Chemical Senses Advance Access published September 3, 2007

Chem. Senses doi:10.1093/chemse/bjm057

Electrophysiological Characterization of Responses from Gustatory Receptor Neurons of *sensilla chaetica* in the Moth *Heliothis virescens*

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Abstract

Discrimination of edible and noxious food is crucial for survival in all organisms. We have studied the physiology of the gustatory receptor neurons (GRNs) in contact chemosensilla (insect gustatory organs) located on the antennae of the moth *Heliothis virescens*, emphasizing putative phagostimulants and deterrents. Sucrose and the 2 bitter substances quinine and sinigrin elicited responses in a larger proportion of GRNs than inositol, KCl, NaCl, and ethanol, and the firing thresholds were lowest for sucrose and quinine. Variations in GRN composition in individual sensilla occurred without any specific patterns to indicate specific sensillum types. Separate neurons showed excitatory responses to sucrose and the 2 bitter substances quinine and sinigrin, implying that the moth might be able to discriminate bitter substances in addition to separating phagostimulants and deterrents. Besides being detected by separate receptors on the moth antennae, the bitter tastants were shown to have an inhibitory effect on phagostimulatory GRNs. Sucrose was highly appetitive in behavioral studies of proboscis extension, whereas quinine had a non-appetitive effect in the moths.

Key words: antennal taste, contact chemosensilla, insect taste, proboscis extension, quinine, sucrose

Introduction

Gustation is an omnipresent sense in virtually all organisms and is used in finding and securing the quality of food, as well as avoiding toxic items. In selecting food and oviposition sites, female insects use gustatory receptor neurons (GRNs) located in contact chemosensilla on various parts of the body (De Boer and Hanson 1987; Ramaswamy 1988; Städler and Roessingh 1991; Bernays and Chapman 1994; Baur et al. 1998; Chapman 2003). In the moth *Heliothis virescens* (Lepidoptera: Noctuidae), contact chemosensilla are located on the antennae (*sensilla chaetica*), the proboscis (*sensilla styloconica*), and the tarsi (Blaney and Simmonds 1990; Jørgensen et al. 2006; Kvello et al. 2006). A contact chemosensillum typically contains 2–4 GRNs with dendrites extending toward the tip of the sensillum hair, and one mechanosensory neuron attached to the hair base (Hallberg 1981; Koh et al. 1995; Ozaki and Tominaga 1999; Kvello et al. 2006), and the antennal *s. chaetica* has 4 GRNs and one mechanosensory neuron (Jørgensen et al. 2006). When the moth antennates, taste stimuli are detected by the GRNs of *s. chaetica* that are especially abundant at the antennal tip. Information from the antennal GRNs is conveyed by their primary axons to the subesophageal ganglion (SOG) (Jørgensen et al. 2006), where it is transmitted to interneurons and motorneurons involved in the proboscis extension reflex (PER). Phagostimulants like sucrose, applied to the antennae, release PER when the moth is hungry and motivated to feed, whereas deterrents inhibit the release of PER. During feeding, GRNs on the proboscis are stimulated and convey information to the tritocerebrum/SOG (Kvello et al. 2006), controlling ingestion. Despite the importance of antennal GRNs in feeding, few studies of these neurons have been performed. The honeybee *Apis mellifera* have particular GRNs on the antennae detecting sucrose, but not the bitter substances tested (Haupt 2004; De Brito Sanchez et al. 2005). Antennal GRNs of the cockroach *Periplaneta americana* seem to detect fruit juices, surface- and tergal extracts, but not sucrose, whereas in *Periplaneta brunnea* they detect sucrose (Hansen-Delkeskamp 1992; Hansen-Delkeskamp and Hansen 1995).

Detection of tastants has evolved differently in various organisms, depending on diet breadth and habitat. Sugars, an important energy source, are detected by particular gustatory cells, present in many species. In mammals, the

2 coupled receptor proteins, T1R2 and T1R3, seem to detect all natural sugars and artificial sweeteners tested (Chandrashekar et al. 2006). The specificity of the insect GRNs involved in sweet taste vary between species (Evans and Mellon 1962; Blaney and Simmonds 1988; Simmonds et al. 1990; Chapman 1998; Schoonhoven and Van Loon 2002). In the blowfly *Phormia regina*, one sugar-responsive GRN responds to all of the feeding stimulants, sucrose, fructose, glucose, sugar alcohols, and some amino acids (Shiraishi and Kuwabara 1970; Dethier 1976), whereas separate GRNs detect sugars, sugar alcohols, and amino acids in lepidopteran larvae (Bernays and Chapman 2000; Glendinning et al. 2000; Schoonhoven and Van Loon 2002). A putative sugar receptor, *Gr5a*, has been identified in *Drosophila* (Dahanukar et al. 2001). In *H. virescens*, a candidate gustatory receptor gene is expressed in cell bodies located at the base of *s. chaetica*, but the specificity of the receptor is not known (Krieger et al. 2002).

In addition to detecting phagostimulants, most animals, including herbivorous insects, possess GRNs responding to a diverse range of deterrents (Dethier 1980; Schoonhoven et al. 1992). Bitter stimuli constitute the largest and most structurally diverse class of gustatory stimuli (Rouseff 1990). In mammals, a family of gustatory receptors, T2R, is involved in bitter taste detection (Adler et al. 2000). In *Drosophila*, the receptor gene, *Gr66a*, is believed to code for a bitter receptor (Thorne et al. 2004; Wang et al. 2004). In addition, various other putative bitter receptors are coexpressed in subsets of *Gr66a*-expressing neurons, implying that several types of GRNs mediate bitter taste. Two putative genes coding for salt receptors are the degenerin/epithelial Na^+ channels PPK11 and PPK19. Ablation of these genes affects electrophysiological and behavioral responses to Na^+ and K^+ salts in *Drosophila* (Liu et al. 2003).

The moth *H. virescens*, a serious pest on monocultures like cotton, tomato, corn, soy beans, grain, and tobacco (Fitt 1989; King and Coleman 1989), is a polyphagous species also preferring other host plants. The females choose between many plant species for nectar feeding and oviposition. The moths are attracted to the host plants by blends of odorants, but the final decision to feed or oviposit requires involvement of the gustatory system (Ramaswamy 1988). Taste substances on the plant surface and the composition of tastants in the nectar determine whether the plant is accepted. In the present paper assaying the physiology of the GRNs on the antennae of female *H. virescens*, we have focused on the following substances of putative importance in host plant selection: sucrose, *myo*-inositol, ethanol, KCl, NaCl, quinine, and sinigrin. The sugar sucrose is present in high levels in lepidoptera-pollinated plant nectar (Baker HG and Baker I 1983), the sugar alcohol *myo*-inositol is detected by specialized GRNs in *H. virescens* larvae (Bernays and Chapman 2000), and the alcohol ethanol is observed to be attractive to *H. virescens* larvae. KCl and NaCl are two important inorganic salts, and quinine and sinigrin are known as bitter substances. The glucosinolate sinigrin is previously found to be nonappetitive for *H. virescens* and other lepidopterans (Blaney and Simmonds 1988; Shields and Mitchell 1995b; Jørgensen et al. 2006). The aim of the present study was to functionally characterize the antennal GRNs in respect to specificity and sensitivity to these substances of putative importance to female *H. virescens*. In addition, we wanted to study GRN composition in the different *s. chaetica* to find out if it was similar or different across sensilla.

Material and methods

Insects and preparation

Heliothis. virescens used in the experiments were received as pupae (Novartis Crop Protection AG, Rosental, Switzerland). The male and female pupae were sorted and hatched with access to 5% sucrose solution in separate climate chambers (Refritherm 200, Struers-Kebolab, Albertslund, Denmark; 22 °C, reversed photoperiod). On the day of the experiment, the adult female moths (1–2 days old) were immobilized with tape and wax between the head with the thorax in Plexiglas holders, exposing the head and the antennae. The antennae were attached to a wax foundation with tungsten hooks so that the leading edge was facing upwards making the *s. chaetica* accessible.

Test substances

The gustatory stimuli used in the experiments were (applied in the following order) KCl, sucrose, the sugar alcohol *myo*-inositol, NaCl (all from Sigma-Aldrich), the glucosinolate sinigrin monohydrate, the alkaloid quinine hydrochloride (both from VWR), and ethanol (Arcus) prepared in dilutions of the electrolyte 0.01 M KCl. The concentration range was from 0.0001 to 0.1 M for KCl, sucrose, inositol, NaCl, and sinigrin (up to 1 M for NaCl). Quinine was applied at 2 concentrations only (0.00001 and 0.001 M) due to putative damage of the cells by this substance. Ethanol was applied at 5% (1 M), 10% (2.2 M), and 20% (4.3 M). Studies of GRN interaction were performed with mixtures of 0.01 M sucrose and quinine (0.00001 and 0.001 M) or 0.01 M sucrose and sinigrin (0.01 and 0.1 M). The experiments started with the lowest concentrations and ended with the highest to avoid adaptation in the cells. The solutions were prepared every 2 weeks and stored at 4 °C. For the behavioral experiments, water, 0.01 and 0.1 M sucrose, and 0.001, 0.01 and 0.1 M quinine dissolved in distilled water were used.

