Peripheral Coding of Bitter Taste in *Drosophila*

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Received 12 October 12, 2002; accepted 11 February 2003

ABSTRACT: Taste receptors play a crucial role in detecting the presence of bitter compounds such as alkaloids, and help to prevent the ingestion of toxic food. In *Drosophila*, we show for the first time that several taste sensilla on the prothoracic legs detect bitter compounds both through the activation of specific taste neurons but also through inhibition of taste neurons activated by sugars and water. Each sensillum usually houses a cluster of four taste neurons classified according to their best stimulus (S for sugar, W for Water, L1 and L2 for salts). Using a new statistical approach based on the analysis of interspike intervals, we show that bitter compounds activate the L2 cell. Bitter-activated L2 cells were excited with a latency of at least 50 ms. Their sensitivity to bitter compounds was different between sensilla, suggesting that specific receptors to bitter compounds are differentially expressed among L2 cells. When presented in mixtures, bitter compounds inhibited the responses of S and W, but not the L1 cell. The inhibition was effective even in sensilla where bitter compounds did not activate the L2 cell, indicating that bitter compounds directly interact with the S and W cells. Interestingly, this inhibition occurred with latencies similar to the excitation of bitter-activated L2 cells. It suggests that the inhibition in the W and S cells shares similar transduction pathways with the excitation in the L2 cells. Combined with molecular approaches, the results presented here should provide a physiological basis to understand how bitter compounds are detected and discriminated. © 2003 Wiley Periodicals, Inc. J Neurobiol 56: 139–152, 2003

Keywords: **taste neurons; bitter compounds; *Drosophila;* electrophysiology; spikes sorting**

INTRODUCTION

Many toxic compounds are reported to taste bitter in humans, and are avoided by many animals, which have developed specialized cells to detect them (Glendinning, 1994). Recently, a family of G-protein-coupled receptors for bitter molecules has been identified in mammals (Adler et al., 2000) and ligands have been found for some of them (Chandrashekar et al., 2000). Although a family of putative taste receptors has been recently identified in *Drosophila*, their function is still largely unknown (Adler et al., 2000; Clyne et al., 2000; Dahanukar et al., 2001; Scott et al., 2001). Some of these genes might encode for receptors tuned to aversive molecules, but the presence of taste neurons specifically excited by bitter compounds was not reported in *Drosophila*. This is not the case for herbivorous insects such as *Lepidoptera*, which possess taste neurons activated by compounds involved in feeding inhibition (Glendinning and Hills, 1997) called "deterrent cells" (Schoonhoven and van Loon, 2002).

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Contract grant sponsor: the French Ministry of Education and Research and by the French Ministry of Foreign Affairs through a Lavoisier program grant delivered by EGIDE (to N.M.).

Contract grant sponsor: a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.T.).

DOI 10.1002/neu.10235

Does *Drosophila* use such specialized cells to detect bitter compounds? Taste sensilla on the proboscis and the tarsal segments of *Drosophila* were found to play a role in determining feeding choice, and some of them have been electrophysiologically characterized (Fujishiro et al., 1984; Wieczoreck and Wolff; 1989; Meunier et al., 2000). Each sensillum houses four taste neurons, which have been named according to their sensitivity to different compounds: S cell for sugars, W cell for water, L1 cell for salts at low concentration, and L2 at high concentration (Singh, 1997). Behavioral observations on the repulsive effect of quinine (Tompkins et al., 1979; Fresquet et al., 1998) and the recent localization in taste sensilla of PBPRP2, a protein that could be a carrier of bitter tastants (Shanbhag et al., 2001), suggest that some sensilla house a neuron excited by bitter substances. However, no physiological data are yet available to support the presence of such bitter-activated neurons in *Drosophila*. In fact, bitter compounds are thought to have an effect only by inhibiting the response to sugars (Morita and Yamashita, 1959; Siddiqi and Rodrigues, 1980). However, the presence of bitter-detecting neurons might have been overlooked because extracellular recordings in *Drosophila* taste sensilla are notoriously difficult to analyze (Fujishiro et al., 1984; Meunier et al., 2000).

In this work, we have evaluated how *Drosophila* detects bitter compounds known to be active on other insects or on humans. Most of these compounds were found to exert a repellent effect on feeding choice behavior in flies when presented in mixture with sugars. We further show that the proboscis extension reflex elicited by a sugar solution stimulating one leg can be inhibited by bitter compounds stimulating the contra lateral leg. This suggests that bitter compounds are detected via a neural pathway different from the detection of sugars. Using electrophysiological methods, we located sensilla housing a neuron responding to these bitter compounds. This neuron was identified as the L2 cell, using a new approach based on the analysis of inter spike intervals. In addition to this excitatory pathway, we found that bitter compounds inhibit the W and S cells, but not the L1 cell. These results open the way to a better understanding of the molecular events underlying bitter taste, thanks to the genetic tools available for *Drosophila*.

MATERIAL AND METHODS

Chemicals

Sucrose, fructose, NaCl, KCl, caffeine, aristolochic acid, and denatonium benzoate were purchased from Sigma-Aldrich Co.; quinine hydrochloride from Tokyo Kasei Chemicals Co; strychnine nitrate, salicin, and berberine sulfate trihydrate from Wako Pure Chemical Industries, Ltd. Solutions were prepared in advance and stored at −20°C. All solutions were prepared as dilutions in 1 m*M* KCl and kept at 4°C for less than 1 week.

Flies

Stocks (*Drosophila melanogaster,* Canton-S) were maintained at 25°C on a standard cornmeal agar medium. Flies aged 2 to 5 days were fed on fresh medium for at least 3 h before experiments.

Behavioral Tests

All behavioral tests were done at 24–25°C.

Behavioral Effects of Bitter Compounds on Feeding. A two-choice preference test was performed as described earlier (Tanimura et al., 1982). Briefly, flies were starved for 20 h, but supplied with water-soaked Kimwipe paper in vials. Flies were introduced into microtest plates equipped with a lid (Nalge Numc) whose wells were alternately filled with two kinds of sugar–agar solutions, each colored with blue or red food coloring. Food coloring at concentrations used have no effect on taste sensitivities and are nontoxic to flies. After 1 h in the dark, the color of fly abdomens was observed under a compound microscope. Flies were offered a choice between wells filled with either 25 m*M* of fructose (blue colored) or 35 m*M* fructose mixed with varying concentrations of a bitter compound (red colored). A preference index (PI) was defined here as the percentage of flies preferring the 35 m*M* fructose side, $(R+M/2)/(B+R+M) \times 100$, where B, R, and M represent the number of flies colored blue, red, and mixed, respectively. Classification was made based on standard mix solutions of the two food dyes. Flies classified as M ingest more than 30% of one colored solution over the other color. The proportion of the mixed category is usually below 20% when PI value is around 50. This test was used to study the repellent effect of quinine, strychnine, caffeine, berberine, salicin, aristolochic acid, and denatonium. For each concentration (ranging from 0.001 to 10 m*M*, depending on the compound), the test was performed on at least six groups of about 50 flies. Using this test, flies have to feed for one hour so that enough amount of colored food is present in the crop of flies. We checked the amount of dye intake overtime for the two-choices test using quinine; 10, 20, 30, 60, 90, and 120 min after flies were introduced into the microtest plates (data not shown). The amount of intake was linear during this period excluding any postingestive effect for this bitter compound.

