The Journal of Experimental Biology **199**, 1523–1534 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0283

IS CHEMOSENSORY INPUT ESSENTIAL FOR THE RAPID REJECTION OF TOXIC FOODS?

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Accepted 28 March 1996

Summary

Herbivorous insects often rapidly reject foods containing toxic plant compounds. While the functional significance of this rejection response is clear, the mechanistic basis is not. The role of peripheral chemoreceptors in the rapid rejection of toxic foods was examined using a model system consisting of nicotine and the tobacco hornworm (*Manduca sexta*), which is a pest of tobacco plants. When offered diets containing naturally occurring concentrations of nicotine, larvae initially fed readily, but abruptly stopped feeding within 30 s. A high percentage of larvae also exhibited toxic responses mediated by the central nervous system (twitching and writhing) to the ingested nicotine within 30 s, indicating that nicotine could have been absorbed within the same time as the rejection response. Two lines of evidence are provided against a role of peripheral chemoreceptors in this rapid rejection response. First, all mouthpart chemoreceptors were ablated from the larvae, and they were then subjected to feeding tests with diets containing either nicotine or a compound (caffeine) that is known to stimulate deterrent taste receptors in *M. sexta*. Whereas the ablations virtually eliminated the rejection response to caffeine, they had no measurable impact on the rejection response to nicotine. Second, sensory recordings from two important gustatory sensilla (the medial and lateral styloconica) failed to demonstrate a plausible role of sensory input from either sensillum in the rapid rejection of nicotine. The most parsimonious interpretation of these results is that the nicotine rejection response was mediated by a rapidly acting post-ingestive mechanism.

Key words: food rejection, toxic response, chemoreception, taste, caterpillar, *Manduca sexta*, Sphingidae.

Introduction

Plant-feeding insects often rapidly reject foods containing toxic plant compounds (e.g. Blaney and Simmonds, 1987; Wrubel and Bernays, 1990; Chapman *et al.* 1991). The functional significance of this rejection response is clear given that many toxic plant compounds are lethal at low doses (Holyoke and Reese, 1987), but the mechanistic basis is not. This is because most investigations into the mechanistic basis of rapid rejection responses have focused on peripheral chemosensory input and have largely ignored the potential contribution of post-ingestive feedback (Frazier, 1992). While chemosensory input alone can cause the rapid rejection of some toxic foods (e.g. Chapman *et al.* 1991; Frazier, 1992), there are other situations where post-ingestive feedback appears to act alone or in conjunction with chemosensory input to elicit a similarly rapid rejection (e.g. Glendinning and Slansky, 1994). Such a post-ingestive response mechanism would help limit consumption of toxic compounds whose threshold concentration for toxicity is below that for chemosensory detection (for examples, see Harley and Thorsteinson, 1967; Detzel and Wink, 1993). In this study, I document a rapid rejection response to a toxic plant compound and then determine the role of peripheral chemosensory input in this response.

For my model system, I used the tobacco hornworm (*Manduca sexta*: Sphingidae) and the toxic compound nicotine. This caterpillar feeds exclusively on the foliage of solanaceous plants (Madden and Chamberlin, 1945; Yamamoto and Fraenkel, 1960), which are rich in a multitude of toxic alkaloids, and thus would be expected to be proficient at handling them. One such compound, nicotine, is the dominant alkaloid in one of the hornworm's primary host plants, tobacco (*Nicotiana* spp.; Saitoh *et al.* 1985). Compared with most other insects, *M. sexta* is relatively insensitive to ingested nicotine (Hansberry and Middlekauff, 1940; Parr and Thurston, 1972). This insensitivity stems in part from constitutive tolerance mechanisms within the Malpighian tubules (Maddrell and Gardiner, 1976), blood–brain barrier (Murray *et al.* 1994) and cholinergic neuropile (Morris, 1984; Trimmer and Weeks, 1989). However, these constitutive mechanisms alone do not permit nicotine-naive, fifth-instar *M. sexta* to ingest naturally

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occurring concentrations of nicotine with impunity; they must first increase their midgut detoxification activities, which requires 30–36 h of intermittent feeding on a nicotine diet (Snyder and Glendinning, 1996). Prior to the full induction of nicotine-metabolizing enzymes, this caterpillar rejects diets containing naturally occurring concentrations of nicotine.

Little is known about the mechanism(s) by which dietary nicotine elicits rejection responses in *M. sexta* or any other lepidopteran caterpillars. In grasshoppers, nicotine stimulates deterrent taste receptors and inhibits sugar receptors, and this in turn elicits rejection (White and Chapman, 1990; Chapman *et al.* 1991). Nicotine also elicits a vigorous electrophysiological response in the taste receptors of several species of lepidopteran caterpillar (Blaney and Simmonds, 1988), but the effect of this response on feeding is unclear. Indeed, when two different species of caterpillar were offered a nicotine diet, they initially fed readily but then abruptly terminated feeding after 75–105 s; many experienced violent twitching and subsequently died within the next 60 s (Glendinning and Slansky, 1994). This sequence of events indicates that the nicotine diet lacked deterrent taste qualities and that the rapid rejection was due to a post-ingestive toxicity mechanism. Here, I examine how nicotine elicits feeding rejection in *M. sexta* by documenting the short-term responses of caterpillars to nicotine diets and then determining whether peripheral chemoreceptors play a role in these responses, using both electrophysiological and chemosensilla ablation techniques.

Materials and methods

Insects and diets

Larvae were obtained from the *Manduca* rearing facility at the Division of Neurobiology, University of Arizona, where they were fed a wheat-germ-based diet and maintained under established protocols at 25 °C with a 16 h:8 h L:D photoperiod (Bell and Joachim, 1976). Larvae of both sexes were used within 3–6 h of completing their moult to the fifth stadium. All larvae were naive to nicotine prior to testing. To control for any potential differences among larvae from different egg batches, individuals from each batch were interspersed across experimental treatments.

