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Perception of Noxious Compounds by Contact Chemoreceptors of the Blowfly, *Phormia regina*: Putative Role of an Odorant-binding Protein

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Abstract

The blowfly, *Phormia regina*, has sensilla with four contact-chemoreceptor cells and one mechanoreceptor cell on its labellum. Three of the four chemoreceptor cells are called the sugar, the salt and the water receptor cells, respectively. However, the specificity of the remaining chemoreceptor cell, traditionally called the 'fifth cell', has not yet been clarified. Referring to behavioral evaluation of the oral toxicity of monoterpenes, we measured the electrophysiological response of the 'fifth cell' to these compounds. Of all the monoterpenes examined, D-limonene exhibited the strongest oral toxicity and induced the severest aversive behavior with vomiting and/or excretion in the fly. D-Limonene, when dispersed in an aqueous stimulus solution including dimethyl sulfoxide or an odorant-binding protein (OBP) found in the contact-chemoreceptor sensillum, the chemical sense-related lipophilic ligand-binding protein (CRLBP), evoked impulses from the 'fifth cell'. Considering the relationship between the aversive effects of monoterpenes and the response of the 'fifth cell' to these effects, we propose that the 'fifth cell' is a warning cell that has been differentiated as a taste system for detecting and avoiding dangerous foods. Here we suggest that in the insect contact-chemoreceptor sensillum, CRLBP carries lipophilic members of the noxious taste substances to the 'fifth cell' through the aqueous sensillum lymph. This insect OBP may functionally be analogous to the von Ebner's grand protein in taste organs of mammals.

Key words: monoterpene, oral toxicity, aversive behavior, bitter taste reception, odorant-binding protein

Introduction

The contact-chemoreceptor sensilla on the labellum of the fly are in the form of a hair housing five sensory neurons, i.e. four contact-chemoreceptors and one mechanoreceptor neuron (Ozaki and Tominaga, 1999). Although three of the four chemoreceptor neurons are termed the sugar, the salt and the water receptor cells after their adequate stimuli, respectively, the fourth neuron has simply been called the 'fifth cell'. Dethier and Hanson reported the responsiveness of the 'fifth cell' to fatty acids (Dethier and Hanson, 1968), but these authors did not change this terminology, because its function had not then been clarified conclusively. Furthermore, Gillary had previously reported that Cs^+ or Rb^+ ions evoke impulses not only of the salt receptor cell but also of the 'fifth cell' (Gillary, 1966). Hence, this cell is sometimes called the 'second salt receptor cell'. However, the 'fifth cell' could be expected to have another function. Dethier showed that the response to some vapors induced vigorous impulses of the 'fifth cell' (Dethier, 1972), suggesting that the 'fifth cell' is sensitive to odorants as well. Ozaki *et al*. showed that the response of the 'fifth cell' to fragrant components of apple juice is depressed in the presence of antibodies raised against chemical-sense-related lipophilic-ligand-binding protein (CRLBP), which is an odorant-binding protein (OBP) commonly found in the olfactory and the contact-chemoreceptor sensilla of the blowfly, *Phormia regina* (Ozaki *et al*., 1995). OBPs are small, water-soluble proteins and widely found in olfactory systems of different animals (Vogt, 1995; Pelosi, 1996; Vogt *et al*., 1999). The OBPs are involved in the first specific biochemical step in odor reception and are thought to carry lipophilic odorants to the olfactory receptor cells through hydrophilic surroundings (Vogt *et al*., 1985; Prestwich *et al*., 1995; Vogt,

1995; Pelosi, 1996; Steinbrecht, 1996, 1999; Ziegelberger, 1996; Kaissling, 1998). Therefore, Ozaki *et al.* suggested that the 'fifth cell', with the help of CRLBP, could be involved in the recognition of volatile, more lipophilic plant compounds.

Phytophagous lepidopterous caterpillars have been shown to have contact-chemoreceptor neurons in the maxillae that are sensitive to deterrent or bitter-tasting compounds (Dethier, 1973; Frazier and Hanson, 1986; Schoonhoven, 1987; Blaney and Simmonds, 1988; Chapman *et al.*, 1991; Glendinning and Hills, 1997). The importance of deterrent neurons had been recognized in phytophagous lepidopterous caterpillars by studying the relationship between food-plant selection behavior of these insects and toxicity of secondary metabolites of plants (Harley and Thoresteinson, 1967; Schoonhoven, 1972; Schoonhoven *et al.*, 1992; Peterson *et al.*, 1993; Bernays and Chapman, 1994; Bernays *et al.*, 2000).

Generally, *P. regina*, has saprophagous larvae and the adults insects visit plants to feed on nectar or sap, among many other potential food sources. During the search for feeding or oviposition sites, the flies will come in contact also with potentially toxic compounds (Dethier, 1993), thus the activation of neurons sensitive to such compounds leading to the avoidance of really toxic compounds could elicit aversive behavior. Recently, Liscia and Solari (Liscia and Solari, 2000) reported for *Protophormia terraenovae* that the 'fifth cell' responds to substances bitter for humans, such as quinine, amiloride, nicotine and caffeine. They also showed that these flies avoided drinking sucrose solution mixed with quinine or amiloride.

In addition to the study of Liscia and Solari (Liscia and Solari, 2000), which indicates a possible function of the 'fifth cell' as a deterrent or a bitter taste receptor cell, here we show that the 'fifth cell' of *P. regina* actually perceives non-polar monoterpenes. We also suggest that the CRLBP might function as a carrier protein for the monoterpenes to the 'fifth cell', which is an indispensable receptor neuron for the 'toxin' detection system in the fly.

Materials and Methods

Flies

The blowflies, *P. regina*, originally donated from Professor Morita's laboratory in Kyushu University, were reared in our laboratory at 22 ± 2°C using the method of Morita (personal communication), and after emergence, fed with 0.1 M sucrose and water.

Preparation of stimuli

The monoterpenes (D-limonene, L-limonene, cineole, citral, β-myrcene) and toluene were purchased from Nakarai Tesuque Corporation, Kyoto, Japan. We directly applied these pure chemicals to several sites of the fly body for the toxicity test.