Tarsal Detection of Bitter Compounds. Flies tested for the proboscis extension reflex were prepared as previously described (Kimura et al., 1986). Flies aged 0–2 day after emergence were maintained on fresh medium for 1 day. Flies were starved for 18–20 h, but supplied with water-

soaked Kimwipe paper in vials. Fixed male flies were then placed in a humidified chamber for 2 h. Before the test, flies were satiated with water. Intervals between stimulations were more than 2 min, to minimize adaptation. First, a drop of either 0.1 m*M* berberine or water was put in contact with a leg and the contact was maintained. As soon as possible (within 0.5 s), a second drop of 250 m*M* sucrose solution was put in contact for less than 1 s with the contralateral leg. We recorded then if this stimulation evoked the extension of the proboscis. The test was performed with at least 10 flies and repeated seven times.

Electrophysiology

Taste Neuron Recording Technique. A decapitated fly was secured to a flat support with insect pins and tape, and electrically grounded via a glass capillary filled with Ringer's solution inserted into the abdomen. To stimulate a sensillum, we covered its tip for less than 2 s with a recording electrode containing both an electrolyte (1 m*M* KCl) and the stimulus (Hodgson et al., 1955). Consecutive stimuli were applied at least 1 min apart to avoid adaptation. Sensilla used in this study are located symmetrically (Fig. 3) and present on both legs. Thus, we could record from four homologous sensilla per preparation, introducing pseudoreplicates. On average, two sensilla 5b, f3b, or f2b were recorded on the same fly while only one sensillum 5s and 4s, was recorded per fly. However, we make sure that data were sampled at least on four different flies. The recording electrode (a glass capillary with a tip diameter of 20 μm) was connected to a TastePROBE amplifier (Marion-Poll and Van der Pers, 1996). The electric signals were amplified and filtered (CyberAmp 320, Axon Instrument, USA; gain: 1000; eighth order Bessel pass-band filter: 1Hz–2800 Hz). Contacting a taste hair with the stimulus electrode triggered data acquisition and storage on a disk (sampling rate 10 kHz, 12 bits; DT2821 Data Translation). These data were then analyzed using Awave (Marion-Poll, 1996). Spikes were detected and analyzed using software interactive procedures of custom-made software dbWave. Spikes originating from the W cell were sorted on the basis of their large amplitude. S and L2 cells, L1 and L2 cells cannot be sorted accurately, whereas W and S cells or W and L2 cells can be easily sorted. Unless otherwise indicated, we evaluated the action-potential frequency by counting spikes during the first second of recording.

Identification of Sensilla Activated by Bitter Compounds. Each taste sensillum from male and female of the last four tarsal segments was screened for an increase of its firing rate in response to aristolochic acid, berberine, denatonium, quinine, strychnine, caffeine, and salicin. The concentrations ranged from 0.01 to 10 m*M*, except for caffeine and salicin, which could be dissolved at up to 100 m*M*. At least four recordings from different flies were performed on each sensillum for each compound and the presence of a response was checked on the oscilloscope.

Sensitivity to Bitter Compounds and Inhibition of W Cell. Each stimulation protocol started with 1 m*M* KCl as a reference for the W cell activity, and was followed by increasing concentrations of a single bitter compound. We recorded responses to berberine, denatonium, quinine, strychnine, and caffeine at concentrations ranging from 0.01 to 10 m*M*, except for caffeine and salicin (up to 100 m*M*). At least nine recordings on at least seven flies were performed for each concentration and each compound tested. In all sensilla responding to bitter compounds, one neuron was consistently responding to water (W cell). Spikes originating from the W cell were easy to separate from the spikes generated by other neurons because of their large amplitude and regularity of firing (Fig. 4). Each stimulation protocol thus allowed us to generate two dose–response curves—one for the neuron activated by bitter compounds and the other for the inhibition of the W cell.

Inhibition of Sugar and Salt Cells. To evaluate the inhibitory effect of bitter compounds on the responses of the S cell, we used 50 m*M* sucrose (used as a reference for the S cell activity), subsequently mixed with increasing concentrations of a bitter compound (aristolochic acid, berberine, denatonium, quinine, strychnine, caffeine, or salicin). We recorded from sensilla 5b (present in males and females) and from sensilla f3b, f2b (present only in females; see Fig. 3 for nomenclature). At least six recordings on at least four flies were performed for each concentration.

To evaluate the inhibitory effects of bitter compounds on the responses of the L1 cell, we stimulated sensilla 5b with 400 m*M* NaCl (used as a reference for the L1 cell activity), subsequently mixed with quinine ranging from 0.01 to 1 m*M* or berberine at 1 m*M*. At least seven recordings were performed for each concentration.

Long-Term Inhibition of S Cell. We checked if bitter compounds had a damaging effect on sensilla 5b ($n = 8$ flies). After a first stimulation with 50 m*M* sucrose, we covered the sensillum with an electrode filled with 5 m*M* quinine for 10 s. This sensillum was subsequently stimulated with 50 mM sucrose 1, 3, 5, 7, 10, 15, 20, 30, and 40 min after the treatment with quinine. The resulting responses were expressed in percent with respect to the response to the first stimulation.

Identification of the Neuron Responding to Bitter Compounds. We recorded responses from sensilla 5b stimulated successively with 0.1 m*M* quinine, 400 m*M* NaCl, and a mixture of both on six flies ($n = 10$). This allowed us to identify the neuron responding to quinine by counting the total frequency of spikes and the ratio of interspike intervals shorter than 4 ms generated during the 0.2–1 s intervals beginning after onset of the stimulation (see Appendix for explanations).

RESULTS

Behavioral Effects of Bitter Compounds on Feeding

Flies were allowed to choose between 25 m*M* fructose and 35 m*M* fructose containing a varying concentration of a bitter compound. In the absence of such compounds, most flies preferred 35 m*M* fructose over 25 m*M* fructose. A high PI indicates that flies preferred the well containing the higher concentration of sugar mixed with bitter compounds to the lower concentration of sugar alone, i.e., that the compound has no or a low inhibitory effect. A PI value of 50% (PI_{50}) indicates that flies have no preference for one of the two wells, i.e., a decrease of the phagostimulatory effect from 35 m*M* fructose by the bitter compound. A low PI indicates that the compound added was deterrent for flies [Fig. 1(A)]. The PI_{50} measured on the curves of Figure 1(A) are reported in Table 1. They ranged from 0.01 m*M* (berberine) to 3 m*M* (caffeine). Some bitter compounds classically active on other insects or humans did not elicit any repellency even at very high concentrations (100 m*M* salicin, 10 m*M* aristolochic acid, both not shown).

Tarsal Detection of Bitter Compounds

The effects of bitter compounds in the two-choice preference test could be due to an inhibition of the responses to sugar but also to the excitation of bitter-activated neurons. We designed a test to check this second hypothesis. One leg of a fly was contacted with 250 m*M* sucrose, while the contralateral leg was already in contact with 0.1 m*M* berberine or water. The proboscis extension reflex induced by the sucrose solution was clearly inhibited (Fig. 2). It indicates that berberine was detected by itself rather than by solely inhibiting the detection of sugars.