Electrophysiology

Electrophysiological recordings from GRNs of *s. chaetica* were carried out using tip recording (Hodgson et al. 1955). The recording electrode (thin-walled borosilicate glass capillaries, Harvard apparatus) was pulled in a 2-step electrode puller (PP-830, Narishige group, Japan) to a tip diameter of approximately 10–20 μm. To avoid crystallization

and concentration changes at the tip, the electrode was filled with the test substance just a few seconds before the start of the recording. The recording electrode containing the test solution was placed over single sensilla hairs for 5 s with an interstimulus interval of approximately 10 min to avoid adaptation. The recording glass electrode was connected to a TastePROBE amplifier (10×, Syntech, Hilversum, the Netherlands) (Marion-Poll and Van der Pers 1996) and the signals were filtered (high pass: 50 Hz and low pass 3000 Hz) using CyberAmp 320 (Axon Instruments, Burlingame, CA). The grounded reference electrode was a 1-mm diameter AgCl coated silver wire placed in the moth abdomen or in the contralateral eye. Analyses of the spikes were performed using the software AutoSpike-32 (Syntech). The analyses were based on properties like waveform and amplitude. Due to changing conductance, the spike amplitudes varied between recordings, so spikes were only classified based on amplitude in cases where they were consistently different in every recording. The annuli were numbered 1–81 from the most proximal to the most distal annulus of the flagellum, and recordings were made from the 4 sensilla on each annulus without preferences. All sensilla between annulus 81 and 55 were described as distally located, between 54 and 27 medially located, and between 26 and 1 proximally located (Figure 1). Only the 3 highest concentrations (0.001, 0.01, and 0.1 M) were included in the dose–response curves to avoid interference of the water cell that was firing at 0.0001 M.

Statistics

For each substance, the proportion of GRNs responding to the substance distally, medially, and proximally on the flagellum was compared using Fisher's exact tests. Differences in response strength distally, medially, and proximally on the flagellum were compared using Kruskal–Wallis tests, and when applicable, 2 × 2 comparisons were performed using Mann–Whitney tests.

Behavior

In order to compare behavioral effects of the appetitive stimulus sucrose and the putative aversive stimulus quinine, PER experiments were performed and presented in a first descriptive approach. Moths were restrained in Plexiglas tubes and starved for 2 days before they were tested for PER by applying different concentrations of the substances to the antennae in the following order: 0.01 M sucrose, 0.1 M sucrose, 0.001 M quinine, 0.01 M quinine, 0.1 M quinine, 0.1 M sucrose again, and water. In order to avoid adaptation, there was a 10-min interval between the stimulations. The number of proboscis elicitations were counted and compared.

Results

Proportion of *s. chaetica* with GRNs responding to the test substances

The results are based on electrophysiological recordings from 132 *s. chaetica* of 11 moths, systematically tested for concentration series of the following 7 substances: KCl, sucrose, inositol, NaCl, sinigrin, quinine, and ethanol. The GRNs that fired in a dose–response manner to a particular substance were considered to be responsive to the substance. In general, excitatory phasic–tonic firing was recorded as responses to all stimuli (Figures 2A,E, 3A,E, 4A,E, 5A,E), except for 0.001 M quinine that elicited an excitatory bursting firing at irregular intervals (Figure 2A). The latency of the cell responding to quinine varied and sometimes extended 4 s. The most active substances were quinine, sucrose,

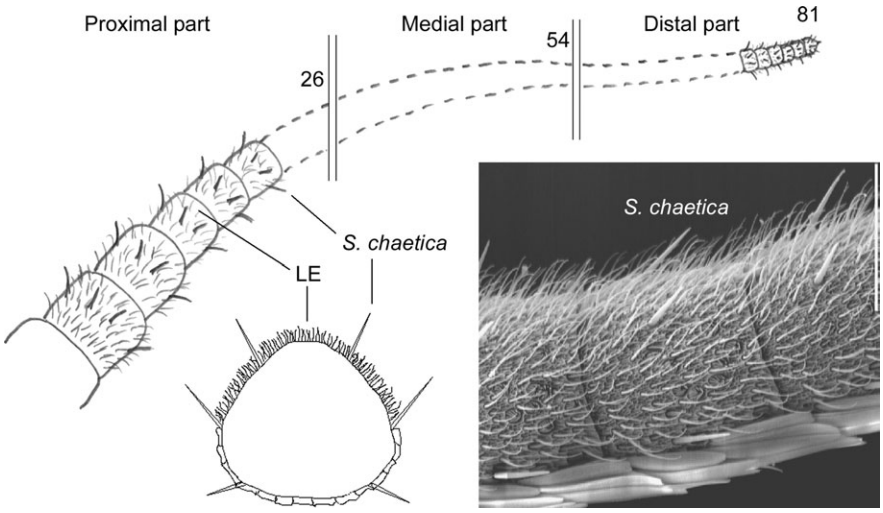

Figure 1 Schematic drawings (longitudinal view and cross section) and scanning electron microscopy image showing the localization of *sensilla chaetica* on the flagellum of *Heliothis virescens*. The numbers indicate the annuli separating the distal, medial, and proximal parts of the flagellum. Scanning electron microscopy is carried out according to Jørgensen et al. (2006). LE, leading edge. Scale bar = 100 μm.