Monoterpenes are mostly non-polar and cannot be easily dissolved in aqueous solutions. Therefore, we dispersed these chemicals in 10 mM NaCl with 0.25, 1 or 4% dimethyl sulfoxide (DMSO) for the aversive proboscis extension reflex tests (aversive PER tests) and the electrophysiological tip-recording experiments. Ten millimolar NaCl was added to every stimulus solution, because the electrophysiological tip-recording method needs an electrolyte in stimulus solutions. We also used 1, 1.25, 5 or 20 μM CRLBP, 20 μM β-lactoglobulin (BLG) or 20 μM bovine serum albumin (BSA) instead of DMSO. CRLBP was purified from the *P. regina* labellar tissue as mentioned below. BLG, BSA and DMSO were purchased from Nakarai Tesuque Corporation, Kyoto, Japan.

Equal volumes (~0.5 ml) of a lipophilic substance (D-limonene, L-limonene, cineole, citral, β-myrcene or toluene) and 10 mM NaCl solution containing a certain concentration of DMSO, CRLBP BLG or BSA were mixed in a glass vial under oxygen-free conditions and centrifuged to separate into aqueous and oil layers. The aqueous layer was recovered and used as a stimulus solution for the aversive PER tests or for electrophysiological experiments after measuring the concentration of the contained lipophilic substance. The resulting concentrations of D-limonene, L-limonene, cineole, citral, β-myrcene or toluene in the stimulus solutions were determined by gas chromatography (GC). This was accomplished by mixing the stimulus solution with n-hexane (v/v 1:3) and separation of the mixture into aqueous and hexane layers. As quantification standard for the GC, dodecane was added to the recovered hexane layer at the concentration of 10 μg/ml. In the chromatograms, the peaks of D-limonene, L-limonene, cineole, citral, β-myrcene and toluene were identified, and their concentrations were calculated by referring to the concentration of dodecane.

Isolation of CRLBP

Lipophilic-ligand-binding protein (CRLBP) was purified from the blowfly labella according to Ozaki's method (Ozaki *et al.*, 1995). The labella of the blowflies were isolated from heads and homogenized in a hand mortar with a small amount of liquid nitrogen for 20 min. The homogenate was mixed with the sample buffer (4.75 mM sodium barbiturate–HCl, 10% glycerol, pH 6.8) and centrifuged at 15 000 r.p.m. for 10 min at 4°C, and the supernatant was applied to the native polyacrylamide gel electrophoresis.

The gel systems of Ornstein (Ornstein, 1964) and Davis (Davis, 1964) were used with some modifications: instead of Tris buffers, we used barbiturate buffers (stacking gel buffer: 9.3 mM sodium barbiturate–HCl pH 6.7; running gel buffer: 91.1 mM sodium barbiturate–HCl buffer pH 8.9; electrode buffer: 41.1 mM sodium barbiturate–glycine buffer pH 8.3); the stacking and the running gels were made of 4.5 and 7.5% acrylamide, respectively. Electrophoresis was carried out at a current of 40 mA for 1.5 h in a cold chamber, so that the temperature of the gel could be adjusted to 20–24°C.

Since the CRLBP is a highly acidic protein (pI = 3.6)

(Ozaki *et al*., 1995), it migrated faster than other proteins. Thus, we could cut the gel, including CRLBP, after electrophoresis without any contamination. The gel piece with CRLBP was put in a dialysis bag with a small amount of electrode buffer and set for horizontal submarine electrophoresis. CRLBP was then electrophoretically eluted out from the gel overnight and dialyzed against distilled water overnight in a cold chamber at 5°C. The concentration of CRLBP in the resulting solution was determined by the CBB protein quantifying method. For storage, we lyophilized the solution to get a dry powder that was kept at –80°C. For experimental usage, we made appropriate concentrations of CRLBP solutions by dissolving the protein in distilled water or buffers.

Toxicity test

The flies, 5–6 days after emergence from a single batch, were immobilized by securing their wings with washing pins. For toxicity test, a 0.3 µl drop of pure D-limonene, L-limonene, cineole, citral, β-myrcene or toluene was directly applied with a glass capillary to the labellum and other body parts (see Figure 1) of 10 flies. Thirty minutes after this treatment (the applied chemicals were allowed to evaporate spontaneously), the flies were classified into three groups of conditions and counted as: killed, handicapped and recovered. The same test was repeated six times for each chemical, and the toxicity was evaluated.

Aversive proboscis extension (PER) test

Flies from a single batch were immobilized 5–6 days after emergence by securing their wings with washing pins. We first gave distilled water to the flies until they were satiated with water, and then subjected them to the test.

We carefully touched the labellar chemosensilla with the stimulus solution in a micropipette tip, so that the flies could only drink sparingly from it. Twenty flies were tested with the same stimulus, and the number of flies showing full extension of a strained proboscis followed by vomiting and/or faecal excretion (see inset of Figure 2A) was counted and expressed as a percentage. This test was repeated six times. It seems likely that the fly can vomit exhaustively by using the full extension of a strained proboscis. Dethier reviewed feeding and aversive behaviors of the blowfly in detail, but did not mention much about this type of PER (Dethier, 1976). However, in order to evaluate a positive aversive behavior against toxins for the fly counting, we selected this type of PER and called it 'aversive PER', discriminating it from a passive aversive-touching response like retracting the proboscis.

Electrophysiological procedure

In electrophysiological experiments, adult flies, 5–6 days after emergence, were used. The electrophysiological response was recorded from the tip of an LL type chemosensillum located on the outer margin of the labellum, using the tip-recording method described first by Hodgson *et al*. (Hodgson *et al*., 1955). If the tip-recording method, which has been widely used for water-soluble stimulants, could also be applied for lipophilic, less conductive substances, it can be expected that the chemoreceptor neurons sensitive to D-limonene would be determined. In order to make the tip-recording method applicable for recording responses to D-limonene, we added DMSO or CRLBP to the aqueous stimulus solution and dispersed D-limonene in it. We stimulated the sensillar tip with the D-limonene-dispersing 10 mM NaCl solution with DMSO or CRLBP in a glass capillary recording electrode. We also examined BLG and BSA instead of CRLBP. The stimulus duration was 30 s and the interval between stimuli was at least 10 min. Evoked impulses were recorded through a band-pass filter (100–2000 Hz). The receptor neurons from which the recorded impulses originate can be determined, because the impulses of the four receptor cells in a sensillum have different amplitudes. The ambient temperature was 20–24°C throughout the experiments.