Properties of Neurons Activated by Bitter Compounds

To find sensilla housing neurons activated by bitter compounds, we investigated a population of sensilla located on the last four tarsal segments of prothoracic legs of females (18 sensilla) and males (28 sensilla). Only three pairs of sensilla housed a neuron activated by bitter compounds at different concentrations. These sensilla, present in both sexes, are located symmetrically on the two terminal tarsal segments (sensilla 5b, 5s, and 4s; see Fig. 3). Sensillum 5b was previously described in our previous article (Meunier et al., 2000). The two others, 4s and 5s, had been overlooked because of their small size and orientation, which made them difficult to record from. Their sensitivity to sugars and salts was similar to the sensillum 5b (data not shown). However, their sensitivity to bitter compounds was different (Table 1): sensilla 5b and 4s responded to quinine but not to berberine (referred thereafter as quinine-activated sensilla),

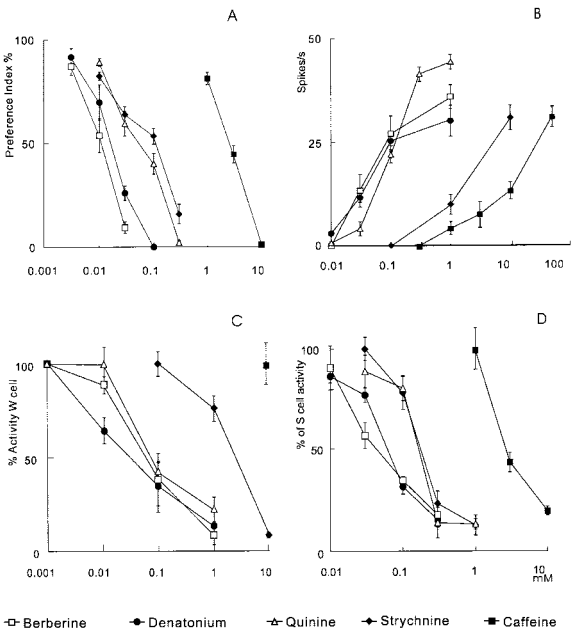

Figure 1 Behavioral and electrophysiological effects of bitter compounds. (A) Behavioral effects: two-choice preference tests were performed between 25 and 35 m*M* fructose mixed with a bitter compound. If flies detect and avoid the bitter compound, they switch their preference from 35 m*M* fructose and start consuming 25 m*M* fructose, thus lowering the value of the Preference Index. Most active bitter compounds were found effective at a micromolar range. At least six tests involving about 50 flies were done for each concentration. Error bars mean ± S.E.M. (B) Electrophysiological responses to bitter compounds of tarsal taste sensilla. All bitter compounds active in behavior were detected by a subset of sensilla (see Table 1 for correlation, $n > 9$, error bars mean ± S.E.M). (C) Inhibition of the firing of the W cell by bitter compounds. Recordings were performed on quinine-activated sensilla ($n > 9$, error bars mean ± S.E.M.). Berberine, which did not elicit any excitation in these sensilla, inhibited the activity of the W cell. Caffeine showed no inhibition even at 100 m*M*, so these data are not included. (D) Inhibition of the response to 50 m*M* sucrose from the S cell by bitter compounds. Sensilla used in this experiment were sensitive to sugar but do not house a neuron activated by bitter compounds (f2b, f3b sensilla on Canton S; $n > 6$, error bars mean ± S.E.M.). Inhibition and behavior curves followed a parallel course. All experiments were conducted on both males and females except (C), where it was only on females.

Table 1 Behavioral and Electrophysiological Evaluation of Sensitivity to Bitter Compounds

Compound	Two-Choice Test[a] Pl_{50}	Activation of L2 Cell[b]	Cl_{50}[c] W	Cl_{50}[c] S
Berberine	0.01	0.05 (*5s*)	0.1	0.04
Denatonium	0.02	0.05 (*5b/5s/4s*)	0.05	0.06
Quinine	0.1	0.1 (*5b/4s*)	0.2	0.2
Strychnine	0.2	3 (*5b/5s/4s*)	3	0.2
Caffeine	3	13 (*5s*)	no	3
Aristolochic acid	no	no	no	no
Salicin	no	no	no	no

All concentrations are in m*M* and "no" indicates no effect.

[a] Concentration of bitter compounds eliciting equal preference between 25 and 35 m*M* fructose mixed with bitter compounds in two-choice preference test.

[b] Concentration of bitter compounds eliciting 20 spikes/s.

[c] Concentration of bitter compounds inhibiting 50% of W and S cell activity.

whereas sensilla 5s responded to berberine but not to quinine (berberine-activated). Denatonium and strychnine were found to be active on both types of sensilla (Table 1). The lowest concentration of bitter compounds activating a taste neuron ranged from 0.01 m*M* for berberine to 1 m*M* for caffeine [Fig. 1(B)]. We expressed their relative effectiveness by extrapolating the concentration eliciting 20 spikes/s from the dose–response curves displayed in Figure 1(B) (Table 1).

The temporal pattern of responses of the neuron activated by bitter compounds was different from those obtained with neurons responding to sugars, water, or salts (Fujishiro et al., 1984; Meunier et al., 2000). With these compounds, responses start immediately with a burst of spikes followed by adaptation.

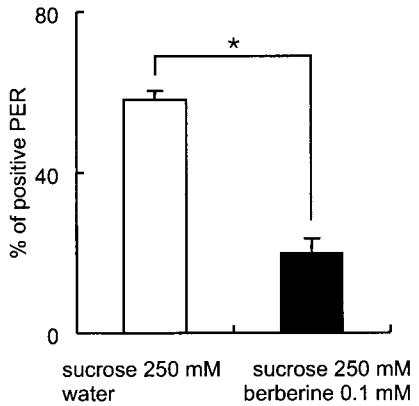

Figure 2 Dual stimulation PER (Proboscis Extension Reflex). One leg was stimulated with either water or 0.1 m*M* berberine while the other leg was stimulated with 250 m*M* sucrose ($n = 7$, error bars mean ± S.E.M.). The stimulation with berberine clearly reduced the positive response to sucrose ($*t_{Student} = 8.86$, $p < 0.001$, $df = 10$), indicating that legs bear taste receptors for bitter compounds.

An example of these responses can be seen in Figure 4 for the stimulation with 10^{-5} *M* of bitter compounds when looking at the firing of the W cell only. With

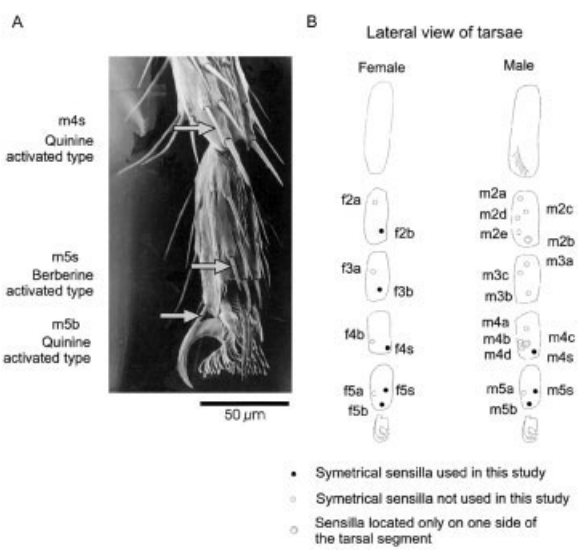

Figure 3 (left) Terminal two segments of a male prothoracic leg. Taste chemosensilla labeled by an arrow on the picture house a neuron activated by bitter compounds. Scale bar: 50 µm. (right) Localization and labeling of tarsal taste sensilla on the last four segments of the prothoracic leg. Sensilla are coded here by three letters describing the sex (f = female, m = male), the tarsal segment number (1 to 5, i.e., from proximal to distal) and their approximate location within a segment (alphabet order from apical to caudal). Two short sensilla were not described previously and are labeled "s," which designs a single short sensillum (20 µm vs. 40 µm for others) present on tarsal segment 4 and 5. We extended the notation used for females to males, by keeping the same suffix for homologous sensilla (f2b and m2b; f3b and m3b). Because sensilla f4s and m4s; f5s and m5s; f5b and m5b display same sensitivity to all compounds tested, we referred them as 4s, 5s, and 5b sensilla in the text.