The rearing diet (henceforth termed the control diet) was used as a substrate for presenting nicotine (Sigma Chemical Co.) in feeding tests. The free base form of nicotine was used because that is what hornworms encounter in tobacco plants. Four different concentrations of nicotine (0, 0.62, 6.2 and 46.2 mmol kg^{-1} nicotine diet: 0, 0.01, 0.1 and 0.75 % fresh mass, respectively) were obtained by heating the control diet to approximately 60 °C, adding the appropriate amount of nicotine, and stirring vigorously for 3 min. These nicotine concentrations fell within the range naturally found in tobacco leaves (Sisson and Saunders, 1982; Saitoh *et al.* 1985; K. Kester, unpublished data).

What are the short-term feeding responses of caterpillars to nicotine?

This experiment (experiment 1) examined how freshly moulted larvae respond to naturally occurring concentrations of dietary nicotine. Because all larvae were naive to nicotine, and because the tests were limited to 2 min, there would have been no induced tolerance.

In each test, a caterpillar was placed in a holding cage (a clear plastic cylinder 7.5 cm in diameter and 10 cm tall with wire-mesh screening on top) and deprived of food for 30 min to standardize its 'hunger' state. Because the inter-meal interval for these caterpillars usually ranges between 15 and 30 min (Reynolds *et al.* 1986), it is unlikely that this food-deprivation period created an extreme state of 'hunger'. Next, a block of control diet (approximately 1 cm×1 cm×0.25 cm) was positioned approximately 0.25 cm from the caterpillar's head. Given the large size of the caterpillars and the characteristic head-rocking motion associated with feeding, feeding could be discriminated from palpating and test-biting relatively easily. Feeding activities as well as any instances of nicotine toxicity were monitored for 2 min after the initiation of feeding, and these observations were recorded on a software-based event recorder. Documented signs of nicotine toxicity in *M. sexta* (Snyder *et al.* 1993) and other insects (Negherbon, 1959; Glendinning and Slansky, 1994) include twitching (i.e. rapid body contortions) and writhing (i.e. slow body twisting).

The behavioural responses of the larvae to the nicotine diets were analyzed in three ways. First, total time spent feeding during the 2 min test was determined; for this analysis, only those larvae that failed to exhibit a toxic response were included. Total time spent feeding was compared across the four nicotine concentrations using a Tukey-type nonparametric multiple comparison of medians (Zar, 1984). Unless indicated otherwise, the alpha level was set at 0.05 across all experiments. In this and all subsequent statistical comparisons, the residual variation of the data was tested for normality and homoskedasticity, and nonparametric tests were used when one or both of these assumptions were violated. Second, the percentage of larvae exhibiting a toxic response (i.e. twitching or writhing) was determined. Third, the time course of the rejection response was determined separately for larvae that either did or did not exhibit a toxic response. To this end, the first 60 s of each larva's feeding test was divided into 10 sequential 6 s bins, and then total time spent feeding was determined during each bin. To determine the earliest bin during which larvae exhibited significantly less feeding (compared with the first time interval), the minimal number of pair-wise comparisons was made to detect a significant difference based on a Wilcoxon matched-pairs signed-ranks test; this turned out to be at most four comparisons in all cases. To control for the use of multiple paired comparisons on the same data set, the Bonferroni correction was used (i.e. the alpha level was divided by the number of comparisons, alpha$\leqslant$0.05/4).

Does nicotine elicit a sensory response in the styloconic sensilla?

My approach (experiment 2) was to examine the nicotine response of the two gustatory sensilla that are known to play a central role in mediating rejection of foods in *M. sexta*: the medial and lateral maxillary styloconica (Waldbauer and

Fraenkel, 1961; de Boer and Hanson, 1987). A range of nicotine concentrations that spanned those used in the feeding tests above was examined.

To record sensory responses from the maxillary styloconica, a non-invasive tip-recording method (see Gothilf and Hanson, 1994) was used. Neural records were processed using a high-impedance preamplifier using a baseline-restoring circuit (George Johnson, Baltimore, MA, USA; see Frazier and Hanson, 1986) and an amplifier-filter with a band stop set at 130–1200 Hz. Neural records were digitized and stored directly onto a computer with SAPID tools (Smith *et al.* 1990).

For each of 10 larvae, one galea was selected, and then the lateral and medial styloconica were stimulated with a control solution (i.e. 100 mmol l^{-1} KCl) and four nicotine solutions (0.1, 1, 5 and 10 mmol l^{-1}); the nicotine was dissolved in the control solution. The number of action potentials generated was quantified from 10 until 1010 ms after contact with a sensillum. To minimize the effect of solvent evaporation at the tip of the recording/stimulating electrode, fluid from the tip was sucked up with a piece of filter paper less than 7 s before each stimulation. At least 2 min was allowed between successive stimulations of the same sensillum.

Previous histological investigations demonstrated that the styloconic sensilla of *M. sexta* each contain four taste receptor neurones (Schoonhoven and Dethier, 1966), and they have been called the salt, inositol, sugar and deterrent receptors on the basis of their respective tuning characteristics (Schoonhoven, 1972; Schoonhoven *et al.* 1992). Whereas the inositol receptors respond almost exclusively to inositol, the salt, sugar and deterrent receptors respond to variety of compounds that humans characterize as salty, sweet and bitter, respectively (Schoonhoven, 1974; Frazier, 1986, J. I. Glendinning, unpublished data).

The activity of different taste receptors was discriminated by analyzing spikes within a neural record on the basis of their amplitude and temporal pattern of firing (see Peterson *et al.* 1993). Using such spike discrimination procedures, I determined which taste receptors within the lateral and medial styloconica responded to nicotine. To identify which nicotine concentrations caused significant stimulation (or inhibition) of a given taste receptor, its firing rate in response to the control solution (i.e. 100 mmol l^{-1} KCl) was compared with that in response to each of the four nicotine concentrations separately, using four paired (two-tailed) *t*-tests (alpha=0.05/4).

Which taste receptor is stimulated by nicotine at low concentrations?

This experiment (experiment 3) critically examined the unexpected conclusion of experiment 2: that 0.1 mmol l^{-1} nicotine stimulates the sugar receptor within the lateral styloconica. My approach was to focus on the sugar, inositol and deterrent receptors within the lateral styloconica because they all express a firing pattern similar to that elicited by 0.1 mmol l^{-1} nicotine (i.e. a phasic–tonic pattern). Each receptor was stimulated with its best stimulus, either alone or mixed with 0.1 mmol l^{-1} nicotine. If nicotine stimulated one of the receptors, then I expected that the mixture would elicit more spikes than the best stimulus alone.