Results

Toxicity of D-limonene

The contact toxicity of D-limonene, L-limonene, cineole, citral, β-myrcene and toluene, when applied to different sites on the fly's body, is summarized in Figure 1, which shows the percentage of flies killed, handicapped and recovered 30 min after application of 0.3 µl of test substance. Application of any of these chemicals produced more or less convulsive

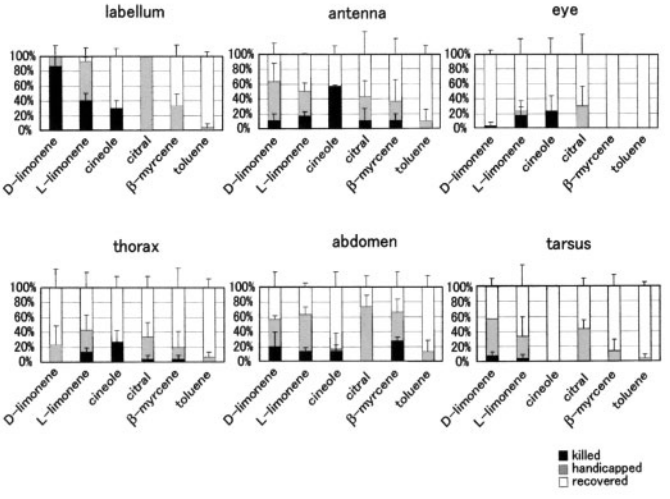

Figure 1 Toxicity of monoterpenes. A 0.3 µl drop of pure D-limonene, L-limonene, cineole, citral, β-myrcene or toluene was applied to the labellum, antenna, eye, thorax, abdomen or tarsus of 20 flies, respectively. Thirty minutes after this treatment, the flies were classified into three groups of conditions and counted as: K, killed (closed columns); H, handicapped (shadowed columns); R, recovered (open columns). The same test was repeated six times for each chemical, and the average percent of flies of each group ± SD (vertical bar) is indicated.

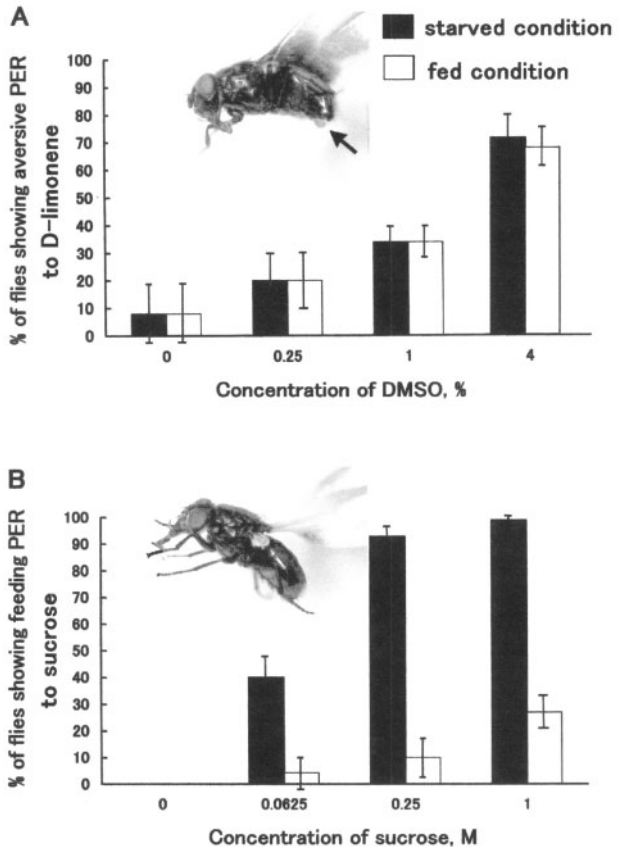

Figure 2 Difference in the starvation effect between the aversive and the feeding PERs. **(A)** Percentage of flies showing aversive PER to D-limonene-dispersing 10 mM NaCl solution with 0, 0.25, 1 or 4 % DMSO (D-limonene concentrations = 8, 14, 24 or 88 μg/ml, see Figure 3) (n = 6); **(B)** Percentage of the flies showing feeding PER to sucrose-containing 10 mM NaCl solution (sucrose concentrations = 0, 0.625, 0.25, or 1 M) (n = 7). The closed and the open columns indicate average percentage of flies with SDs (vertical bars) under starved and fed conditions, respectively. Insets show aversive PER to D-limonene (A) and the feeding PER to sucrose (B). Aversive PER is accompanied by excretion of faeces (arrow).

shaking and paralysis of wings and legs. However, the strength of the locomotive disorder depended on the individual chemical. After application of toluene, for example, 100% of flies were alive and more than 87% of them recovered normal locomotive activity within 30 min, regardless of the application site. Citral and β-myrcene evoked paralysis of legs lasting 30 min in a significant percentage of flies, but these substances seemed less toxic than D-limonene, L-limonene or cineole. The strength of the locomotive disorders also depended on the application sites. Cineole, when applied to the antennae, and D-limonene and L-limonene, when applied to the labellum, showed the strongest toxicity. After application of D-limonene, L-limonene and cineole to the labellum, the death rates were 87, 40 and 30% respectively. These death rates were regarded as indicators for relative oral toxicities of the chemicals applied to the labellum.

It can be concluded that D-limonene produced the severest oral toxicity of all examined chemicals. The toxicity of the other compounds was in the following order: D-limonene > L-limonene > cineole > citral > β-myrcene > toluene.

Aversive PER to monoterpenes

When we touched the labellar sensilla with D-limonene-dispersing solution, the fly showed aversive PER (inset of Figure 2A), in which the proboscis was extended between the prothorathic legs in a protective movement. An irritated movement of labellum and abdomen were usually observed prior to the aversive PER, and vomiting and excretion of faeces were often induced as well. These behaviors can help the fly to avoid intake of toxic substances.