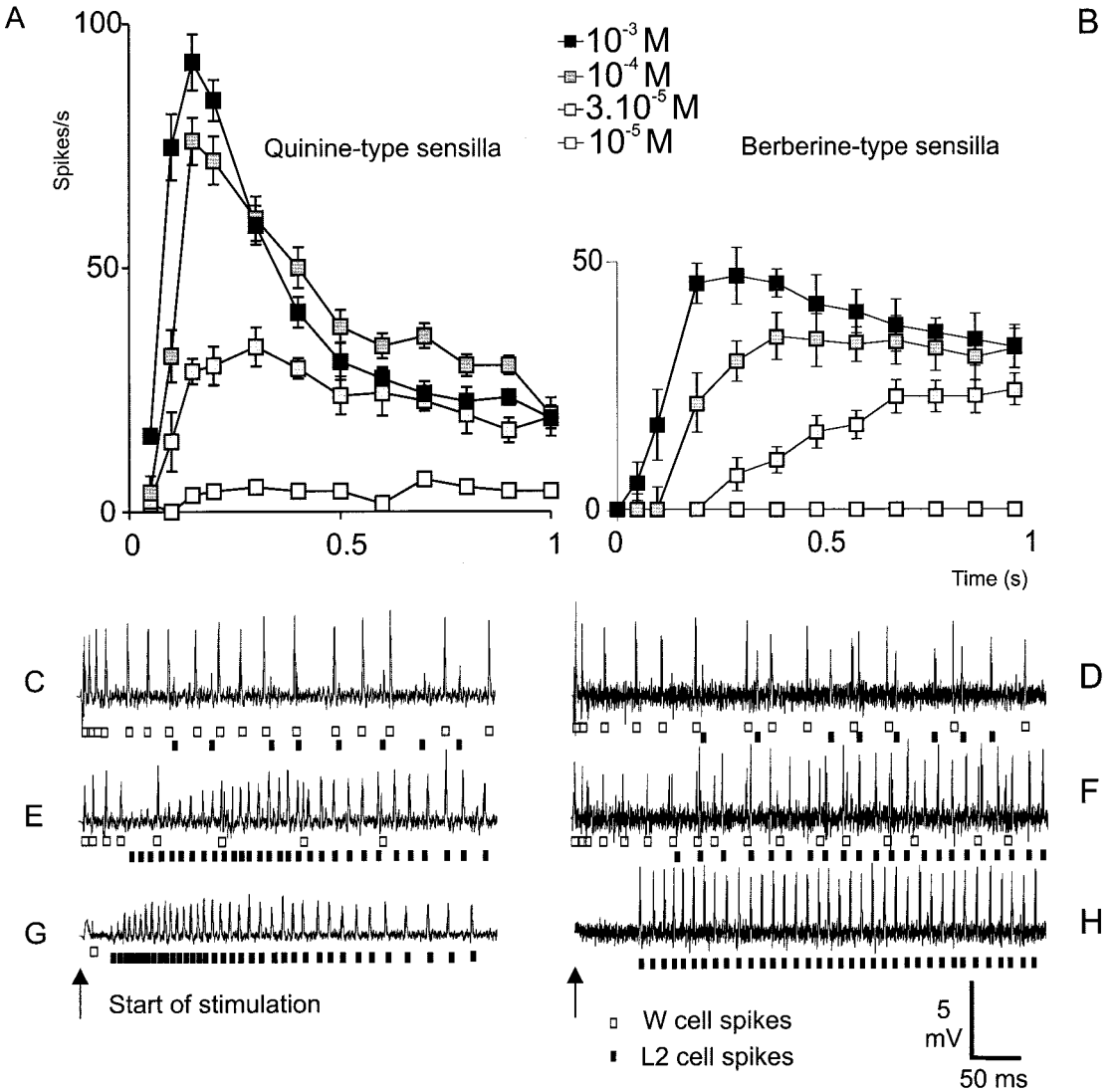

Figure 4 Temporal firing patterns of neurons activated by quinine (left column) and berberine (right column). (A,B) Poststimulus histogram during stimulation on sensilla responding to one of these two compounds but not to the other. Quinine- and berberine-activated sensilla show different time courses of adaptation. (C–H) Typical responses to increasing concentrations of quinine and berberine [(C,D): 10^{-5} *M*, (E,F): 10^{-4} *M*, (G,H): 10^{-3} *M*]. Unlike S, L1, and W cells that fire immediately after contact with a burst of spikes (see the response of the W cell at 10^{-5} *M*), these neurons respond after a variable latency that shortens at higher concentrations.

bitter compounds, we always observed a latency of at least 50 to 200 ms between the onset of the stimulation and the beginning of the discharge (Fig. 4, 10^{-3} *M*). Furthermore, the firing frequency first increased before adaptation occurred. At higher concentration of bitter compounds, this latency shortened and the response peak occurred sooner (Fig. 4).

Berberine- and quinine-activated sensilla differed in other respects than their selectivity. First, the responses of the quinine-activated sensilla to bitter compounds exhibited a shorter rise time than those of berberine-activated sensilla (Fig. 4). Second, the quinine-activated sensilla responded to bitter compounds with a faster adaptation rate than berberine-activated sensilla at all concentrations. For example, quinine-activated sensilla were adapted to 50% of the peak action potential frequency after 400 ms with quinine 0.1 m*M*, while less than 30% of adaptation was reached in berberine-activated sensilla at the same concentration after 1 s (Fig. 4).

Effects of Bitter Compounds on W Cell

This neuron is active when stimulated with water and is inhibited by an increase of osmolarity (Evans and Mellon, 1962). All behaviorally active bitter compounds inhibited the neuron activated by water with the exception of caffeine [Fig. 1(C)]. The study of inhibition was performed on the quinine-activated sensilla that also house a neuron activated by water (Meunier et al., 2000). Interestingly, although berberine was not eliciting spikes from any neuron in quinine-activated sensilla, it did inhibit the activity of the W cell in a dose-dependent way. A few spikes from the W cell usually remained in the very beginning of the stimulation even at high concentration of bitter compounds (Fig. 4).

Effects of Bitter Compounds on S Cell

We checked if the bitter compounds also inhibited firing of the S cell. Spikes fired by the neurons responding to sugars and bitter compounds were of similar amplitude and we were unable to sort them accurately.

For this reason, we first used sensilla f3b and f2b, which house a neuron excited by sugars (Meunier et al., 2000) but not a bitter-activated one. For each bitter compound tested previously, we determined the concentration necessary to inhibit 50% (inhibitory concentration 50% IC_{50}) of the response to 50 m*M* sucrose [Fig. 1(C)]. At high concentration of all bitter compounds tested (Fig. 5), we were still able to detect spikes of the S cell. Interestingly, these spikes occurred always during the first 50 ms of stimulation. This matches the latency noted earlier, in the response of the bitter-activated neurons [Fig. 5(C)].