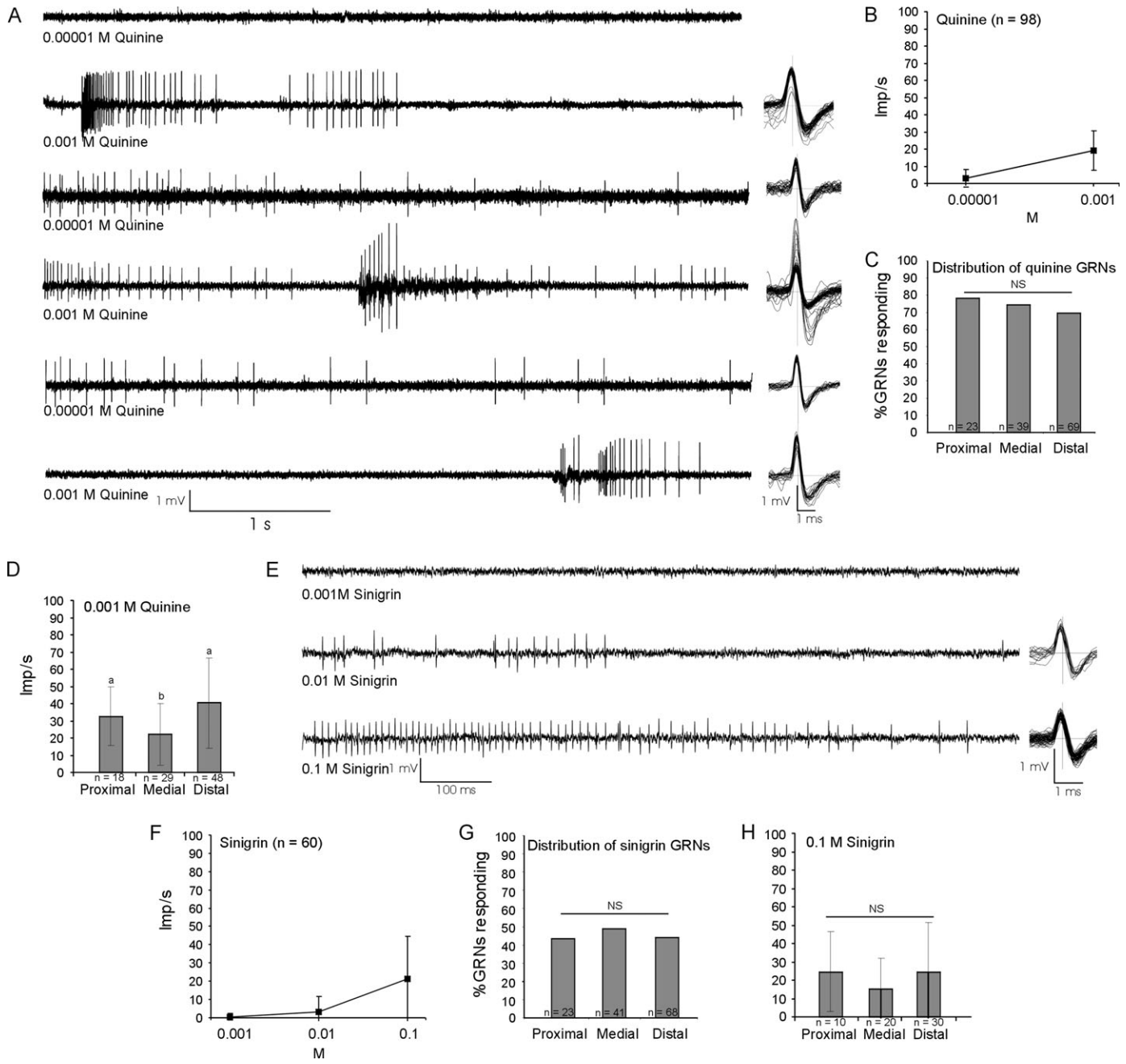

Figure 2 Response properties of GRNs in *sensilla chaetica* of *Heliothis virescens* responding to the 2 bitter substances sinigrin and quinine. **(A)** Responses and spike analyses of 3 different sensilla to quinine, illustrating the variation of the bursting response to 0.001 M quinine. The spike amplitude increased during bursts. (The response properties of the sensillum in the 2 upper traces to other substances are shown in Figure 6B.) **(B)** Dose–response curve of average firing to quinine. **(C)** Distribution of quinine-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $P > 0.05$). **(D)** Response strength to 0.001 M quinine of the GRNs located along the flagellum. Different letters indicate significant differences (Mann–Whitney tests, $P < 0.05$). **(E)** Example of responses and spike analyses of one GRN to sinigrin. There was no response to 0.001 M. (The response properties of this sensillum to other substances are shown in Figure 6A.) **(F)** Dose–response curve of average firing to sinigrin. **(G)** Distribution of sinigrin-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $P > 0.05$). **(H)** Response strength to 0.1 M sinigrin of the GRNs along the flagellum. The letters NS indicate no significant differences (Mann–Whitney tests, $P > 0.05$). The error bars show the standard deviation.

and sinigrin eliciting GRN responses in a larger proportion of *s. chaetica*; quinine in 74% (98 of 132), sucrose in 65% (85 of 130), sinigrin in 46% (60 of 131), KCl in 39% (48 of 124), NaCl in 35% (24 of 84), ethanol in 31% (29 of 95), and inositol in 25% (32 of 128). Complete recordings at all concentrations were missing in some sensilla, causing the difference in numbers of tested sensilla. The distribution of these GRNs differed along the flagellum (Figures 2C,G, 3C,G,

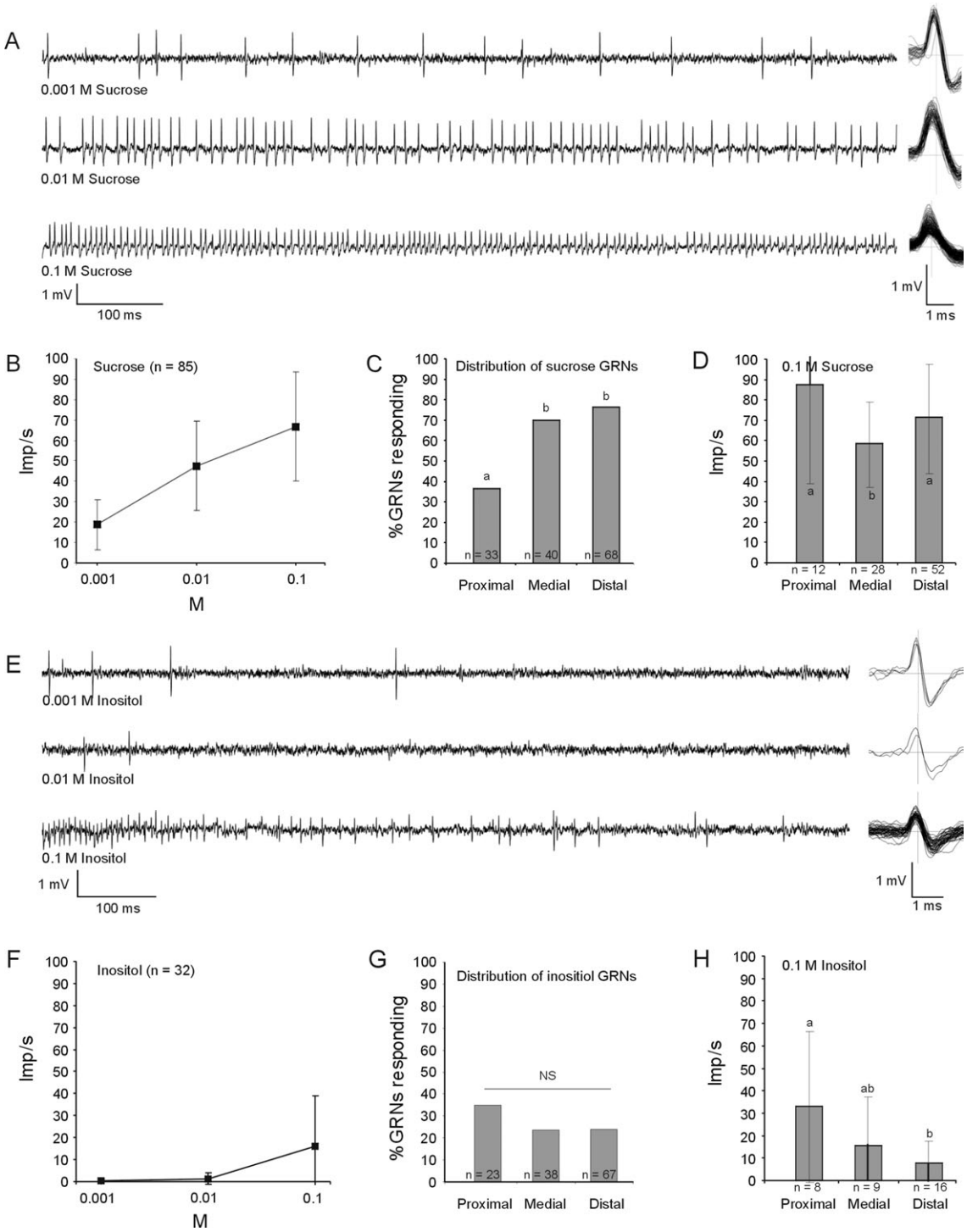

Figure 3 Response properties of GRNs in *sensilla chaetica* of *Heliothis virescens* to sucrose and the sugar alcohol inositol. **(A)** Example of responses and spike analyses of one GRN to sucrose. Sucrose elicited spikes with relatively high amplitude and broad spike shape. The spike amplitude of the sucrose GRN decreased as the firing frequency increased. **(B)** Dose–response curve of the average firing to sucrose. **(C)** Distribution of sucrose-responding GRNs along the flagellum. Different letters indicate significant differences (Fisher's exact test, $P < 0.05$). **(D)** Response strength to 0.1 M sucrose of the GRNs along the flagellum. Different letters indicate significant differences (Mann–Whitney tests, $P < 0.05$). **(E)** Example of responses and spike analyses of one GRN to inositol. The upper trace shows spikes elicited by the water-responsive GRN (no response to inositol), and the spike analyses show that this is a different GRN than the small amplitude GRN responding to inositol. **(F)** Dose–response curve of average firing to inositol. **(G)** Distribution of inositol-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $P > 0.05$). **(H)** Response strength to 0.1 M inositol of the GRNs along the flagellum. Different letters indicate significant differences (Mann–Whitney tests, $P < 0.05$). The error bars show the standard deviation.

