum responses in extracellular recordings. These results suggest that Ca^{2+} probably did not contribute in a major way to the early steps of the transduction mechanisms in salt receptor neurons as is the case in sugar receptor neurons. It is quite likely that such a difference is due to different transduction mechanisms between the sugar and salt receptor neurons.

Regional stimulation of the distal dendritic membrane, presumed to contain the transduction mechanisms, would confirm our results. Only the distal dendritic membranes encounter taste stimulants when taste cells are in their natural environment, that is, within a sensillum. However, it was difficult to identify which of the processes extending from a bipolar cell included the distal dendritic tip; the bipolar cells that once showed Ca^{2+} response to a taste stimulant tended to become unresponsive to subsequent stimulations. Additionally, the length of a bipolar cell process has occasionally prevented us from successfully applying taste stimulants to the distal dendritic membrane (also see Figure 2). The distal part of the processes could occasionally not be observed under the optimum magnification for calcium imaging that we used. Solving these problems would make our cell culture method an even more powerful tool for examining the transduction mechanisms of GRNs.

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