The best stimuli for the sugar, inositol and deterrent receptors are, in respective order, glucose, inositol and caffeine. Two concentrations of each best stimulus were used; one elicited approximately 50 % and the other 75 % of its target receptor's maximal firing rate (J. I. Glendinning, unpublished data). The concentrations were 50 and 100 mmol l^{-1} for glucose, 0.5 and 1 mmol l^{-1} for inositol, and 0.1 and 1 mmol l^{-1} for caffeine.

For each stimulus (e.g. glucose), the protocol was as follows. One lateral styloconica was selected and stimulated with four solutions: each glucose concentration alone (i.e. 50 and 100 mmol l^{-1}) and each mixed with 0.1 mmol l^{-1} nicotine. To determine whether nicotine significantly enhanced the response of the sugar receptor, the instantaneous firing rate of the sugar receptor was calculated in response to each solution (i.e. the number of spikes during each successive 100 ms interval). Repeated-measure analyses of variance (ANOVAs) were calculated on the instantaneous firing rate data, separately for each glucose concentration. Thus, there were two repeated factors: the presence or absence of 0.1 mmol l^{-1} nicotine and time (the 10 sequential 100 ms bins). The same procedure was repeated with inositol and caffeine. Sample sizes ranged between 10 and 12 caterpillars for each stimulus.

Does ablating all mouthpart chemoreceptors affect the nicotine response?

In experiments 2 and 3, the potential role of chemosensory input from the styloconic sensilla in the nicotine rejection response was examined. However, the ability to predict feeding behaviour on the basis of sensory responses from these sensilla is limited by the fact that *M. sexta* possesses additional mouthpart chemosensilla, which may have contributed to the rejection response. There are olfactory receptors on the antennae and maxillary palp, and gustatory receptors on the maxillary palp and epipharynx (Schoonhoven, 1972). In the present experiment (experiment 4), I addressed this problem by surgically ablating all known mouthpart chemosensilla (using a procedure that does not interfere with feeding *per se*; de Boer and Hanson, 1987), and then determining how this influenced feeding responses to either the control or the 46.2 mmol kg^{-1} nicotine diet. I reasoned that if ablated larvae responded to the nicotine diet in a manner that was indistinguishable from that of non-ablated larvae (described in experiment 1), then this would provide compelling evidence against a role of chemosensory input in the rapid rejection of nicotine.

However, there is an alternative interpretation of this hypothesized result: the persistence of the nicotine rejection response after chemoreceptor ablation may be due to the recruitment of alternative sensory mechanisms. To address this possibility, a parallel experiment was carried out using another feeding inhibitor: caffeine. Because caffeine strongly stimulates the deterrent taste receptors of *M. sexta* (see experiment 3), and because stimulation of deterrent taste receptors is thought to inhibit feeding in *M. sexta* (Peterson *et*

al. 1993), I expected that ablation of all mouthpart chemoreceptors would eliminate the rejection response to caffeine. Such a result would provide strong evidence against the possibility that ablated larvae recruit alternative sensory mechanisms for detecting nicotine in the diet, at least over the time course of this experiment.

Ablation procedure

Established procedures were employed for ablating all mouthpart chemosensilla from larval *M. sexta* (Hanson and Dethier, 1973; de Boer and Hanson, 1987). All ablations were performed during the second day of the fourth stadium so that behavioural observations could be made on freshly moulted, fifth-instar larvae. Immediately following the operation, larvae were returned to the artificial diet; most recommenced feeding within 3–4 h. After moulting to the fifth instar, which occurred 3–4 days after the operation, the success of each operation was assessed. Larvae with successful operations lacked any evidence of functional chemosensilla and had incision sites that were completely sealed with a new layer of cuticle. All larvae with unsuccessful operations (i.e. ones that seemed feeble or unusually small, had incomplete ablations, and/or had other signs of surgical complications) were discarded; only 2 % of the ablated larvae fell into this category.

Nicotine tests

Within 3–6 h of moulting, each ablated larva was subjected to one of two types of feeding tests. Each type of test involved a within-subject design, where the larva was offered two test diets in sequence, under a no-choice protocol. In the first, I deprived the larvae of food for 30 min, recorded feeding responses to the control diet for 2 min (trial 1a), fed it the control diet *ad libitum* for 30 min, deprived it of food again for 30 min, and then recorded feeding responses to the 46.2 mmol kg^{-1} nicotine diet for 2 min (trial 1b). The second type of feeding test was identical in all respects except that a control diet was offered for both trials (in this case, trials 2a and 2b). This latter feeding test was necessary to control for any unexpected effects of the ablations, which might have caused larvae to reduce feeding during trial 2b in the absence of nicotine. All other details concerning the experimental conditions and data collection procedures were as in experiment 1.

Two types of comparisons were made between trials 1a and 1b or 2a and 2b, using a Wilcoxon matched-pairs signed-rank test (one-tailed). Total time spent feeding across the entire 2 min of each test (alpha=0.05), and across successive 6 s bins during the first 60 s of both trials, were compared. For the latter comparison, the earliest bin during which larvae exhibited significantly less feeding on the nicotine diet (as compared with the corresponding time interval on the control diet) was determined; this required no more than four pair-wise comparisons in all cases (alpha=0.05/4). All other statistical details are as in experiment 1.

Caffeine tests

These tests were identical to those with nicotine in all respects except that both ablated and non-ablated larvae were tested. A caffeine concentration that strongly inhibited feeding in *M. sexta* (30.9 mmol kg^{-1} caffeine diet; =0.6 % fresh mass) during preliminary feeding trials was determined.

Results

What are the short-term feeding responses of caterpillars to nicotine?