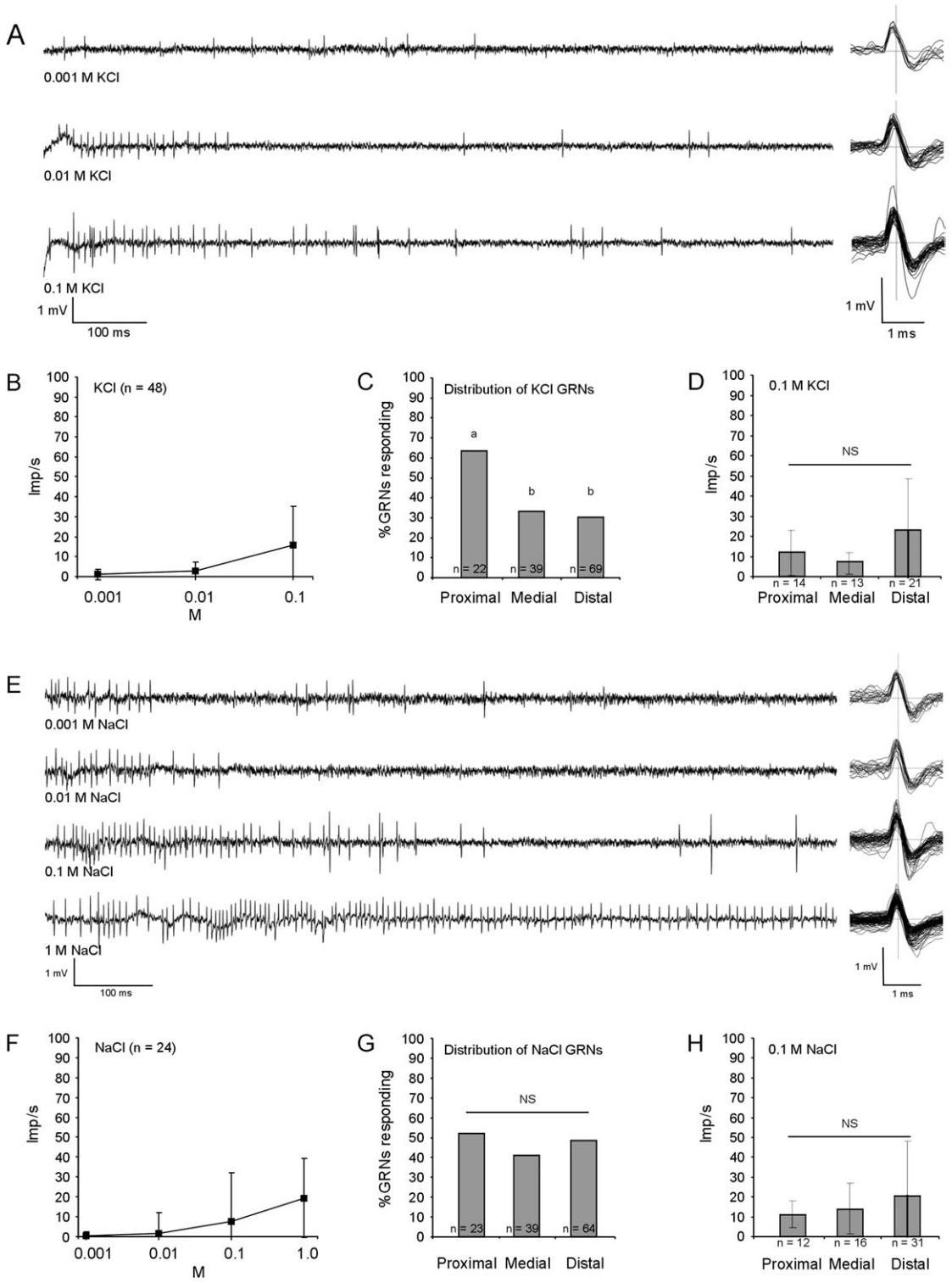

Figure 4 Response properties of GRNs in *sensilla chaetica* of *Heliothis virescens* to the 2 inorganic salts KCl and NaCl. **(A)** Example of responses and spike analyses of one GRN to KCl. The GRN responding to KCl had small amplitude. **(B)** Dose–response curve of average firing to KCl. **(C)** Distribution of KCl-responding GRNs along the flagellum. Different letters indicate significant differences (Fisher's exact test, $P < 0.05$). **(D)** Response strength to 0.1 M KCl of the GRNs along the flagellum. The letters NS indicate no significant differences (Mann–Whitney tests, $P > 0.05$). **(E)** Example of responses and spike analyses of one GRN to NaCl. The spike amplitude of the NaCl-responding GRN was small, whereas the additional cell firing at 0.1 M and had high amplitude, and was not considered a real response. **(F)** Dose–response curve of average firing to NaCl. **(G)** Distribution of NaCl-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $P > 0.05$). **(H)** Response strength to 0.1 M NaCl of the GRNs along the flagellum. The letters NS indicate no significant differences (Mann–Whitney tests, $P > 0.05$). The error bars show the standard deviation.

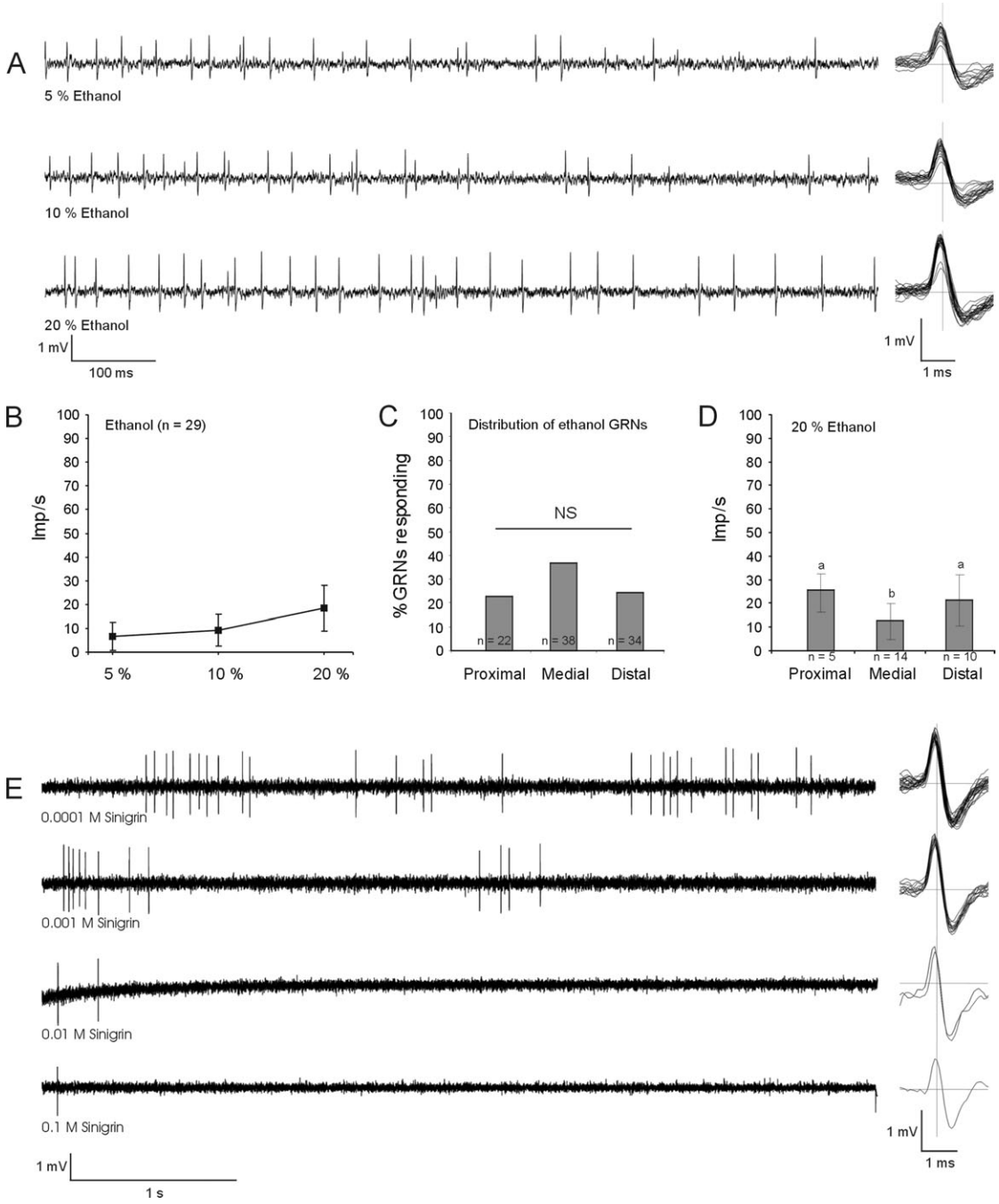

Figure 5 Response properties of GRNs in *sensilla chaetica* of *Heliothis virescens* responding to ethanol and water. **(A)** Example of response and spike analyses of one GRN to ethanol. The response to the 2 lower concentrations showed smaller spike amplitudes than to the highest concentration. **(B)** Dose–response curve of average firing to ethanol. **(C)** Distribution of ethanol-responding GRNs along the flagellum. The letters NS indicate no significant differences (Fisher's exact test, $P > 0.05$). **(D)** Response strength to 20% ethanol of the GRNs along the flagellum. Different letters indicate significant differences (Mann–Whitney tests, $P < 0.05$). **(E)** Example of a water-responsive GRN and spike analyses during stimulation with 0.0001, 0.001, 0.01, and 0.1 M sinigrin. There was no excitatory response to sinigrin, but the water-responsive GRN was inhibited with increasing concentrations of sinigrin. The error bars show the standard deviation.