The inset of Figure 2B shows the feeding PER to stimulation of the labellar sensilla with sucrose solution. The fly extends the proboscis towards the pipette tip filled with sucrose solution and opens the labellar lobes ready to suck. Thus, the aversive and the feeding PERs can be discriminated from each other by careful observation. The difference in the starvation effect between the aversive and the feeding PERs strongly suggested that these PERs are totally different in their behavioral meanings. Forty flies hatched from the same batch were divided into two groups of 20 flies each: one group was fed with 0.1 M sucrose for 5 days after emergence until the PER test, and the other group was fed with 0.1 M sucrose until 36 h prior to the test and starved after that (water was continuously supplied to both groups). Five days after emergence, the PER test to D-limonene dispersed in 10 mM NaCl solutions with 0, 0.25, 1 or 4% DMSO was carried out in both groups at the same time. The ratio of flies showing the aversive PER increased as the concentration of DMSO was increased (72% of flies showed aversive PER at 4% DMSO), but no significant difference was found between the fed (open columns) and the starved groups (closed columns) (Figure 2A). On the other hand, the ratio of flies showing feeding PER increased as the concentration of sucrose was increased, and it was obviously higher in the starved group (closed columns) than that in the fed flies (open columns) at every sucrose concentration (Figure 2B). Significant differences in the ratio of flies showing feeding PER between the starved and the fed groups were confirmed either at 0.0625, 0.25 or 1 M sucrose by unpaired t-test ($P < 0.01$).

We carried out the PER test to D-limonene, L-limonene, cineole, citral, β-myrcene or toluene-containing solutions, respectively, and the ratios of the flies showing aversive PER are indicated in Figure 3 (open columns).

The concentrations (μg/ml) of examined chemicals (indicated above the columns in Figure 3) varied with the concentration of DMSO. These concentrations might be underestimated if the chemicals could not completely be transferred to the hexane layer for GC measurement (see Materials and methods). β-Myrcene was difficult to dissolve in 10 mM NaCl either with or without DMSO; cineole,

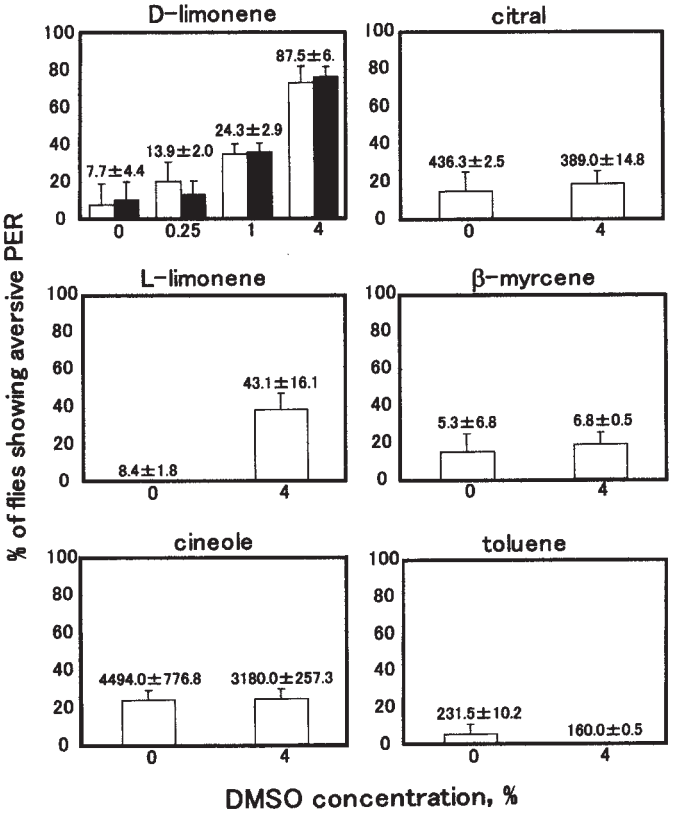

Figure 3 Aversive PER to monoterpenes solubilized with DMSO. Average percentage of flies showing aversive PER to D-limonene, L-limonene, cineole, citral, β-myrcene or toluene solubilized with 0, 0.25, 1 or 4 % DMSO (open columns) ± SD (vertical bar) is indicated, respectively. The PER test was repeated six times. Average concentration (μg/ml) of the solubilized chemical in each test solution ± SD is expressed above the corresponding columns. D-Limonene application to flies with the olfactory input cut is shown in the closed columns.

citral, and toluene were, however, easily dissolved in 10 mM NaCl even in the absence of DMSO. Figure 3 indicates that D- and L-limonene are hardly dissolved without DMSO, but when dispersed with DMSO, could induce aversive PER more effectively than cineole, citral and toluene. In order to exclude purely olfactory repellent effects, we carried out also aversive PER tests to D-limonene with flies whose olfactory input was eliminated by removing their antennae and maxillary palps (closed columns). We found no significant differences as compared to intact flies (open columns). We conclude that olfactory cues are not as important as gustatory cues for the aversive PER in *P. regina*.

Thus, the order of gustatory effectiveness of the examined chemicals in the aversive PER was as follows: D-limonene > L-limonene > cineole, citral or toluene (the effect of β-myrcene was not evaluated due to the weak dispersion of this compound).

In subsequent tests we used, instead of DMSO, CRLBP, which is an intrinsic lipophilic-ligand-binding protein purified from the labellar tissue of *P. regina*, to disperse D-limonene

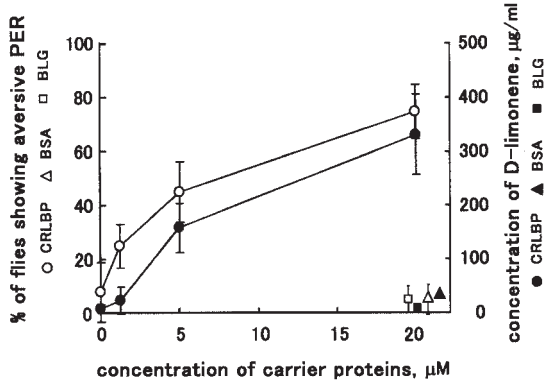

Figure 4 Aversive PER to D-limonene in the presence of putative carrier proteins. Average percentage of flies showing aversive PER to D-limonene solubilized with 0, 1.25, 5, or 20 μM CRLBP, 20 μM BLG or 20 μM BSA (open symbol) ± SD (vertical bar) and average concentration of D-limonene solubilized in each test solution (closed symbol) ± SD (vertical bar) are plotted. For convenience, the symbols of BLG and BSA at 20 μM are plotted alongside each other. The PER test was repeated six times per data point.

in aqueous solutions. As with DMSO, the ratio of flies showing the aversive PER to the D-limonene-dispersing solution with CRLBP was increased as the concentration of CRLBP was increased (Figure 4). In the presence of 20 μM CRLBP, 74.7% of flies showed aversive PER. Dispersion of D-limonene with BLG or BSA was far less effective.