After initiating feeding on the nicotine diets, caterpillars exhibited two types of response to the nicotine. Some stopped feeding abruptly and became quiescent (i.e. exhibited a rejection response) and others started to twitch or writhe (i.e. exhibited a toxic response). Among caterpillars that did not exhibit a toxic response, I observed a significant and robust decrease in total time spent feeding with increasing nicotine concentration (Fig. 1A). Among all caterpillars, I observed a

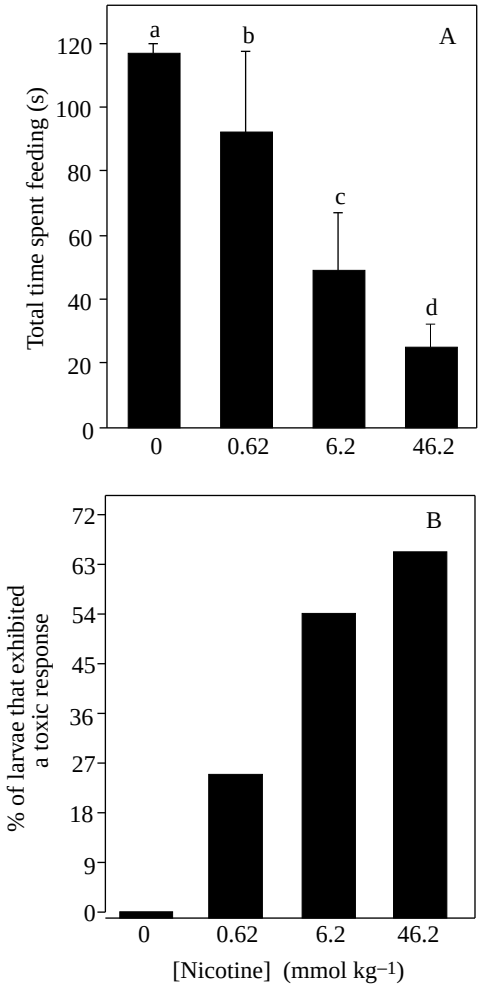

Fig. 1. Larval responses to diets containing different concentrations of nicotine during a 2 min feeding test. (A) Total time spent feeding across the 2 min test (median + median absolute difference) in those larvae that did not exhibit a toxic response (i.e. twitch or writhe). Different letters (a, b, c and d) indicate significant differences among medians (Tukey-type nonparametric multiple comparison of medians; $P \leqslant 0.05$). (B) Percentage of larvae that exhibited a toxic response. N=29–33 larvae per nicotine concentration

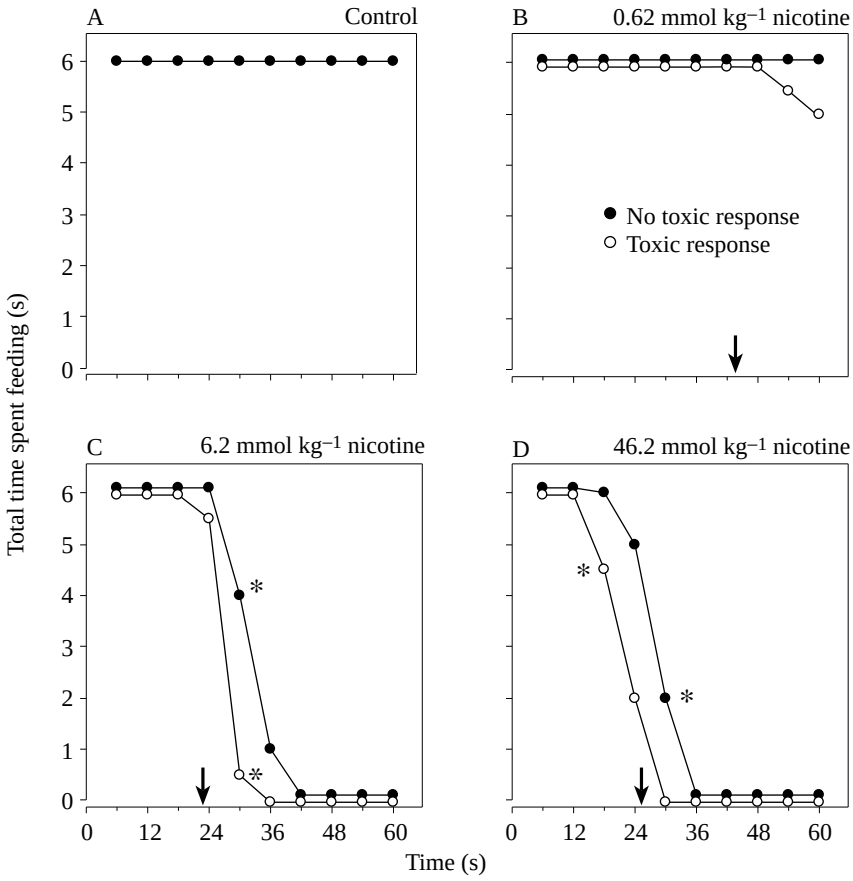

Fig. 2. Total time spent feeding on diets containing 0 (A), 0.62 (B), 6.2 (C) or 46.2 mmol kg^{-1} nicotine (D) during 10 successive 6 s time intervals. These time intervals correspond to the initial 60 s of the 2 min feeding test. Results for larvae that did or did not exhibit a toxic response are presented separately in each panel; the median latency of the toxic response is indicated by an arrow on the *x*-axis in B–D. Asterisks indicate the earliest time interval during which larvae exhibited significantly less feeding (compared with the first time interval); separate statistical comparisons were made for each line within C and D (Wilcoxon matched-pairs signed-ranks test; $*P<0.05/4$). See Fig. 1 for sample sizes.

marked increase in occurrence of toxic responses (i.e. twitching and writhing) with increasing nicotine concentration (Fig. 1B).

The time course of the rejection and toxic responses was extremely rapid on diets with $\geqslant$6.2 mmol kg^{-1} nicotine (Fig. 2). Whereas the caterpillars initially fed readily on these high-nicotine diets, their rate of feeding (i.e. total time spent feeding per 6 s bin) decreased precipitously and significantly within 30 s, irrespective of whether they exhibited a toxic response. Further, visual inspection of Fig. 2C,D reveals a striking temporal coincidence between the time courses of the rejection and toxic responses (see arrows on *x*-axes) to the three nicotine diets. In contrast, nearly all caterpillars offered the control and 0.62 mmol kg^{-1} nicotine diets fed throughout the initial 60 s of the test (Fig. 2A,B).