4C,G, 5C), the proportion of sensilla with GRNs responding to sucrose increased significantly from the base to the tip of the flagellum (all parts: Fisher's exact test, $P < 0.001$; 2×2 comparisons by Fisher's exact tests: distal vs. medial, $P = 0.5$; distal vs. proximal, $P < 0.001$; medial vs. proximal, $P = 0.005$), whereas the opposite was observed for KCl (all: Fisher's exact test, $P < 0.019$; 2×2 comparisons by Fisher's exact tests: distal vs. medial, $P = 0.83$; distal vs. proximal, $P < 0.011$; medial vs. proximal, $P = 0.032$). The number of GRNs responding to the other substances was

approximately equal along the flagellum (Fisher's exact tests, $P > 0.544$ in all cases).

Sensitivity of the GRNs

The sensitivity varied between the GRNs in different sensilla, both in respect to threshold concentrations and response strength. Quinine, the only substance tested at 0.00001 M, elicited responses in 51% of the quinine-responsive GRNs at this concentration. The average firing frequency was 2.9 imp/s, increasing to 18.2 imp/s at 0.001 M (Table 1, Figure 2B). Due to the bursting firing, the response to quinine is given as imp/s during the bursting period. At higher concentrations than 0.001 M, quinine caused noise, and the spikes disappeared in all GRNs within the sensilla. Even hours after stimulation with higher concentrations of quinine, the recordings showed only irregular noise to the other test substances. Therefore, tests with quinine were only performed twice in each sensillum at concentrations causing no damage. Like the quinine-responsive GRNs, the GRNs responding to sucrose showed a high sensitivity; all activated by 0.001 M sucrose with an average firing frequency of 18.8 imp/s (Table 1, Figure 3B). At the highest concentration of sucrose (0.1 M), the average firing frequency was 66.8 imp/s, the strongest average response measured (Table 1, Figures 2B,F, 3B,F, 4B,F, 5B). The individual sensitivities of these GRNs showed variations from 3 to 133 imp/s as responses to 0.1 M sucrose. The other test substances had higher threshold concentrations than 0.001 M (Table 1, Figures 2B,F, 3B,F, 4B,F, 5B), and the dose–response curves showed an overall lower response to these substances compared with sucrose.

Differences in the GRN response strength to the individual substances were evident along the flagellum. Sucrose, quinine, and ethanol elicited significantly stronger responses distally and proximally than medially on the flagellum (Figures 2D, 3D, 5D) (Sucrose [all parts]: Kruskal–Wallis, $P = 0.019$; 2×2 comparisons, Mann–Whitney: distal vs. medial, $P = 0.03$; proximal vs. medial, $P = 0.02$; distal vs. proximal, $P = 0.119$; ethanol [all parts]: Kruskal–Wallis, $P = 0.010$; 2×2 comparisons, Mann–Whitney: distal vs. medial, $P = 0.026$; proximal vs. medial, $P = 0.007$; distal vs. proximal, $P = 0.310$; quinine [all parts]: Kruskal–Wallis, $P = 0.001$; 2×2 comparisons, Mann–Whitney: distal vs. medial, $P < 0.0001$; proximal vs. medial, $P = 0.019$; distal vs. proximal, $P = 0.395$). Inositol elicited significantly stronger firing proximally than distally (all parts: Kruskal–Wallis, $P = 0.07$; 2×2 comparisons, Mann–Whitney: distal vs medial, $P = 0.388$; proximal vs. medial, $P = 0.277$; distal vs. proximal, $P = 0.019$) (Figure 3H), whereas KCl, NaCl, and sinigrin elicited approximately equal firing at all parts of the flagellum (Kruskal–Wallis, $P > 0.207$ in all cases).

Comparison of responses between individual *s. chaetica*

In 76 sensilla, complete recordings were obtained for all concentrations of each substance. Comparison between the individual response profiles of the sensilla showed variations (Table 2). Separate sensilla showed responses to the 2 inorganic salts in 2 populations of 18 and 16 sensilla, respectively, whereas 12 other sensilla showed responses to both salts. The 2 bitter substances also elicited responses in different sensilla, 29 only to quinine and 8 only to sinigrin, while 29 others showed responses to both. In addition, individual variations were observed between responses to sinigrin and the 2 salts; GRNs in 13 sensilla responding only to KCl, in 21 only to sinigrin, and in 16 to both. Fifteen sensilla showed responses only to NaCl, 24 only to sinigrin, and 12 to both. In addition, responses to the 2 phagostimulants sucrose and inositol showed individual variations between sensilla; in 37 sensilla responses appeared only to sucrose, in 10 only to inositol, and in 9 to both. Comparison between inositol and ethanol showed 6 sensilla with responses to both, 14 only to inositol and 15 only to ethanol.

Table 1 Average GRN responses to 2 concentrations (0.001 and 0.1 M) of sucrose, sinigrin, KCl, inositol, and NaCl, in addition to 1 and 4.3 M ethanol and 0.00001 and 0.001 M quinine

Substance	Lowest concentration (imp/s)	Highest concentration (imp/s)	% GRNs responding to the lowest concentration
Sucrose	18.8	66.8	100
Sinigrin	0.5	21.2	17
Inositol	0.4	16.3	31
KCl	1.0	15.8	17
NaCl	0.3	7.7	10
Ethanol	6.6	18.6	93
Quinine	2.9	18.2	51

The percentage of the GRNs responding to 0.001 M solution (1 M for ethanol and 0.00001 M for quinine) for the 7 substances is also shown.

Analysis of single GRN responses

Spike analysis were performed in order to separate spikes originating from different GRNs. Overall, a definite identification of the neuron types across recordings was difficult due to the change of recording electrodes with varying conductance. In spite of this, some general features appeared. The GRNs responding to KCl, NaCl, inositol, and sinigrin always had smaller spike amplitudes (less than 1 mV) than the GRNs responding to sucrose, water, quinine, and ethanol (Figures 2A,E, 3A,E, 4A,E, 5A,E, 6). The spikes of the GRNs responding to sucrose were broader than those of the other cells (Figures 3A, 6). The GRN responding to quinine showed a gradual increase in spike amplitude during a burst, and the response to sinigrin differed from the quinine response both in spike amplitude and temporal firing pattern (Figures 2A,E, 6B). Concerning the 2 salts, 2 GRNs with different spike amplitudes seemed to be involved in the

Table 2 Response properties of 76 *sensilla chaetica*, allowing comparison of the responses to different substances by individual sensilla

Individual moth	*s. chaetica* of annulus #	KCl	Sucrose	Inositol	NaCl	Sinigrin	Quinine	Ethanol
1	80	–	+	–	–	–	–	–
	77	–	+	–	–	–	+	–
	76	–	+	–	+	–	+	–
	75	+	+	–	–	–	–	–
	74	+	+	–	+	+	–	–
	73	–	+	+	+	+	–	+
	72	–	+	–	–	+	+	–
	71	–	–	–	–	+	+	+
	70	–	+	–	+	+	–	+
	69	–	+	+	+	–	+	–
	68	–	–	–	+	–	–	–
2	58	–	+	–	+	–	+	–
	57	–	+	–	–	–	+	–
	56	+	–	–	–	–	+	–
	55	–	+	–	+	+	+	–
	54	–	+	–	+	+	+	–
	53	–	+	+	–	+	+	–
	52	–	+	–	–	+	–	+
	51	–	+	–	+	+	+	+
	50	–	+	+	–	+	+	–
	49	–	+	–	–	+	+	–
	48	+	+	–	–	+	+	–
	47	–	+	–	–	+	+	–
3	36	–	+	–	–	–	+	+
	35	–	+	–	–	–	+	–
	34	–	–	–	+	–	+	–
	32	–	–	+	–	+	+	–
	31	–	+	+	–	–	+	+
4	31	–	+	–	–	–	–	–
	29	–	–	+	–	+	+	–
	28	–	–	–	–	–	+	–
	27	–	–	+	–	–	–	–
	26	+	–	–	–	–	+	–
	24	–	–	–	–	+	–	+
	23	–	–	+	–	+	–	+
	22	–	–	+	+	–	+	+
	21	+	–	–	–	–	–	–
	20	–	–	–	+	–	+	–
	19	–	–	–	–	+	+	–
	18	–	–	–	–	–	+	–
5	36	–	+	–	–	–	–	+
	35	+	+	–	–	+	–	–