Electrophysiological response of the 'fifth cell' to D-limonene

Since D-limonene is highly toxic and effectively induced aversive behavior of *P. regina* as mentioned above, we carried out electrophysiological experiments to find which taste receptor neuron responded. We mainly used D-limonene for stimulation, because the other monoterpenes did not induce such sensitive responses from the 'fifth cell' (Data not shown).

The impulses of the four functionally different chemoreceptor neurons in a labellar sensillum were discriminated from each other by their amplitudes; the 'fifth cell' stimulated with 0.5 M CsCl, 0.74 ± 0.08 mV (n = 20); the water receptor cell with 10 mM NaCl, 0.49 ± 0.08 mV (n = 20); the sugar receptor cell with 0.5 M sucrose, 1.04 ± 0.04 mV (n = 20); the salt receptor cell with 0.5 M NaCl, 1.29 ± 0.11 mV (n = 20).

In the labellar sensillum of *P. regina*, up to 4% pure DMSO did not induce any obvious impulses except for the impulses from the water receptor cell (see Figure 5A bottom). Figure 5A (top) shows a recording from the LL type of labellar sensillum to D-limonene-dispersing 10 mM NaCl solutions with 4% DMSO. The small impulses appeared immediately after the beginning of the stimulation and gradually diminished by adaptation, while the larger impulses appeared after a significant latency. Referring to the standard amplitudes of impulses (mentioned above), it is

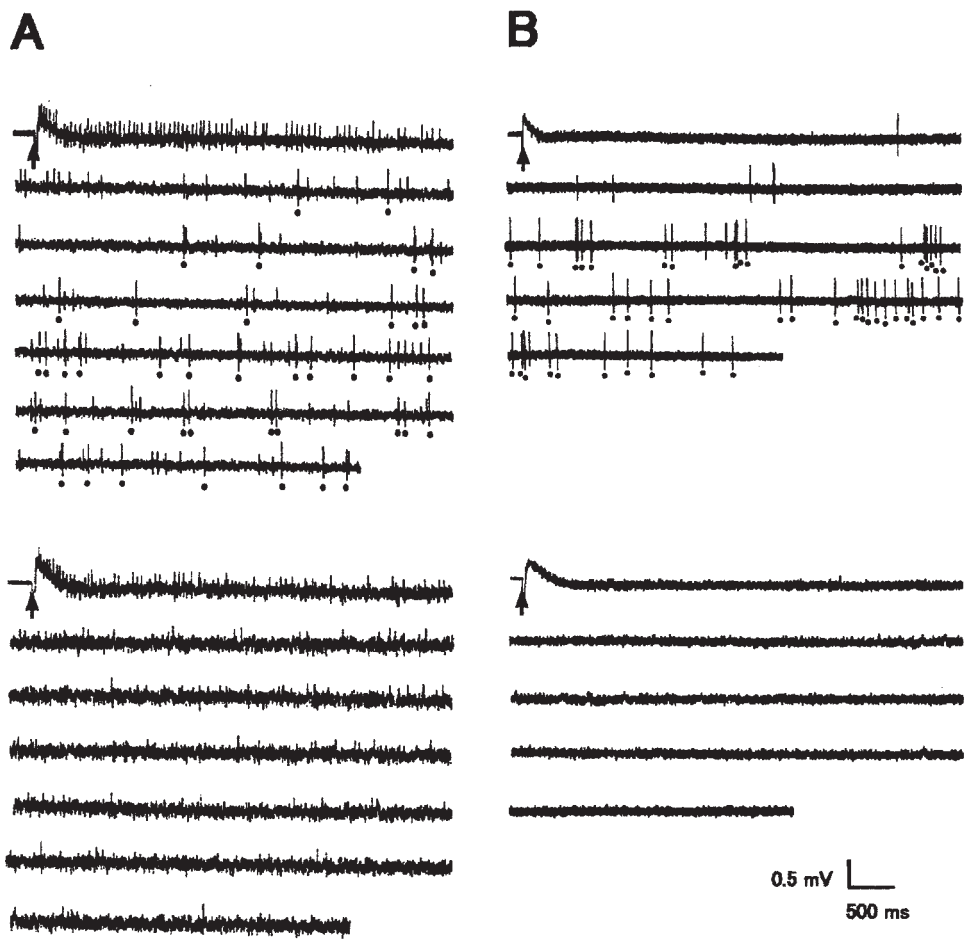

Figure 5 Electrophysiological recordings in response to D-limonene dispersed with DMSO. **(A)** Record from an intact sensillum to D-limonene-containing 10 mM NaCl solutions with 4% DMSO (D-limonene concentration = 90 μg/ml) (top) and the control record to 10 mM NaCl solution with 4 % DMSO (bottom). **(B)** Record from a sensillum having an inactive water receptor cell, to D-limonene-containing 10 mM NaCl solution with 4% DMSO (D-limonene concentration = 90 μg/ml) (top) and the control record to 10 mM NaCl solution with 4% DMSO (bottom). In Figures 5 and 6, arrows indicate the beginning of stimulation, and the impulses of the 'fifth cell' are pointed by dots.

tentatively defined that the small (0.53 ± 0.03 mV, n = 10) and the large impulses (0.76 ± 0.11 mV, n = 10) are derived from the water receptor cell and the 'fifth cell', respectively. In this record, ~8 s latency is seen until the first appearance of the 'fifth cell' impulse, probably because it takes time for D-limonene molecule to be transferred from the DMSO-containing environment in the stimulus solution to an intrinsic carrier protein, CRLBP in the receptor lymph. Figure 5A (bottom) is the control record in the absence of D-limonene. The impulses of the 'fifth cell' diminish, and only the impulses of the water receptor cell are seen. It is therefore concluded that the 'fifth cell' responds to D-limonene. Among the examined sensilla, we found some (2 in 56 trials) in which, for unknown reasons, the water receptor cell generated no impulses. Figure 5B (top) shows the impulse caused by D-limonene-dispersing 10 mM NaCl solution with 4% DMSO in one such sensilla. Only the impulses of the 'fifth cell' (0.74 ± 0.05 mV, n = 10) can be clearly seen. Here we estimated responding neurons based only on the impulse amplitudes, hence we use the traditional term 'fifth cell' as a tentative name for the D-limonene-sensitive neuron.