The median latency (and range) for the toxic response were 43 s (28–85 s), 23 s (7–47 s) and 26 s (5–59 s) on the 0.62, 6.2 and 46.2 mmol kg^{-1} nicotine diets, respectively (Fig. 2). In one case, a larva thrashed within 5 s of initiating feeding on the 46.2 mmol kg^{-1} nicotine diet. Although the intensity of the toxic response varied among caterpillars, I scored only those instances that were unambiguous; i.e. those that lasted at least 1 s and involved either violent twitching or lateral thrashing of the entire body. The duration of the toxic responses varied between 1 and 10 s, during which time the larvae usually become physically separated from the diet cube. Once the toxic response subsided, many larvae reinitiated feeding within the 2 min test period. However, the percentage that reinitiated feeding decreased with increasing nicotine concentration (71, 44 and 35 % on the 0.62, 6.2 and 46.2 mmol kg^{-1} nicotine diets, respectively; data not shown).

Does nicotine elicit a sensory response in the styloconic sensilla?

Spike discrimination

I could reliably assign spikes from neural records to specific receptors by examining the first 1 s of the sensory response. For instance, Fig. 3B–D illustrates typical responses of the inositol, sugar and deterrent receptors in the lateral styloconica (for examples in the medial styloconica, see Peterson *et al.* 1993). In these traces, note that only a single unit (or receptor) is discharging consistently and frequently, and that the spike amplitude and temporal pattern of firing (e.g. extent of the phasic response) of each unit differ across traces. Fig. 3A illustrates the typical response to salts such as KCl. In contrast to the traces in Fig. 3B–D, this response contains two units, discharging at a similar frequency. By analyzing this trace on the basis of inter-spike interval, it is evident that one of the units has a phasic–tonic pattern of firing and the other an irregular pattern of firing (see Fig. 4A). It is certain that the latter unit is the salt receptor because this irregular temporal pattern persists across a wide range of KCl and NaCl concentrations (0.05–0.75 mol l^{-1}; J. I. Glendinning, unpublished data). It is likely that the unit with the phasic–tonic

firing pattern in Fig. 4A is the sugar receptor because of the similarity of its spike amplitude with that of the sugar receptor in Fig. 3C.

Sensory responses to nicotine

The neural records from mixtures of KCl and nicotine and those from KCl alone did not differ in the units firing. Spike amplitudes were similar across the nicotine concentrations (i.e. 0, 0.1, 1, 10 and 50 mmol l^{-1}), and all sensory responses contained two active units; one had an irregular firing pattern and the other a phasic–tonic pattern (e.g. see Fig. 4B). Thus, given that KCl appears to stimulate the sugar and salt receptors, it is likely that the stimulatory effects of nicotine involved these same two receptors. On the basis of this inference, I compared the firing rate of the presumed sugar and salt receptors across the nicotine series. The only significant effect was a twofold increase in the firing rate of the lateral sugar receptor in response to 0.1 mmol l^{-1} nicotine (Figs 4B, 5). Unexpectedly, the nicotine concentrations that elicited rejection in experiment 1 (10–50 mmol kg^{-1} nicotine diet) failed to stimulate or inhibit any receptors significantly. These data provide no obvious mechanisms whereby the medial and lateral styloconica could have mediated the nicotine rejection responses in experiment 1.

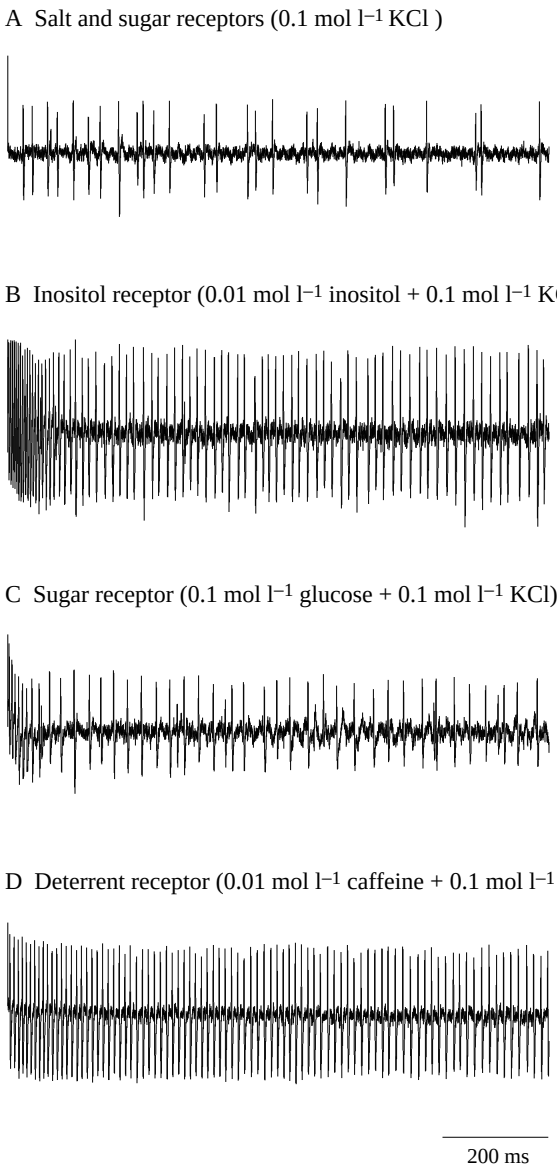

Fig. 3. Typical responses of the four gustatory receptors within the lateral styloconic sensilla. The traces illustrate the response of (A) salt and sugar receptors to 100 mmol l^{-1} KCl, (B) an inositol receptor to 10 mmol l^{-1} inositol + 100 mmol l^{-1} KCl, (C) a sugar receptor to 100 mmol l^{-1} glucose + 100 mmol l^{-1} KCl, and (D) a deterrent receptor to 10 mmol l^{-1} caffeine + 100 mmol l^{-1} KCl. A resolution of trace A into separated spike trains is presented in Fig. 4A. Note that in traces B and C, there is a rapidly discharging, large unit (the inositol and sugar receptors, respectively), and a slowly discharging, smaller unit (the salt receptor responding to KCl).

Which taste receptor is stimulated by nicotine at low concentrations?