With 10 m*M* quinine, there was a clear inhibition of the response elicited by 100 m*M* sucrose on the quinine-activated sensilla. When the S cell stops firing, the neuron activated by bitter compounds starts to discharge [Fig. 5(D)]. In this case, we could thus see a correlation between the inhibition of the S cell and the activity elicited by bitter compounds.

Long-Term Inhibition of the S Cell by Bitter Compounds

Bitter compounds elicited erratic bursts of action potentials at concentrations higher than 1 m*M*, often with a latency of at least 1 s [Fig. 6(A)]. This kind of response was variable, present in all types of sensilla and obtained with quinine, denatonium, and strychnine but neither with berberine nor caffeine.

To examine possible damage to the taste neurons, we recorded the responses of sugar-sensitive sensilla to 50 m*M* sucrose before and after exposing these sensilla to 5 m*M* quinine during 10 s. Immediately after this treatment, the responses to sugar disappeared. The responses were progressively restored,

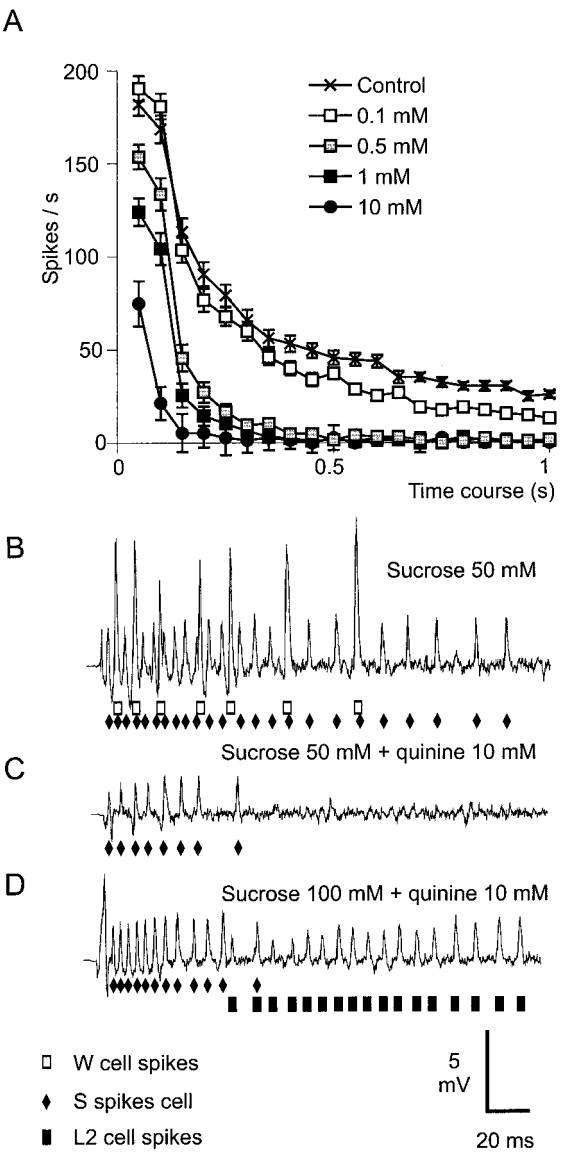

Figure 5 Effect of bitter compounds on the S cell. (A) Poststimulus histogram of a 1-s stimulation with 50 m*M* sucrose mixed with increasing concentrations of quinine. Even at the highest concentration, some spikes from the S cell were fired at the beginning of stimulation (sensilla f2b,f3b, $n > 6$, error bars mean ± S.EM.). (B,C) Typical recording from sensilla housing a neuron activated by sugar but not a bitter-activated one (sensilla f2b and f3b). The S cell is inhibited with delay. (D) Sample record from sensilla 5b housing a neuron activated by sugars and a quinine-activated one. Spiking activity of the S cell stops when the L2 cell starts firing, allowing to sort spikes.

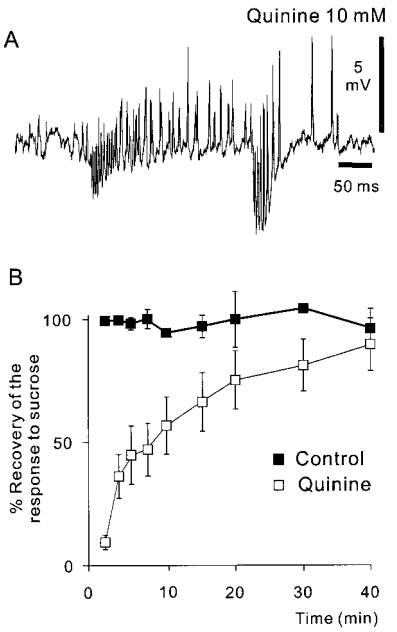

Figure 6 (A) Responses obtained with high concentrations of bitter compounds are characterized by an erratic activity, burst of spikes, and sudden baseline drops. Sample trace 1 s after the onset of stimulation (sensilla 5b). (B) Recovery of sugar response after treatment with quinine 5 m*M* for 10 s. Stimulation with 50 m*M* sucrose. Fifty percent recovery is reached after 8 min ($n = 8$, error bars mean ± S.E.M.).

following an exponential function [50% recovery was reached after 8 min: Fig. 6(B)].

Effects of Bitter Compounds on L1 Cell

To check if bitter compounds affected the response to salts, we used a mixture of different concentrations of quinine (or berberine) with 400 m*M* NaCl. This was evaluated on quinine-activated sensilla (which do not house a neuron activated by berberine). With quinine, the total number of spikes elicited was similar to the sum of those elicited by each compound separately (Fig. 7). We observe no difference in the response to NaCl 400 m*M* in the presence of berberine 1 m*M* (data not shown).

Origin of Spikes Elicited by Bitter Compounds

Because it was not possible to use only spike shapes to elucidate which neuron was activated by bitter compounds, we addressed this problem by looking at the temporal properties of the spike trains. When only one nerve neuron is active in a recording, consecutive spikes are separated by the silent period [Fig. 8(A); quinine 0.1 m*M*]. By silent period we mean a time interval following a spike during which a second spike is generated with zero or very small probability. If a second neuron is firing, both neurons can discharge independently, and thus interspike intervals (ISIs) shorter than the silent period can occur [Fig. 8(A); NaCl 400 m*M* + quinine 0.1 m*M*]. Then, all ISIs shorter than this period reflect firing from at least two neurons (in the following, we call these ISIs "doublets"). Using computer simulations, we showed that the proportion of doublets in a two-neuron recording depends on the firing frequency of each neuron. The curve giving the occurrence of the doublet frequency as a function of the respective neuron firing frequencies can be estimated and plotted (see Appendix and Fig. 9). This model allows one to estimate the respective firing frequencies of two neurons firing independently, knowing the total frequency of spikes present in the recordings and the proportion of ISIs shorter than the silent period. Thus, only the silent period value needs to be choosen. In all recordings

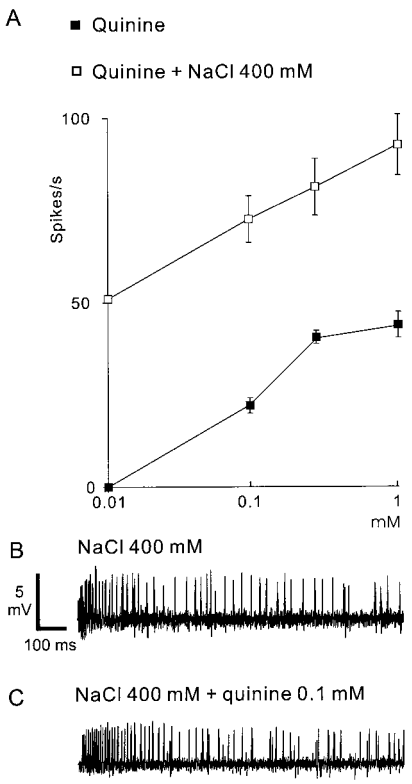

Figure 7 Effect of bitter compounds on the L1 cell. The sensilla used here were activated by quinine and salts (sensilla 4s and 5b). (A) Dose–response curves run parallel, indicating that quinine does not affect the response to salts (sensilla 5b, $n > 7$, error bars mean ± S.E.M.). (B,C) Sample record from sensilla 5b.