Figure 6A shows the impulses generated by D-limonene dispersed in 10 mM NaCl solution with 1 μM CRLBP. The impulses of the water receptor cell (0.53 ± 0.04 mV, n = 13) and the 'fifth cell' (0.79 ± 0.10 mV, n = 25) can be seen. These impulses are qualitatively similar to those in Figure 5A, in which DMSO was used to disperse D-limonene in the stimulus solution. This implies that CRLBP can help D-limonene to be dissolved in an aqueous environment. In the control record from the same sensillum stimulated with 10 mM NaCl solution containing 1 μM CRLBP (Figure 6B), only the impulses of the water receptor cell did appear. Figure 6C shows four types of nerve impulses recorded in the LL type of labellar sensillum: (a) the impulses of the 'fifth cell' (to 0.5 M CsCl); (b) the water receptor cell (to 10 mM NaCl); (c) the sugar receptor cell (to 0.1 M sucrose); and (d) the salt receptor cell (to 0.5 M NaCl). D-Limonene,

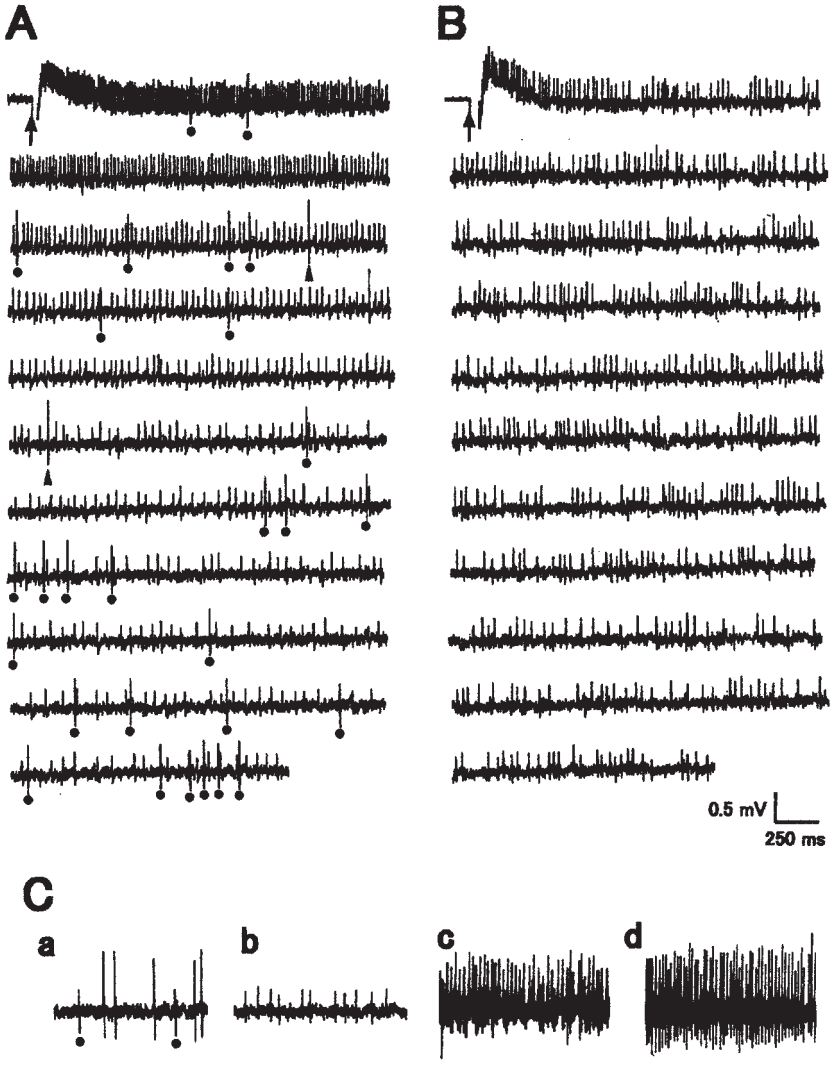

Figure 6 Taste response of an intact sensillum to D-limonene dispersed with CRLBP. **(A)** Record to D-limonene-containing 10 mM NaCl solution with 1 μM CRLBP (D-limonene concentration = 20.5 μg/ml). **(B)** Control record to 10 mM NaCl solution with 1 μM CRLBP. The impulses of the water receptor cell and the fifth cell were sometimes generated at the same time, resulting in a pseudo large impulse having an additive amplitude (arrowhead). **(C)** Standard impulses of the four functionally different contact-chemoreceptor neurons in the sensillum are indicated: (a)–(d) show the impulses to 0.5 M CsCl, 10mM NaCl, 0.1 M sucrose and 0.5M NaCl, respectively.

when dispersed with BLG or BSA instead of CRLBP, evoked fewer impulses from the 'fifth cell', at least for 30 s after the beginning of stimulation (data not shown). This suggests that BLG or BSA are acting differently to CRLBP in the taste sensillum of *P. regina* (see Discussion).

Except for the cases as shown in Figure 5B, we usually observed that the 'fifth cell' and the water receptor cell impulses were generated by stimulation with D-limonene-containing solutions. However, the time course of impulse generation in the 'fifth cell' was not always constant. The duration of the latency varied, and the peak of the 'fifth cell' activity, at which the highest impulse frequency was observed, appeared at different times after the beginning of stimulation. At the present time, it is unknown why such differences appeared among the sensilla. In fact, these differences made it difficult to investigate the concentration–response relationship of the 'fifth cell' activity, and so we selected the number of impulses generated for 5 s at the peak of the cell activity and plotted these in Figure 7A. Figure 7A shows such concentration-response relationships of D-limonene dissolved with DMSO (closed circles) and with CRLBP (open circles), respectively. The D-limonene concentration dependency of the aversive behavior is also indicated in Figure 7B.