Table 2 Continued

Individual moth	*s. chaetica* of annulus #	KCl	Sucrose	Inositol	NaCl	Sinigrin	Quinine	Ethanol
	34	+	+	–	+	+	+	–
	33	–	+	–	–	–	–	–
	32	–	–	–	–	–	–	+
	31	+	–	–	–	–	+	+
	30	+	+	–	–	+	+	+
	29	–	–	–	–	–	+	–
	28	+	–	+	+	–	+	–
	27	+	–	+	+	+	+	–
	26	–	+	+	–	+	+	–
	25	+	–	+	–	+	+	–
	24	+	–	+	–	+	+	–
6	60	+	+	+	–	+	+	–
	59	+	+	–	+	+	+	+
	58	–	+	+	+	+	+	–
	57	–	+	–	+	–	+	+
	56	+	+	–	+	–	+	–
	55	–	+	–	+	–	+	–
	54	+	+	–	–	–	+	+
	53	–	+	–	–	+	+	–
	52	+	+	–	+	–	+	–
	51	+	+	+	+	–	+	+
	50	+	+	–	+	–	+	–
	49	+	+	–	+	+	–	+
	47	+	+	–	+	+	+	–
	46	–	+	–	+	+	+	–
7	18	+	–	–	+	–	–	–
	17	+	–	–	–	+	+	–
	16	+	+	–	–	+	+	–
	15	+	+	+	–	–	+	+
	14	+	–	–	–	+	+	+
	13	+	–	+	–	–	+	–
	12	+	–	–	–	+	+	–
	11	–	+	–	–	–	+	–
	10	–	–	–	–	–	+	–

Firing in a dose–response manner was considered as response (+). No response (–).

responses to both KCl and NaCl (Figure 6). Figure 6A shows activity of the small amplitude GRN and Figure 6B of the larger amplitude GRN. Firing of both as well as of only one of them appeared in the recordings. The small amplitude GRN fired vigorously to 0.1 and 1 M NaCl, whereas the large amplitude GRN often displayed a low-frequency firing at all NaCl concentrations. Stimulation with KCl showed a similar response pattern. Variations considered

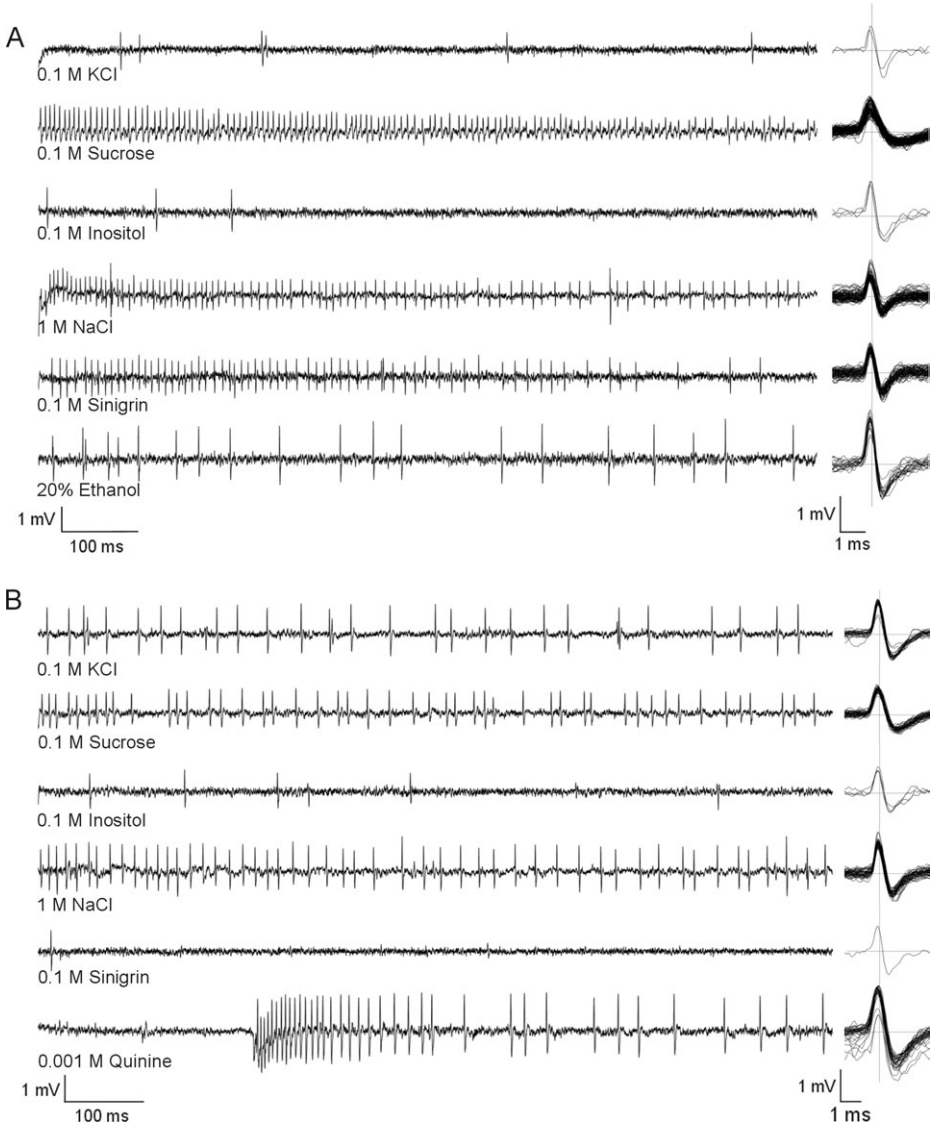

Figure 6 Response properties of 2 different *sensilla chaetica* of *Heliothis virescens* to the highest concentrations of various substances. **(A)** Responses and spike analyses of GRNs on annulus 72 to KCl, sucrose, inositol, NaCl, sinigrin, and ethanol. (The response properties of this sensillum to 3 concentrations of sinigrin are shown in Figure 2E.) The spike shape of the sucrose-responsive GRN was broader then the other GRNs. KCl, NaCl, and sinigrin might be detected by the same small amplitude GRN, whereas ethanol seemed to be detected by a large amplitude GRN. **(B)** Responses and spike analyses of GRNs on annulus 60 to KCl, sucrose, inositol, NaCl, sinigrin, and quinine. (The response properties of this sensillum to two concentrations of quinine are shown in the two upper traces of Figure 2A.) Again, broad-shaped spikes of one GRN were elicited by sucrose. One GRN seemed to respond to the 2 salts. There was no response to sinigrin, and 2 different GRNs seemed to be responding to quinine and inositol.

not to be real responses were occasionally seen in different recordings, as exemplified in Figure 4E (third trace) where a large amplitude GRN appeared, that did not fire in response to stimulation with the other concentrations of NaCl. Peculiarly, the response to ethanol consistently showed larger spike amplitudes (2 mV) at the highest concentration than at the 2 lowest (Figure 5A), possibly due to the fat-soluble properties of ethanol. Another GRN, probably a water-responsive GRN, appeared with large spikes and tonic firing during stimulation with the lowest concentration (0.0001 M) of all substances (Figure 5E, upper trace) and occasionally to 0.001 M (Figure 3E, upper trace). The spikes of this GRN usually disappeared at higher concentrations, when the other GRNs were activated. In a few cases where no excitatory response to the test substance was observed, this water GRN showed decreased firing with increasing concentration of the substance, exemplified in Figure 5E.

The different compositions of GRNs in individual sensilla are exemplified in Figure 6. Both recordings show responses to sucrose, NaCl, KCl, and inositol. Responses to sinigrin and ethanol are evident in the recordings shown in Figure 6A, whereas response to quinine is seen only in

the recordings shown in Figure 6B. Based on the analysis of spike amplitudes and waveforms, it seems that the response to KCl, NaCl, and sinigrin originate from the same GRN; in Figure 6A from the small amplitude GRN and in Figure 6B from the larger amplitude GRN. The characteristic broad spikes are elicited by the sucrose GRN, whereas the spikes elicited by inositol originate from a third GRN. In addition, ethanol elicits tonic firing of large amplitude spikes of one GRN in Figure 6B. Another GRN responds in a bursting manner to quinine (Figure 6A).

Responses to mixtures of sucrose and bitter substances

Comparisons of the responses to sucrose and the mixtures of sucrose and the 2 bitter substances, quinine and sinigrin, were performed in order to study possible interactions between phagostimulatory and deterrent GRNs. Stimulation with mixtures of sucrose and quinine were performed in 92 sensilla with separate GRNs responding to sucrose and quinine (Figure 7A–C). The average responses to the initial and final stimulation with 0.01 M sucrose were approximately equal, 54 and 53 imp/s, respectively. The average firing decreased to 39 and 14 imp/s, respectively, when 0.00001 and 0.001 M quinine was mixed with the 0.01 M sucrose solution. In addition, the bursting response to quinine was not seen when quinine was mixed with sucrose, implying a mutual inhibition of the quinine- and sucrose-responsive GRNs. The latency of the GRN responding to quinine was long and inconsistent during stimulation with quinine alone (Figure 2A), whereas the latency of inhibition of the sucrose-responsive GRN was immediate, impairing the sucrose response from the start of the stimulation period (Figure 7A).