The central finding of this experiment was that 0.1 mmol l^{-1} nicotine significantly enhanced the response of the sugar receptor to 50 mmol l^{-1} but not 100 mmol l^{-1} glucose (Table 1; Fig. 6C,D),

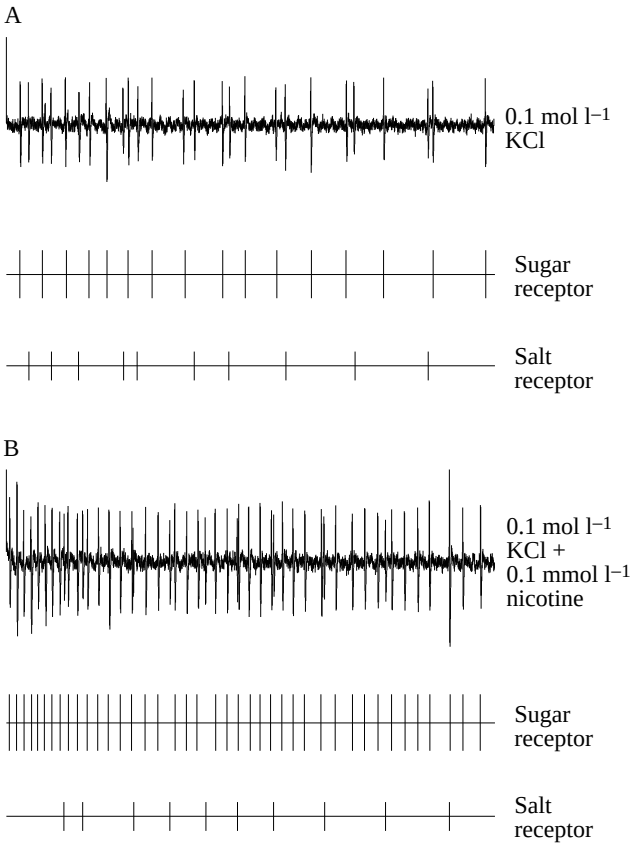

Fig. 4. Illustration of how sensory responses from the same lateral styloconicum to 100 mmol l^{-1} KCl (A) and a mixture of 100 mmol l^{-1} KCl and 0.1 mmol l^{-1} nicotine (B) were resolved. In each panel, the neural record is at the top, and the inferred location of spikes from the sugar and salt receptors are below. Note that the addition of nicotine increased the firing rate of the sugar but not of the salt receptor. Each trace corresponds to a 1 s stimulus period.

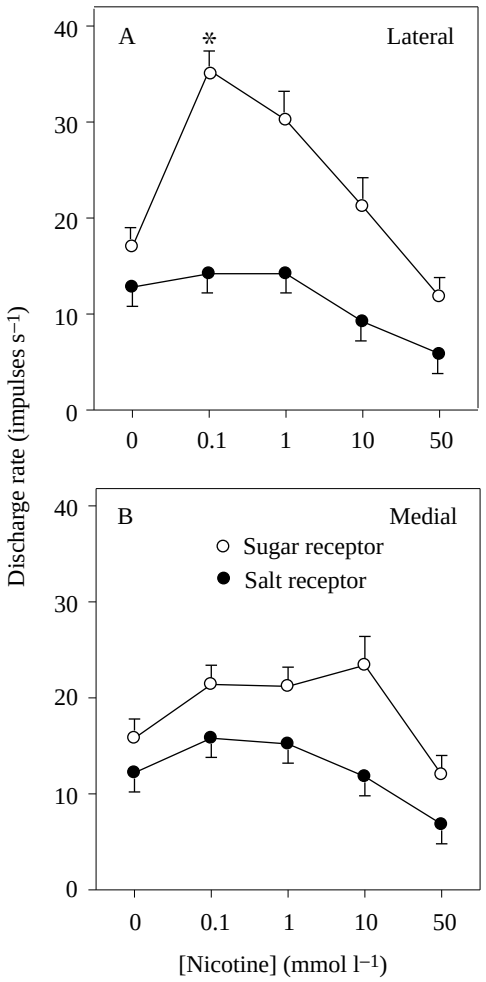

Fig. 5. Firing rates (impulses s^{-1}) of the sugar and salt receptors within the lateral (A) and medial (B) styloconica in response to a concentration series of nicotine (mean ± S.E.M.; N=10 per styloconica). All solutions contained 100 mmol l^{-1} KCl. The only nicotine concentration that caused a significant increase in firing rate (compared with the response to 0 mmol l^{-1} nicotine) was 0.1 mmol l^{-1}, and this involved the sugar receptor in the lateral styloconica (Wilcoxon matched-pairs signed-rank tests; *$P \leqslant 0.05/4$).

thereby corroborating the finding of the previous experiment. Visual inspection of Fig. 6C, together with the nonsignificant interaction between nicotine concentration and time (Table 1), indicates that the stimulatory effect of nicotine occurred during both the phasic and tonic portions of the response.

I did not find that 0.1 mmol l^{-1} nicotine enhanced the response of the inositol receptor (to 0.5 or 1 mmol l^{-1} inositol) or the deterrent receptor (to 0.1 or 10 mmol l^{-1} caffeine) (Table 1; Fig. 6A,B,E,F). The only significant effect of nicotine on either of these receptors was inhibitory; it reduced the response of the inositol receptor to 0.5 mmol l^{-1} inositol. Visual inspection of Fig. 6A, together with the significant interaction between nicotine concentration and time for 0.5 mmol l^{-1} inositol (Table 1), indicates that the inhibitory effect of nicotine occurred primarily during the phasic portion

Table 1. *Results of two-way repeated-measure ANOVAs to determine whether the presence of 0.1 mmol l^{-1} nicotine enhanced the responsiveness of the inositol, sugar or deterrent receptor within the lateral styloconica (see data in Fig. 6)*

			Source of variation		
Receptor	Best stimulus	Concentration (mmol l^{-1})	Presence of nicotine	Time	Interaction
Sugar	Glucose	50	9.3*	61.4*	2.5
		100	1.1	53.8*	1.7
		d.f.	1, 99	11, 99	9, 99
Inositol	Inositol	0.5	17.8*	64.8*	3.0*
		1	1.1	107.0*	0.5
		d.f.	1, 99	11, 99	9, 99
Deterrent	Caffeine	0.1	1.0	6.7*	0.3
		10	0.2	13.3*	1.9
		d.f.	1, 81	9, 81	9, 81

Each receptor was stimulated with two concentrations of its best stimulus, and the response to each of these concentrations was analyzed separately. In each analysis, the main effects were the presence of 0.1 mmol l^{-1} nicotine (yes or no) and time (10 consecutive 100 ms bins), and the response variable was instantaneous discharge rate; *$P \leqslant 0.05$.

of the response (i.e. the first 100 ms). Indeed, the instantaneous firing rate during the initial 100 ms differed significantly between the responses to 0.1 mmol l^{-1} nicotine alone and to 0.1 mmol l^{-1} nicotine plus 50 mmol l^{-1} glucose (paired t-test; t_{11}=3.29; P<0.05).