Discussion

The 'fifth cell' and its role in the taste system of the fly

We can hypothesize that the sense of taste in insects is also categorized into some fundamental tastes, four functionally

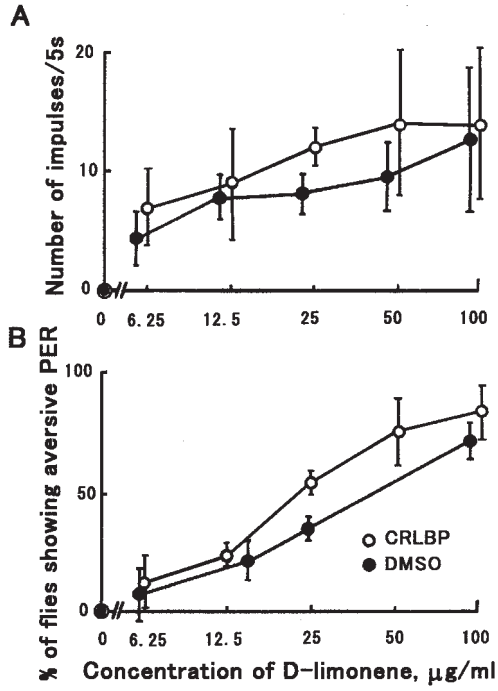

Figure 7 Concentration–response relationship for D-limonene. **(A)** D-Limonene concentration dependency of the electrophysiological response of the 'fifth cell'. **(B)** D-Limonene concentration dependency of the aversive behavioral response of the fly. Average percentage of flies showing aversive PER ± SD (vertical bar) is plotted against D-limonene concentration. Stimulus solutions were prepared by dilution with 10 mM NaCl plus 4% DMSO, starting from 90 μg/ml D-limonene-dispersing 10 mM NaCl solution with 4% (closed circles) or by dilution with 10 mM NaCl plus 5 μM CRLBP, starting from 100 μg/ml D-limonene-dispersing 10 mM NaCl solution with 5 μM CRLBP (open circles). The D-limonene concentration of the starting solution was adjusted to be 90 or 100 μg/ml by diluting the D-limonene-satiated solution with 10 mM NaCl plus 4% DMSO or 5 μM CRLBP, respectively. The number of tests is five per data point.

differentiated taste receptor cells in a sensillum may primarily be responsible for such fundamental tastes. The electrophysiological action of the sugar receptor cell, which responds not only to sugars but also to nucleotides (Amakawa *et al.*, 1992) or amino acids (Shiraishi and Kuwabara, 1970), induces feeding PER in the hungry flies (Dethier, 1976). This implies that the sugar receptor cell of the fly is responsible for a more general sign of food intake rather than a sign of sugars. Both water and salts are indispensable supplements. The fly chooses which one to take in order to optimize its internal water–salt balance. The feeding PER induction to water or salt may be regulated by the central nervous system referring to the internal requirement (Dethier, 1976; Moss and Dethier, 1983).

As for the 'fifth cell', how does its activity reflect on the behavior of the fly? Prior to the present paper, Liscia and Solari reported that the 'fifth cell' of *P. terraenovae* responded to some substances that humans find bitter (Liscia and Solari, 2000). In addition to the electrophysiological results, Liscia and Solari showed that the fly preferred pure sucrose solution to mixtures of sucrose plus quinine or amiloride. These results might easily lead to the conclusion that the 'fifth cell' of the fly has a functional analogy to the bitter taste receptor cell of humans. In *P. regina*, however, it has been reported that alkaloids inhibit the responsiveness of the sugar receptor cell (Dethier and Bowdan, 1989, 1992). Recently, Sadakata *et al.* discussed the inhibitory effects of amiloride on the sugar receptor cell of the fleshfly, *Boettcherisca peregria*, but did not show any response to amiloride itself (Sadakata *et al.*, 2002). If substances that taste bitter to humans have inhibitory effects on the sugar receptor cell in *P. terraenovae*, such inhibitory effects could explain the behavioral results of Liscia and Solari (Liscia and Solari, 2000), independently of the 'fifth cell' activity. In order to indicate a direct contribution of the 'fifth cell' activity to insect behavior, it is necessary to evaluate the behavior induced by the electrophysiological activity of the 'fifth cell'.

In our study, the toxicity of D-limonene and other monoterpenes (Figure 1) and their gustatory effectiveness estimated by the aversive PER test (Figure 3) showed the same order. Therefore, it seemed convenient to use D-limonene and other monoterpenes as warning taste stimuli, which would activate the 'fifth cell'. However, monoterpenes purified as essential oils were not directly applicable for the electrophysiological tip-recording method. The sidewall recording method (Morita and Yamashita, 1955) would have been an alternative, but the aversive movement of the labellum, which often occurrs even in the isolated head preparation, prevented us from inserting an electrode into the sidewall of a fine sensillum and from keeping it stable during impulse recording. Thus, we attempted the tip-recording method, in which monoterpene-containing electrolytes were used as the stimuli, and carried out the behavioral tests with the same stimuli. Such stimuli evoked the impulses of the water receptor cell as well (Figures 5A top and 6A). However, it is thought that it is not the impulses of the water receptor cell, but rather those of the 'fifth cell' that trigger the aversive reactions, because the impulses of the water receptor cell by themselves did not induce any aversive reactions from the fly.

If the activation of the 'fifth cell' triggers the aversive PER, the electrophysiological latency should be shorter than the behavioral latency (Dethier, 1968). Inspecting the impulse recordings to D-limonene-containing solutions in detail, 2–15 s latencies are seen. Sometimes, the aversive PER occurred later than 20 s after the beginning of stimulation. However, there was some overlap and we could not measure the electrophysiological and the behavioral latencies in the same animal at the same time. As for concentration dependency, there is little inconsistency between the electrophysiological activity of the 'fifth cell' and the aversive reaction of the fly (Figure 7). Thus, all of our results tend to suggest that the 'fifth cell' is a warning cell to induce aversive reaction from the fly.