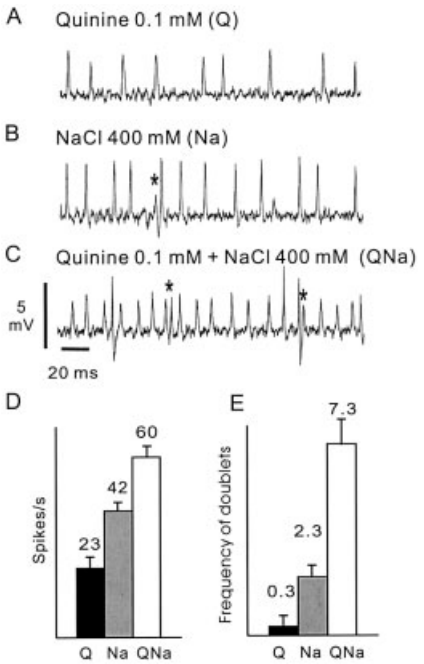

Figure 8 Interspike intervals (ISIs) shorter than the silent period were used to estimate the respective firing frequencies of two neurons and to identify which neuron responds to bitter compounds. (A,B,C) Sample records. Stars indicate ISIs lasting less than 4 ms. (D) Average spiking activity from 0.2 to 1 s after the onset of stimulation ($n = 10$, error bars mean ± S.E.M.). (E) Frequency of ISIs shorter than the silent period (4 ms), counted in the same time interval ($n = 10$, error bars mean ± S.E.M.). NaCl at 400 m*M* elicits mainly spikes from the L1 cell and only a few of the L2 cell. After addition of quinine, which elicits spikes either from the L1 or L2 cells, the percentage of ISIs lasting less than 4 ms almost triple. This indicates that quinine stimulates the L2 cell according to the model developed in Appendix.

used, the highest total frequency is 60 spikes/s (Table 2). This value correspond to a mean ISI of more than 16 ms. We choose 4 ms for the duration of the silent period because it is much lower than the mean ISI. Thus, even if we considered that all 60 spikes/s were coming from the same neuron, we should not observe a significant amount of doublets that are wrongly counted as coming from the firing of two different neurons. The model does not allow a direct assignment of the component frequencies to either L1 or L2. This is the reason why Table 2 gives only the frequencies for the least and most active neurons.

To determine which neuron was responding to the bitter compounds, we compared the responses of quinine-activated sensilla that also respond to salts (Meunier et al., 2000) to different stimuli: 400 m*M* NaCl, 0.1 m*M* quinine and a mixture of both. The total spike frequency and the proportion of ISI shorter than 4 ms (doublets) were measured. NaCl elicited 42 spikes/s and 2.3 doublets/s; according to the model one neuron fires eight spikes/s and the other 34 [see Fig. 8(B), 9(B), and Table 2]. Because NaCl at this concentration is known to stimulate mostly L1 and marginally L2 (Singh, 1997), the most active neuron

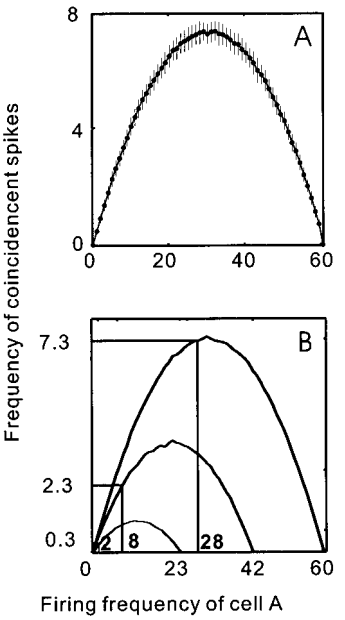

Figure 9 Computer simulation giving the number of doublets, i.e. spikes separated by an interval shorter than the silent period Δ, in the global recording with the same electrode of two neurons A and B located in the same sensillum. (A) Frequency of doublets per second d as a function of the firing frequency of one of the neurons (f_A of neuron A, for example) for a given overall firing frequency $f = f_A + f_B$,. Each neuron is modeled as a random generator firing spikes according to Poissonian or Gaussian processes with various standard deviations (σ_A and σ_B). In all cases the frequency of doublet spikes d is the same parabolic-like function of the firing frequency f_A. Thus, only the curve for one of the Gaussian processes is shown here, the other curves are superimposed. Parameters: $\Delta = 4$ ms, $f = 60$ spikes/s, $\sigma_A = f_A/2$, $\sigma_B = f_B/2$; curves are based on the mean of d in $n = 100$ simulated trains of 1000 spikes each (i.e., train durations of $1000/f$ s). Error bars mean ± S.E. The doublet frequency is maximum when the two neurons fire at the same rate and is minimum when only one neuron is firing. (B) Same as (A) for processes at various overall firing frequencies f simulating the responses to quinine 0.1 m*M* ($f = 23$, lower curve), NaCl 400 m*M* ($f = 42$, middle curve), quinine 0.1 m*M* + NaCl 400 m*M* ($f = 60$, upper curve). The experimentally obtained d values (0.3, 1.3, and 7.3 doublets/s) were projected on the corresponding curve to get the firing frequency of the least active neuron (the frequency of the other neuron is such that the sum of frequencies of both neurons is f). Parameters: $f = 23$, 42, and 60 spikes/s, $n = 100$.

Table 2 Estimated Value of the Firing Frequencies f_A and f_B of Two Undistinguishable Neurons Based on the Total Firing Frequency f and the Frequency of Doublets d (See Appendix)

Stimulus	f[a]	d[b]	f_A[c]	f_B[d]
400 m*M* NaCl	42	2.3	34	8
0.1 mM Quinine	23	0.3	21	2
Mixture	60	7.3	32	28

[a] Frequency (spikes/s) of both neurons A and B together.
[b] Frequency (doublets/s) of doublets in the record A+B.
[c] Frequency (spikes/s) of the most active neuron A according to Figure 9.
[d] Frequency (spikes/s) of the least active neuron $f_B = f - f_A$.

can be identified as L1 and the other as L2. According to the model, the high frequency of 7.3 doublets/s found with the mixture of 400 m*M* NaCl and quinine means that one neuron was firing at about 28 spikes/s and the other at 32 spikes/s. The most parsimonious interpretation of these rates is that quinine did not change the firing of L1 (which remained about 33 spikes/s) but instead increased the firing of L2 from 8 to 28 spikes/s. Thus, bitter compounds stimulate L2 cell. Finally, quinine alone gave only 0.3 doublets per second for a total of 23 spikes/s which, according to the model, can be analyzed as two spikes/s from one neuron (L1) and 21 spikes/s from the other (L2). If the action of NaCl and quinine on L2 were additive, one would expect $28 - 8 = 20$ spikes/s with quinine alone. This prediction is very close to the observed 21 spikes/s, indicating that the results obtained by the model are consistent.