The effect of time was significant for all receptors and solutions (Table 1; Fig. 6). This reflects the fact that all receptors exhibited a phasic–tonic response to their respective stimuli, although the extent of the response differed across receptors.

Does ablating all mouthpart chemoreceptors affect the nicotine response?

Among caterpillars that did not exhibit a toxic response, total time spent feeding (median ± median absolute difference) was 92±15 s on the control diet and 25±10 s on the 46.2 mmol kg^{-1} nicotine diet; these medians differed significantly from one another (d.f.=18, z=–3.74, $P \leqslant 0.05$). In contrast, when control diet was offered twice in succession, total time spent feeding was 84±26 s and 89±18 s; these medians did not differ significantly from one another (d.f.=22, z=–1.03; P>0.05). No caterpillars exhibited a toxic response to the control diet, but 35 % of them did to the 46.2 mmol kg^{-1} nicotine diet.

The time course of the rejection and toxic responses to the 46.2 mmol kg^{-1} nicotine diet in ablated caterpillars was rapid (Fig. 7). Consumption of the 46.2 mmol kg^{-1} nicotine diet decreased significantly within 30 s of initiating feeding, irrespective of whether the caterpillars exhibited a toxic response. That the median latency of the toxic response was

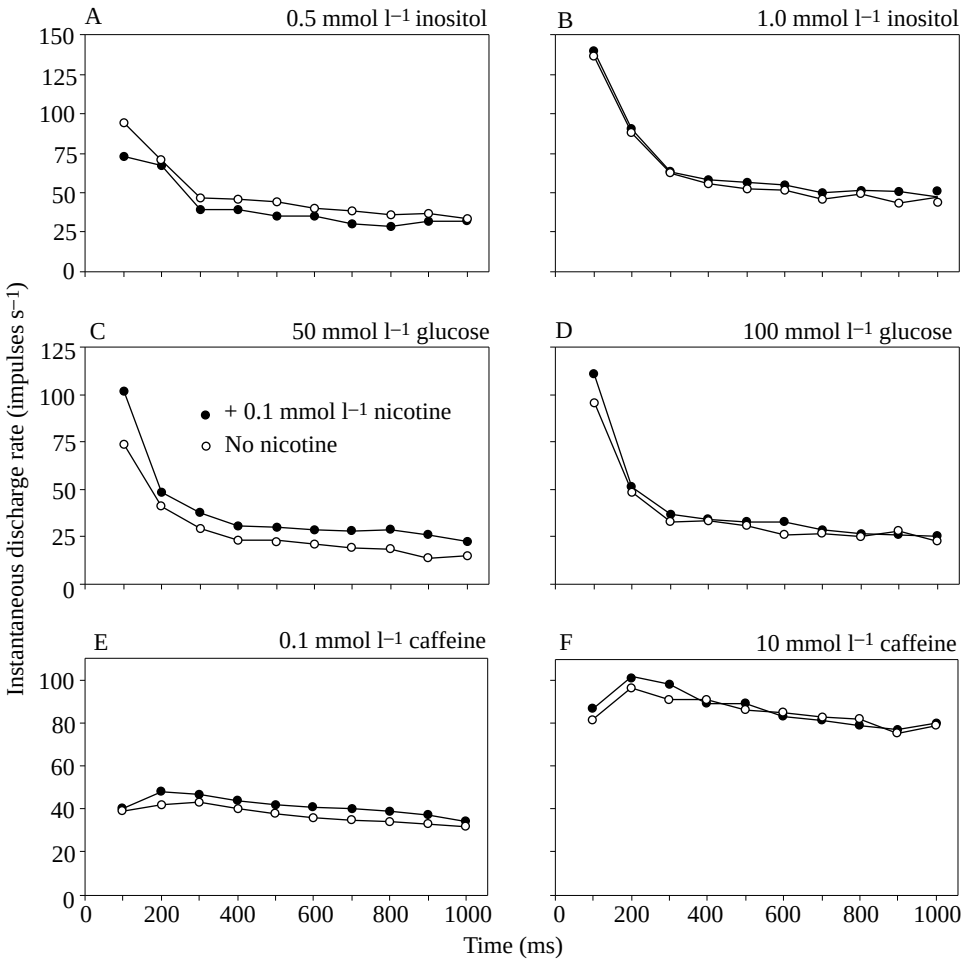

Fig. 6. Effect of 0.1 mmol l^{-1} nicotine on the response properties of the inositol, sugar and deterrent receptors within the lateral styloconica. Results are presented as mean instantaneous firing rates of the inositol receptor to 0.5 (A) and 1.0 mmol l^{-1} inositol (B), of the sugar receptor to 50 (C) and 100 mmol l^{-1} glucose (D), and of the deterrent receptor to 0.1 (E) and 10 mmol l^{-1} caffeine (F). In each panel, responses to these stimuli with (filled circles) and without (open circles) 0.1 mmol l^{-1} nicotine are compared. All solutions contained 100 mmol l^{-1} KCl. See Table 1 for the statistical analysis of these results. The units on the *y*-axes differ among compounds.

34 s (range=13–59 s) illustrates the remarkable temporal coincidence between the time course of the rejection and toxic responses to the nicotine diet. Thus, the rejection response of larvae with ablated mouthpart chemoreceptors was nearly identical to that of larvae with intact mouthpart chemoreceptors (see experiment 1).