Nevertheless, aversive behavior is not always induced when the 'fifth cell' generates impulses. Dethier (Dethier, 1976) and Ozaki *et al*. (Ozaki *et al*., 1995) observed that apple juice stimulation of the taste sensillum of *P. regina* elicited the impulses of both the sugar and the 'fifth cell'. In the case of apple juice, the fly did not show the aversive PER but the feeding PER. Unknown components of apple juice, which stimulate the 'fifth cell', may have weak toxicity. As has been discussed in the mammalian bitter taste receptor cell (Garcia and Hankins, 1975), the 'fifth cell' of *P. regina* might also respond to potentially toxic substances, even if they are not really toxic. This seems reasonable, considering the relationship between deterrence and toxicity of plant secondary compounds for phytophagous insects (Bernays, 1991). Monoterpenes, some of which were used in the present work, belong to one of the biggest groups of secondary metabolites of plants. Plants use the toxicities of these compound to defend themselves against herbivores, but some insects counter such toxins by evolving detoxification systems to protect themselves. Thus, the sensitivity of a contact-chemoreceptor neuron to phagostimulants may be diverse (Bernays and Chapman, 2000).

In fact, the 'fifth cell' responds to quite different structures of molecules, the toxicities of which have not been investigated in detail. This is one of the main reasons why the 'fifth cell' has not been given a name according to its function. Considering the study of Liscia and Solari (Liscia and Solari, 2000) and the present work together, however, we can now define this insect taste receptor cell as a deterrent cell, or more precisely a warning cell for gustatory suspicion of toxicity. It is noticed that this cell is functionally analogous to the mammalian bitter taste receptor cell, although all substances bitter for humans or other mammals may not be recognized as deterrents in insects.

CRLBP, an odorant-binding protein in the taste system and its role

In 1995, a unique type of OBP, called CRLBP, was found by Ozaki *et al.* to be widespread in the olfactory and taste organs of *P. regina*. Their findings were reminiscent of Dethier's observation (Dethier, 1972) that the 'fifth cell' of *P. regina* responds to some vapors, and that the 'fifth cell' could be involved in the recognition of volatile, more generally lipophilic substances. Odor perception by contact-chemoreceptor neurons reported in other insects (Städler and Hanson, 1975; Städler, 1984) may also involve lipophilic substance transport by CRLBP or its relatives.

Prior to the discovery of CRLBP, Schmale *et al*. (Schmale *et al*., 1990) and Bläker *et al*. (Bläker *et al*., 1993) reported a gustatory homolog of mammalian OBP, von Ebner's gland protein (VEGP), and predicted that VEGP could be a carrier protein for some lipophilic, probably bitter substances. Considering the role of the 'fifth cell' discussed in the present paper, CRLBP seems to be functionally analogous to VEGP, although mammalian and insect OBPs have quite different structures. VEGP has a lipophilic inside pocket of β-barrel type but CRLBP does not. Because VEGP could not be obtained commercially, we examined BLG, which is also a mammalian lipophilic ligand-binding protein that has a β-barrel type inside pocket like VEGP. However, we found that BLG, which hardly solubilized D-limonene, did not replace CRLBP (Figure 4). The ligand-binding spectrum of BLG may be different from that of CRLBP, probably because of their different structures. Presumably, mammalian and insect OBP families convergently appeared in different phylogenetic branches of animal evolution, so that they have different ligand-binding spectra and are not functionally compatible with each other.

As shown in Figure 4, 20 μM BSA solubilized 30.1 ± 1.5 μg/ml D-limonene, while 1.25 μM CRLBP solubilized 23.5 ± 3.4 μg/ml. Nevertheless, D-limonene with 20 μM BSA did not elicit aversive PER as effectively as with 1.25 μM CRLBP. Possibly BSA would not pass D-limonene to the receptor molecule on the 'fifth cell' as perfectly as CRLBP, the lipophilic ligand carrier in the taste and the olfactory organs of *P. regina* (Ozaki *et al.*, 1995). When comparing the data of Figure 4 and 7, we find that 4% DMSO consistently solubilized ~90 μg/ml of D-limonene and elicited ~70% aversive PER. However, there is a quantitative inconsistency on the effect of CRLBP; Figure 4 shows that 5 μM CRLBP solubilized ~158 ± 19 μg/ml of D-limonene to elicit 45% aversive PER; Figure 7 shows that 100 μg/ml D-limonene solubilized with the same amount of CRLBP elicited 85% aversive PER. The most likely explanation for this inconsistency is structural and/or functional differences in the purified CRLBP, as we used different batches in the two experiments.

It was reported that the CRLBP is a major protein component in the labellar contact-chemosensilla in *P. regina* (Ozaki *et al.*, 1995), but we do not exactly know its concentration in the sensilla. Considering that the concentration of the PBP of *Antheraea polyphemus* was measured to be 10 mM in the olfactory sensilla (Klein *et al.*, 1986; Kaissling, 1998), we can roughly estimate the intrinsic concentration of the CRLBP to be in the same range. The CRLBP concentrations used in this study were 1000 times lower, because of technical difficulties for large-scale purification of CRLBP from the fly tissue. If we could use the intrinsic concentration of CRLBP, the gustatory sensitivity of the 'fifth cell' and the aversive PER sensitivity of the fly might be increased and/or the electrophysiological and the behavioral latencies might be shortened.

The results of the present study suggest that the taste receptor molecules of the 'fifth cell' to which CRLBP passes its ligands might resemble olfactory receptors. In insect olfactory sensilla, the ligand-solubilizing effect of OBPs has been electrophysiologically demonstrated by Van den Berg and Ziegelberger (Van den Berg and Ziegelberger, 1991), and recently a contribution of OBPs to receptor specificity has been shown in addition (Pophof, 2002). Our study in

Phormia is the first electrophysiological study on the role of an OBP in an insect taste sensillum.

Which receptor molecules on the 'fifth cell' can receive a ligand from CRLBP? How does the CRLBP bind or release the ligand for the receptor molecule? In order to answer these questions and understand the warning mechanism of the 'fifth cell', especially for lipophilic toxins, the ligand-binding spectrum of CRLBP and its carrier-receptor relation should precisely be investigated.

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