In 44 other sensilla, sinigrin elicited the same pattern of inhibition when stimulating with the mixtures of 0.01 M sucrose and 2 different concentrations (0.01 and 0.1 M) of sinigrin (Figure 7D–F). Because of sensitivity differences, higher concentrations of sinigrin than quinine were used in the mixtures. The initial and final stimulation with sucrose elicited an average firing of 41 imp/s, whereas the mixtures with increasing concentrations of sinigrin elicited decreased firing (27 and 9 imp/s). These series of stimulations with single compounds and mixtures of sucrose and the 2 bitter substances imply that both sinigrin and quinine elicit excitatory responses in separate GRNs and cause inhibition of the sucrose-responsive GRN.

Behavior

Behavioral effects of the phagostimulant sucrose and the putative deterrent quinine were assayed by applying different concentrations to the antennae of 47 starved moths. The initial stimulation with 0.01 and 0.1 M sucrose elicited PER in 88 and 93% of the moths, respectively. When 0.001 M quinine was applied to the antennae, 60% of the moths showed proboscis extension. This number decreased to 30 and 16%, respectively, when the quinine concentration was enhanced to 0.01 and 0.1 M. In the subsequent stimulation with 0.1 M sucrose in the same group of insects, 77% of the moths extended their proboscises. Finally, 65% of the moths showed proboscis extension to water.

Discussion

The results of the present study have shown that the moth *H. virescens* has GRNs responding to all 7 selected tastants, with strongest responses to sucrose and quinine. In addition, sucrose- and quinine-responsive GRNs were present in a majority of the *s. chaetica*. However, the GRN composition of individual sensilla varied to a great extent, showing no distinct sensillum types or distribution of specific types to particular locations. This absence of sensillum types might appear because of the limited number of test substances as well as varying sensitivities of the GRNs. Other biologically relevant tastants might have elicited stronger responses, particularly in the weakly activated GRNs. The varying sensitivities of the GRNs might have enhanced the impression of variability of the responses, disabling a classification of sensillum types.

We based our choice of test substances on their statuses as general phagostimulants or deterrents as well as expected relevance to *H. virescens*. Sucrose, an important energy source and the most prominent component in the nectar of lepidoptera-pollinated plants (Baker HG and Baker I 1983), is a well-known phagostimulant and relevant for *H. virescens* during nectar feeding, as evidenced by the strong responses in numerous GRNs in our study. When the moth searches for food or oviposition sites, it antennates, tapping the surface rapidly with the antennal tip. Approaching a flower, the whole flagellum of *H. virescens* is in contact with the interior of the flower, whereas the tip is touching the nectar source. This behavior in combination with the vital importance of sugar might be reflected in the relatively large number of specific sucrose-responding GRNs at the antennal tip. Particular GRNs for sucrose are also found in other insects (Dethier 1976; Blaney and Simmonds 1990; Hiroi et al. 2002; Haupt 2004; Thorne et al. 2004). The second expected phagostimulant, the sugar alcohol inositol, is ubiquitous in plants, a key structural component of phospholipids, involved in osmoregulation and phosphate storage in animals, as well as being a second messenger probably in all insects (Loewus 1990). Its phagostimulatory effect is well known in many insect species, including the tobacco hawkmoth *Manduca sexta* (Dethier 1976; Bernays and Chapman 1994; Chapman 2003). We found no evidence for a general phagostimulatory GRN type responding both to sucrose and inositol, as reported in the fleshfly *Sarchophaga bullata* (Shimada 1987). The different spike shapes of the responses, as well as responses to only one of them in some sensilla, indicated that separate GRNs were activated by the 2 substances. Overall, the weak firing

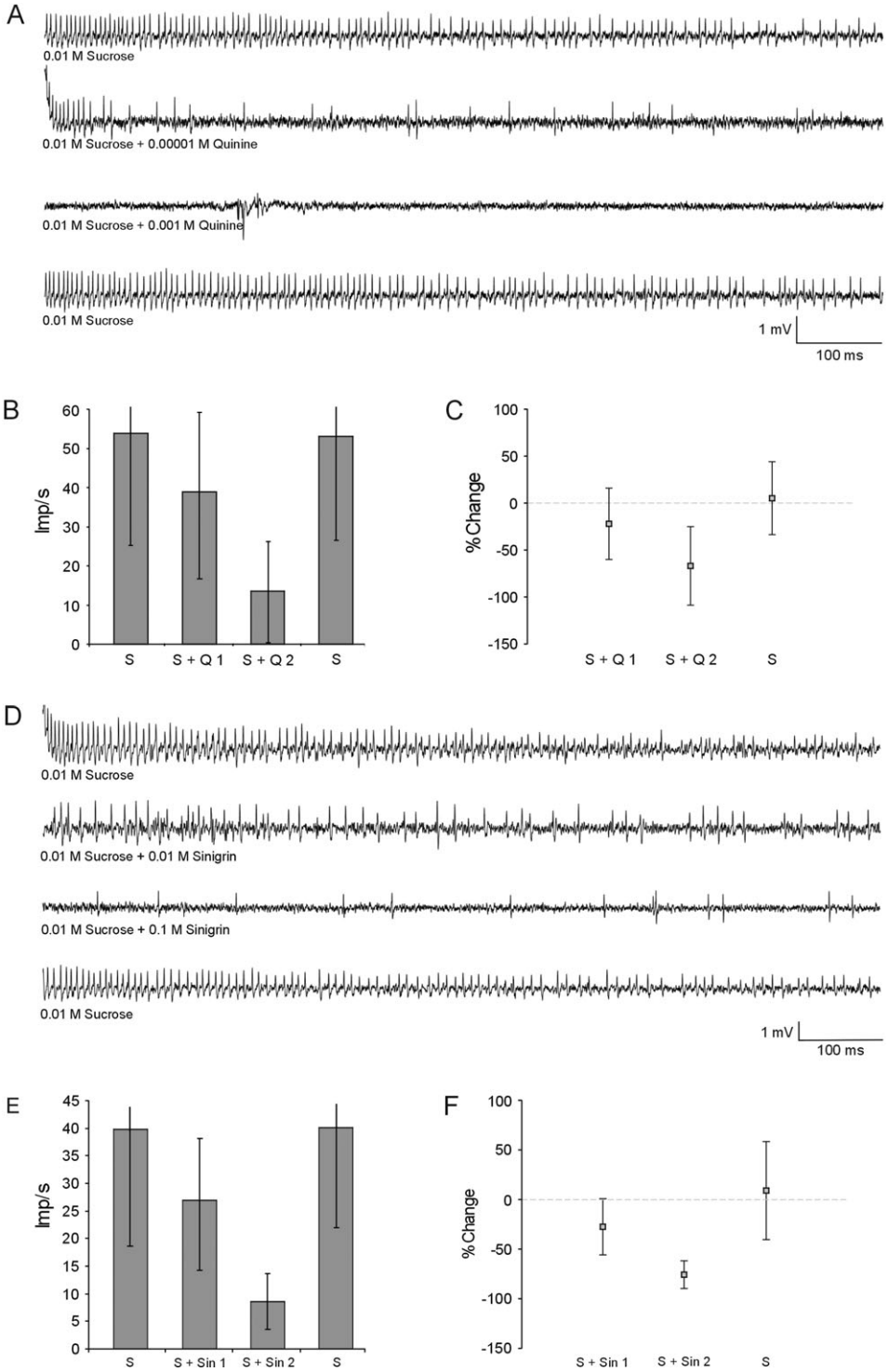

Figure 7 Responses to sucrose and mixtures of sucrose and bitter stimuli. **(A)** Response properties when stimulating one *sensilla chaetica* of *Heliothis virescens* with sucrose and mixtures of sucrose and quinine. There was a mutual inhibition of the quinine- and sucrose-responsive GRNs. **(B)** Average responses (imp/s) of 92 sensilla elicited by sucrose and mixtures of sucrose and quinine, showing inhibition of the sucrose-responding GRN by quinine. **(C)** The percentual change from the initial stimulation with sucrose, when stimulating with the quinine mixtures and the final stimulation with sucrose. **(D)** Response properties when stimulating one *s. chaetica* with sucrose and mixtures of sucrose and sinigrin. Sinigrin inhibited the sucrose-responsive GRN. **(E)** Average responses (imp/s) of 44 sensilla elicited by sucrose and mixtures of sucrose and sinigrin, showing that sinigrin inhibited the sucrose-responsive GRN. **(F)** The percentual change from the initial stimulation with sucrose, when stimulating with the sinigrin mixtures and the final stimulation with sucrose. S: 0.01 M sucrose, Q 1: 0.00001 M quinine, Q 2: 0.001 M quinine, Sin 1: 0.01 M sinigrin, Sin 2: 0.1 M sinigrin. The error bars show the standard deviation.

of few GRNs during stimulation with inositol, imply that no specific inositol GRN was present in these moths. In contrast, lepidopteran larvae, including *H. virescens*, have GRNs vigorously responding to inositol (Dethier and Kuch 1971; Shields and Mitchell 1995a; Schoonhoven et al. 1998; Bernays and Chapman 2000), implying that ingestion of inositol is more important for larvae than adults, although both need inositol due to its overall importance in the cells. One might speculate whether the nectar of the host plants is devoid of inositol, while it is present in leaves, explaining the absence of specialized inositol GRNs in adults. In addition, inositol might be more vital to growing and developing larvae than to adults, or adults synthesize inositol easier than larvae, diminishing the need to acquire it through ingestion.