DISCUSSION

A Limited Number of Chemosensilla House a L2 Cell Responding to Bitter Compounds

Among all sensilla that have been electrophysiologically characterized so far in *Drosophila* (Fujishiro et al., 1984; Wieczoreck and Wolff, 1989; Meunier et al., 2000; Hiroi et al., 2002), no bitter-activated neuron has been found yet. From the available evidences, it was commonly assumed that *Drosophila* could detect bitter substances mostly through inhibition of the responses to sugars (Tanimura and Kikuchi, 1972; Siddiqi and Rodrigues, 1980). However, recent molecular evidence indicate that taste neurons express a family of about 56 putative Gr taste receptors (Clyne et al., 2000; Kim and Carlson, 2002), expressed across different hairs (Dunipace et al., 2001; Scott et al., 2001). Thus, we can expect some taste bristles to have a different sensitivity than those already characterized and to be able to detect bitter compounds with specific neurons.

Accordingly, using a behavioral test based on a modified proboscis extension reflex, we have found that bitter compounds can be detected by tarsal taste sensilla, through a mechanism different from the inhibition of the sugar response. We characterized six sensilla housing a bitter-activated neuron on the tarsal segments. Using a novel approach based on the study of interspike intervals, we showed that these bitter-activated neurons belong to the L2 type. This result is consistent with earlier studies showing that L2 cells are activated by high concentrations of salts, which are also repellent for flies (Singh, 1997). These six sensilla are lateroventral, on the last two tarsal segment. They are thus in direct contact with the substratum when the fly stands or walks on a surface and are most likely involved in the early steps of food sensing and rejection behaviors.

Among all putative gustative receptors whose expression pattern has been published (Dunipace et al., 2001; Scott et al., 2001), the distribution of the expression of *Gr32a* (referred as *Gr32D1* in Scott et al., 2001) most closely matches the localization of bitter-activated L2 cells described in this study. *Gr32a* is expressed in the S type sensilla of the proboscis that are difficult to access for recordings (Hiroi et al., 2002) and in the six sensilla on the last two tarsae (Scott et al., 2001; T. Inoshita, personal communication) that we identified as housing a neuron activated by bitter compounds. Our physiological data provide a reference to analyze if the expression of this putative *Gr* is involved in the detection of bitter substances.

Relative Importance of the Effects of Bitter Compounds on Taste Neurons

In addition to the activation of the L2 cell, bitter compounds inhibit the activity of the S and W cells, but not of the L1 cell. Although S cell inhibition was observed in various insects such as *Diptera* (Dethier and Bowdan, 1992), *Lepidoptera* (Schoonhoven and van Loon, 2002), and even vertebrates (Formaker et al., 1997; Ogawa et al., 1997), it has not received a detailed attention. How do these effects contribute to the food rejection behavior? By using a differential stimulation with bitter compounds and sugar in the proboscis extension reflex, we showed that neurons activated by bitter compounds can counteract the activation of contralateral S cells by sugars. In addition, we found a good match between the range of bitter

compounds active on the behavior and on the bitter-activated L2 cells.

However, some discrepancies exist between the sensitivity range of these L2 cells and the behavioral effects (Table 1). For example, quinine and strychnine elicit 20 spikes/s of the L2 cell at different concentrations (0.1 and 3 m*M*, respectively), whereas the inhibition of feeding behavior in the two-choice test occurred within the same concentration range (0.1 and 0.2 m*M*, respectively). Furthermore, a very good match seems to exist between the behavioral tests and the S cell inhibition (Table 1): (1) the order of effectiveness of bitter compounds was identical in both cases; (2) the concentrations of the PI_{50} in two-choice test and the IC_{50} for S cell inhibition were similar. For example, the concentration of strychnine inhibiting feeding behavior (PI_{50} = 0.2 m*M*) is similar to that inhibiting S cell (IC_{50} = 0.2 m*M*) while L2 cells are less excited by strychnine (activation of 20 spikes/s at 3 m*M*) (Table 1); (3) only the five behaviorally active compounds inhibited the S cell. These data strongly indicate that both bitter-activated L2 cells and the inhibition of S cells contribute to the repellency of bitter compounds.

Damage Induced by High Concentration of Bitter Compounds

We observed an erratic baseline and irregular firing of action potentials following a contact with some bitter compounds at concentrations in the millimolar range. Comparable effects were described in the blowfly (Dethier and Bowdan, 1992) and in other insects (Schoonhoven and van Loon, 2002). We also observed that S cells became less responsive after such a contact. Previous studies of bitter taste transduction on vertebrates suggest that amphiphilic molecules like bitter compounds could directly interact with the transduction pathway of taste neurons by inhibiting a phosphodiesterase (Rosenzweig et al., 1999) or by activating G proteins (Naim et al., 1994). These authors found that for quinine, the EC_{50} (concentration giving half maximum activity) for potentiating GTPase activity of G proteins was 4 m*M*. This value is close to the concentration of quinine inducing an erratic firing and inhibiting for a long time the S cell in *Drosophila*. These observations suggest that the irregular bursting activity and the following inhibition of the S cell could be due to a direct action of some bitter compounds on the transduction pathway.

Possible Transduction Pathway for the Inhibition Caused by Bitter Compounds

As already shown in vertebrates (Chandrashekar et al., 2000), recent studies in *Lepidoptera* suggest the existence of distinct receptors to bitter compounds in insects (Glendinning and Hills, 1997; Glendinning et al., 2002). The fact that the sensitivity to bitter compounds is different among distinct sensilla (i.e., quinine- and berberine-activated types) supports the hypothesis of separate receptors in *Drosophila.* Are these putative receptors involved only in activation of the L2 cell, or could they also be involved in the inhibition of the W and S cells that we observed in *Drosophila*?

The W cell is generally considered as being unspecifically inhibited by an increase in osmolarity (usually by osmolarity higher than 0.1 osm/L; Evans and Mellon, 1962; Fujishiro et al., 1984). The IC_{50} of the W cell by bitter compounds was very low (2×10^{-4} *M* for quinine, i.e., 2.2×10^{-3} osm/L considering that all compounds tested were mixed with 1 m*M* KCl). This indicates that bitter compounds act specifically on the W cell. Amphiphilic molecules such as bitter compounds are known to cross the membrane. The simplest explanation for this inhibition is that such molecules modify the membrane properties of taste neurons unspecifically (Koyama and Kurihara, 1972). This explanation cannot be applied to *Drosophila* because (1) L1 cell sensitivity to salts was not modified by the presence of quinine, and (2) caffeine did not inhibit the W cell even at high concentration. Another hypothesis is that the W and S cells are laterally inhibited by the activity of the L2 cell. This does not hold true either, as we observed an inhibition of the W and S cells in sensilla in which no L2 cell was responding (sensilla f2b and f3b). The most likely explanation is that specific receptors for bitter compounds are also involved in the inhibition of these two neurons.