As putative deterrents, we selected quinine and sinigrin. The prototypical bitter substance, the alkaloid quinine, is used in studies of many organisms, and the glucosinolate, sinigrin, is a nonappetitive tastant for *H. virescens* and other lepidopterans (Blaney and Simmonds 1988; Shields and Mitchell 1995b; Jørgensen et al. 2006). In a recent study of adult *H. virescens*, we have shown an aversive effect of both quinine and sinigrin in a conditioning context (Jørgensen et al. 2007). As shown in the present study, the bitter substances were detected by specific GRNs, corresponding to results obtained in studies of other insect species (Glendinning and Hills 1997; Bernays and Chapman 2000; Chapman 2003; Meunier et al. 2003; Thorne et al. 2004). The presence of bitter GRNs on insect antennae has not previously been found, in spite of particular search for them on the antennae of honeybees (De Brito Sanchez et al. 2005). Separation of the responses by the 2 quinine and sinigrin GRN types in *H. virescens* was based on the different response patterns, bursting and phasic–tonic, respectively, as well as responses to only one of the substances in some sensilla (Figure 2, Table 2). The bursting activity with long latency elicited by quinine in the GRNs is previously described in several insect species (Dethier 1980; Chapman et al. 1991; Schoonhoven et al. 1992). In humans, a long latency of the perception of bitter taste is known, which is proposed to be caused by a slow and long-lasting binding to the receptor (Rouseff 1990). An alternative interpretation of the responses to quinine and sinigrin in the present study might be that they originate from the same GRN, where the different temporal response patterns result from the involvement of 2 receptor types and possibly different excitatory transduction pathways, as suggested in *M. sexta* (Glendinning and Hills 1997). Coexpression of different bitter receptor proteins in the same GRN is shown in molecular studies of *Drosophila* (Thorne et al. 2004; Wang et al. 2004). Having several receptor types for different bitter substances in subsets of bitter-responsive GRNs increase the ability of the insect to discriminate the components in mixtures of bitter substances in plants and allow differentiation between toxic and harmless constituents, possibly eliciting different behaviors of acceptance or rejection. The behavioral experiments in this study showed a nonappetitive dose–dependent effect of quinine from 60% response at 0.001 M to 16% at 0.1 M concentration. This is in contrast to the highly appetitive effect of 0.001 and 0.01 M sucrose, eliciting response in 88 and 93% of the moths. Possibly, there is a hard-wired labeled line arrangement from the gustatory receptors to the brain driving the 2 different behaviors, as shown in mammals, by expressing bitter receptors in sugar gustatory cells, resulting in phagostimulatory behavior toward bitter substances (Mueller et al. 2005).

In nature, feeding animals, especially herbivores, encounter complex mixtures of nutrient and other substances. The responses of the GRNs are thus greatly affected by interactions between chemicals (Schoonhoven et al. 1992; Smith et al. 1994; Chapman 2003). In particular, the suppression of phagostimulant GRN activity by bitter substances, for example quinine, is a widespread phenomenon in several species (Dethier and Bowdan 1989, 1992; Chapman et al. 1991; Formaker et al. 1997; De Brito Sanchez et al. 2005). In the present study, quinine and sinigrin caused excitatory responses of particular GRNs as well as inhibition of the sucrose- and water-responsive GRNs (Figures 2A,E, 7), similar to the results obtained from GRNs on the prothoracic legs of *Drosophila* (Meunier et al. 2003). Feeding is positively correlated to activity in phagostimulatory GRNs, and negatively correlated to activity in deterrent GRNs, suggesting that quinine and sinigrin inhibit feeding both by exciting the deterrent GRNs and inhibiting the sucrose GRNs in *H. virescens* moths. In *H. virescens* larvae, a clear negative correlation has been found between the firing rate of the sinigrin-responsive GRNs and the amount of food consumed (Bernays and Chapman 2000; Bernays et al. 2000). In addition, behavioral studies of adult *H. virescens*, assaying PER during tarsal stimulation, showed an increasing inhibition of PER when stimulating with mixtures of sucrose and increasing concentrations of quinine (Ramaswamy et al. 1992). How the 2 kinds of information (phagostimulatory and aversive) is transmitted to second-order neurons in the insect central nervous system, like ventral unpaired median neurons or motorneurons, is an interesting question in future studies. In our study, there also seemed to be an inhibition of the bitter-responsive GRNs by the sucrose GRN because no spikes from these GRNs were observed when stimulating with the mixture. This kind of mutual inhibition is also observed in the parabranchial nucleus in hamsters (Smith et al. 1994). In addition, we demonstrated interactions between the water-responsive GRNs and the GRNs responding to the test substances. Suppression of water-responsive GRNs by other substances is previously shown in the fly *Phormia terranovae* (Rees 1970). In our study, neither quinine nor sinigrin caused any damage to the GRNs, shown by the similar firing to the initial and final sucrose stimulation.

The 2 inorganic salts, KCl and NaCl, are in general important in regulating the osmotic equilibrium in all organisms.

K^+ is the major cation in plants and present in high concentration in lepidopteran haemolymph (Dethier 1977). The responses to the inorganic salts in our study seem to originate from 2 GRNs eliciting small and large spike amplitudes, respectively. The GRN that often fired vigorously with small amplitude spikes to high salt concentrations might be the same GRN responding to sinigrin. Several GRN types involved in the response to inorganic salts, as well as deterrent receptors detecting high concentrations of salts are previously reported in other insects (Dethier and Hanson 1968; Bernays and Chapman 2001; Chapman 2003; Hiroi et al. 2004; Marella et al. 2006). In our study, the GRNs fired weakly to low salt concentrations and often vigorously to higher concentrations, which might influence feeding behavior, eliciting feeding, or avoidance, respectively, as shown in an early study of the blowfly (Dethier 1968). This seems to be reflected in the nutritional needs; low levels of salts being satisfactory, whereas high concentrations threaten the osmotic equilibrium. There is no evidence that insects ever suffer from salt deficiency in nature, possibly reflected by the weak overall salt responses. The stronger average firing to KCl than to NaCl might reflect the moths' common exposure to KCl in plants. We expected the 2 salts to be detected by the same GRN type, but some sensilla had GRNs responding to only one of the salts, indicating involvement of separate GRNs in salt detection. In contrast, 2 types of channels in the same GRN accept different cations in *Drosophila* (Siddiqi et al. 1989), suggesting a possible discrimination of salts by the same GRNs.

Ethanol was included because according to our observations, it seems to be attractive to the *H. virescens* larvae. The highest concentrations of ethanol and quinine elicited peculiar response properties, ethanol causing tonic firing of larger spikes than at lower concentrations, and quinine spikes of increasing amplitude during the bursts. Possibly, high concentrations of these substances act on the GRN membranes. One in vitro study of the amphiphilic quinine have shown that it permeate cell membranes directly, bypassing the receptors, and activate G-proteins (Naim et al. 1994). Ethanol is fat soluble, and might also act directly on the GRN membranes, causing large amplitude spikes. However, ethanol did not elicit responses in the sucrose or inositol-responding GRNs, in contrast to recordings from monkey chorda tympani nerves showing that ethanol stimulate sweet-best fibers and at high concentration some salt-best fibers (Hellekant et al. 1997).

Recordings from *s. chaetica* in the present study showed responses to more than 4 substances in each sensillum (Figure 6). Because *s. chaetica* have only 4 GRNs, it implies that at least one GRN responded to more than one substance, like the mammalian afferent gustatory fibers (Smith and Davis 2000). However, specific GRNs responding to sucrose and quinine were found, which by activation elicited appetitive and nonappetitive behavioral responses, respectively. Like in mammals, this might be a hard-wired arrangement where phagostimulants and deterrents elicit different innate behaviors.

Funding

Norwegian Research Council (project number 157936/v40).

Acknowledgements

We thank Marit Stranden for comments on the manuscript and Novartis Crop Protection AG, Rosental, Switzerland for kindly providing the insects.

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Accepted August 1, 2007