The latency of the L2 cell response is correlated with the latency preceding the W and S cell inhibition (Figs. 4 and 5). This is particularly striking when sensilla responding to sugars and bitter compounds were stimulated with a mixture of both [Fig. 5(C)]. In this case the inhibition of the S cell started at soon as the L2 cell was activated. These results suggest that L2, S, and W cells share a similar mechanism for detecting bitter compounds—one leading to the inhibition of the neuron activity, the other one to activation via a different transduction pathway.

Compared Encoding of Bitter Compounds between *Drosophila* and Other Models

In vertebrates, although some receptors and transduction pathways of bitter compounds were characterized

(Lindemann, 2001), the correlation of physiological data to behavior is made difficult by the complex organization of the taste system. Only few electrophysiological studies are available about taste receptor cells *in vivo* due to the difficulty of such recordings (Gilbertson et al., 2001).

In insects, taste organs are easier to access (Hodgson et al., 1955) and neurons activated by bitter compounds are subject to considerable interest because they are thought to play a key role in food-selection behavior, especially for insects feeding on plants (Glendinning et al., 2001). In *Lepidoptera*, the activity of such neurons is correlated with feeding inhibition and they were thus called "deterrent cells" (Schoonhoven and van Loon, 2002). Our results in *Drosophila* are consistent with data from *Lepidoptera*. In both cases, micromolar concentrations of bitter compounds elicit a characteristic response from such neurons, including an initial latency (50 to 200 ms), and they inhibit the S cell. Likewise, millimolar concentrations of bitter compounds elicit a bursting and erratic firing (Schoonhoven and van Loon, 2002). The convergence between the response pattern observed in *Drosophila* and *Lepidoptera* is surprising, considering the data recently presented on the blowfly (Liscia and Solari, 2000). In this case, the neuron responding to bitter compounds was activated at best ca. 15 spikes/s and discharged without the typical temporal pattern reported here and in *Lepidoptera*.

Interestingly, we found that bitter compounds inhibited the W cell in *Drosophila*, and that the inhibition of the S cell occurred at a lower threshold than the excitation of the bitter-activated neuron for some compounds. This indicates that inhibition may be an important part of bitter taste coding as recently suggested in the leech (Li et al., 2001). Because sophisticated molecular genetic techniques can be applied, *Drosophila* is a good model organism to investigate the molecular mechanism of chemicals senses. Coupled with the similar properties of bitter compounds sensitivity in other animal models, the results presented here should provide a good physiological basis for a better understanding of how bitter substances are detected and discriminated.

APPENDIX

Estimating Individual Spike Frequencies from Two Simultaneously Recorded Neurons

In this Appendix two neurons A and B, firing undistinguishable action potentials, recorded simultaneously from the same sensillum, are considered. The analysis is based on the doublets of spikes in the overall recorded spike train. Two spikes are forming a doublet when the time interval between them is less than the silent period. In this case, the first spike of the couple is known to come from one of the neuron and the second spike from the other neuron. We show below that the firing frequencies of the neurons, f_A and f_B, can be estimated knowing only their observed overall firing frequency $f = f_A + f_B$, and the number of intervals between spikes shorter than the silent period of the neurons (called here number of doublet d).

Model

Neuron A (respectively B) is assumed to fire spikes at random, according either to a Poisson process of mean interspike intervals $1/f_A$ (respect. $1/f_B$), or to a Gaussian process of same mean ISIs and standard deviation σ_A (respectively σ_B). The ISIs of the Poissonian model neuron are distributed according to an exponential distribution, with many short ISIs and a few long ones, and those of the Gaussian model neuron are distributed according to a bell-shaped normal distribution. The ISIs of a Poissonian neuron are purely random, whereas those of a Gaussian neuron are more regular, the degree of regularity depending on the choice of the standard deviation. By analogy with real neurons, σ_A (respectively σ_B) was chosen proportional to $1/f_A$ (respectively $1/f_B$) for Gaussian neurons. For Poissonian neuron, the standard-deviation is always equal to the square root of the mean (this is a property of the Poisson process). These two kinds of neurons were simulated by pseudorandom generators, whose outputs follow exponential and Gaussian distributions respectively, except that all generated intervals shorter than the silent period were removed. Then, two spike trains of the same kind (Poissonian or Gaussian) and the same duration were generated, one for each neuron, and the number of doublets per second and per spike d between them was determined.

The overall spike trains with mean frequency f can be produced by any combination of mean firing frequencies f_A and f_B. At one extreme $f_A = 0$ and $f_B = f$ (only neuron B is active), at the other extreme $f_A = f$ and $f_B = 0$ (only neuron A is active), all intermediate values with $f = f_A + f_B$ being possible. For this reason, d was plotted as a function of f_A ranging from 0 to f spikes per second (so, the firing of the other neuron is $f_B = f - f_A$). Four curves were drawn for each value of f_A: one describing a couple of Poissonian neurons and the three others describing a couple

of Gaussian neurons at three different standard deviations (see Fig. 9). To smooth out the effect of random fluctuations, 100 pairs of spike trains were generated for each set of parameter values, and as many values of d were counted.

RESULTS

Figure 9(A) shows the mean value of the frequency of doublet d as a function of f_A for $f = 60$ spikes/s:

1. For a given combined frequency f the number of doublets per second d is a parabolic-like function of the mean firing frequency f_A of neuron A. The maximum of d is reached for $f_A = f_B = f/2$ and its minima are $d(0) = d(f) = 0$.
2. The value of d at any f_A, depends on f but not of the spike distribution. We found almost the same values of d for the Poissonian and the three Gaussian distributions tested. This result suggests that the relation between the frequency of doublet and f_A for a given value of f is independent of the firing process of the model neuron.

Figure 9(B) shows the mean value of the frequency of doublets d versus f_A for $f = 23$, 42, and 60 spikes/s, which mimic the combined response of L1 and L2 cells for Quinine 0.1 m*M*, NaCl 400 m*M*, and Quinine 0.1 + NaCl 400 m*M*, respectively. These curves allow one to estimate the respective frequencies f_A and f_B by reporting the experimental value of d on each corresponding curve. The estimates obtained are given in Table 2 for the three experimental conditions used (quinine, NaCl, and the mixture of both). Note that the model gives the component firing frequencies but does not assign them to a specific neuron. This means that the least active neuron [f_A in Fig. 9(B) and Table 2] is not necessarily the same when the stimulus or the concentration is modified. The assignment of neurons A and B in Table 2 to L1 and L2 cells must be based on complementary evidence (see Result sections).

Limitations

This model presents three limitations. First, it is restricted to recordings from two neurons. The taste sensilla studied here contain four neurons. However, the bitter compounds inhibited the responses from the W and S cells in those sensilla. Thus, bitter-elicited spikes could only originate from the L1 and L2 cells and the first condition of the model is fulfilled. Second, the model is based on the assumption that spiking frequencies are stationary during the observation period. In this study, we considered only the interval during which the responses to salt and to quinine were stable, i.e., the 200–1000-ms interval after stimulation (Meunier et al., 2000). Last, the simulations were made using model neurons firing independently, thus excluding phase shift problems. This is not always true because *Locusta* taste neurons were found in some cases to have negative interactions (White et al., 1990). However, our results are consistent with our model and thus we can assume that it is not a problem here.

We are grateful to Drs. Harry Itagaki, Xavier Grosmaitre for critical readings of the manuscript, Makoto Hiroi for helpful discussions, Dominique Tauban for her help in S.E.M., and two anonymous referees for their suggestions.

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