In contrast to the rejection response to nicotine, that to caffeine was virtually eliminated by ablating the mouthpart chemoreceptors. Among non-ablated caterpillars, total time feeding was 70±26 s on the control diet and 33±14 s on the 30.9 mmol kg^{-1} caffeine diet; these medians differed significantly from one another (d.f.=24, z=–4.18, $P \leqslant 0.05$). Among ablated caterpillars, total time spent feeding was 77±36 s on the control diet and 71±26 s on the caffeine diet; these medians did not differ significantly from one another (d.f.=24, z=–1.45; $P>0.05$). The difference in caffeine responsiveness between non-ablated and ablated caterpillars is even more pronounced when one looks at the initial feeding response. Non-ablated caterpillars rejected the caffeine diet during the first 6 s of the feeding test, whereas the ablated ones fed readily and consistently throughout the initial 60 s (Fig. 8). Caffeine did not elicit a toxic response in any of the caterpillars. Taken together, these results indicate that the larvae do not recruit alternative sensory mechanisms for assessing food quality following the ablation of mouthpart chemoreceptors.

Discussion

Role of chemosensory input in the rapid rejection of nicotine

I provide several lines of evidence against a role of peripheral chemoreceptors in mediating the rapid rejection of nicotine. The strongest came from the chemoreceptor ablation studies: whereas the ablations virtually eliminated the rejection response to a compound that is known to stimulate deterrent taste receptors in *M. sexta* (caffeine), they had no effect on the rejection response to nicotine. The second line of evidence was that sensory recordings from the medial and lateral styloconica failed to reveal a plausible role of gustatory input in the rapid rejection of nicotine. The only nicotine concentration that produced a significant sensory response (positive or negative) was 0.1 mmol l^{-1}, and this concentration was more than an order of magnitude below that required to elicit rejection robustly (i.e. $\geqslant$6.2 mmol kg^{-1}). Moreover, the sensory response to 0.1 mmol l^{-1} nicotine involved the sugar receptor, and stimulation of this receptor is thought to stimulate rather

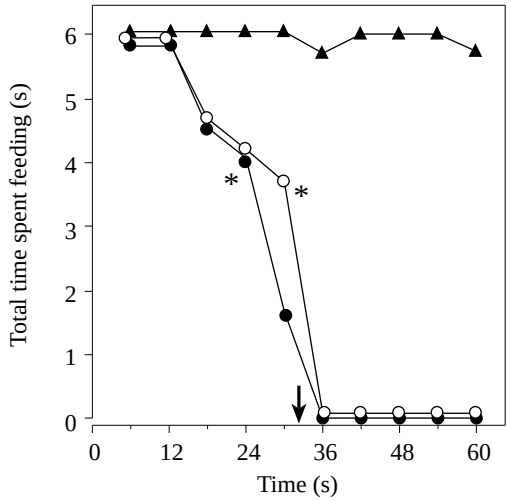

Fig. 7. Total time spent feeding by ablated larvae on the control diet (filled triangles) and then the 46.2 mmol kg^{-1} nicotine diet (open and filled circles) during 10 successive 6 s time intervals. These time intervals correspond to the initial 60 s of the 2 min feeding tests (i.e. trials 1a and 1b; see text). Results for those larvae that either did (open circles) or did not (filled circles) exhibit a toxic response to the nicotine diet are presented separately; the median latency of the toxic response is indicated by an arrow on the *x*-axis. Asterisks indicate the earliest time interval during which larvae exhibited significantly less feeding (compared with the corresponding time interval on the control diet); separate comparisons were made for larvae that either did or did not exhibit toxic responses (Wilcoxon matched-pairs signed-ranks test; *P<0.05/4). N=19 larvae.

than inhibit feeding (Schoonhoven, 1974). Finally, I did not observe any instances of irregular firing patterns (or bursting activity) in response to nicotine stimulation, which is another postulated gustatory mechanism for food rejection (Schoonhoven *et al.* 1992).

In the absence of deterrent chemosensory input, the most likely explanation for the rejection response is that nicotine triggered a rapidly acting post-ingestive mechanism for detecting nicotine. Even though there are other examples of toxic compounds eliciting behavioural rejection through a post-ingestive mechanism (e.g. Bernays and Lee, 1988; Glendinning and Slansky, 1995), the time course of these responses was relatively slow; i.e. across successive meals, not within the same meal. The rapid detection mechanism described herein should help *M. sexta* regulate the intake of nicotine (and perhaps of other toxic alkaloids present in its solanaceous food plants) to physiologically tolerable levels while the animal is inducing the necessary detoxification enzymes (e.g. Snyder and Glendinning, 1996). Further, because many plant compounds are toxic at concentrations that fail to elicit taste-rejection (Harley and Thorsteinson, 1967; Detzel and Wink, 1993), one would expect that insects lacking a rapidly acting, post-ingestive detection mechanism would be more likely to ingest lethal doses of these compounds. There is some experimental support for this hypothesis: when two species of noctuid caterpillars (*Spodoptera frugiperda* and *Anticarsia gemmatalis*) were offered a 20 mmol kg^{-1} nicotine diet, they all fed readily but subsequently died within 3 min (Glendinning and Slansky, 1994). If these caterpillars had detected the nicotine sooner, they might have avoided death.

Even though the physiological basis of the rapid rejection response remains obscure, the results of this and other studies offer several insights. Because smearing a small block of 46.2 mmol kg^{-1} nicotine diet on the ventrum of freshly moulted, fifth-instar *M. sexta* (so as to simulate standing on the diet) does not produce toxic effects within 3 min (J. I. Glendinning, unpublished data), it is unlikely that the primary route of entry for nicotine was diffusion through the external cuticle or the tracheal system after the nicotine had volatilized. Further, because the toxic response occurred within 10 s in several instances, it is unlikely that there would have been sufficient time for ingested nicotine to have moved through the foregut and then diffused across the midgut tissues. A more likely scenario is that nicotine diffused through the cuticle lining the cibarial cavity and foregut during mastication and ingestion, and then entered the haemolymph. That this cuticle is permeable to nicotine is suggested by the observation that pure nicotine diffuses across the external cuticle of *M. sexta* after a median latency of 32 s (range 6–84 s; J. I. Glendinning,

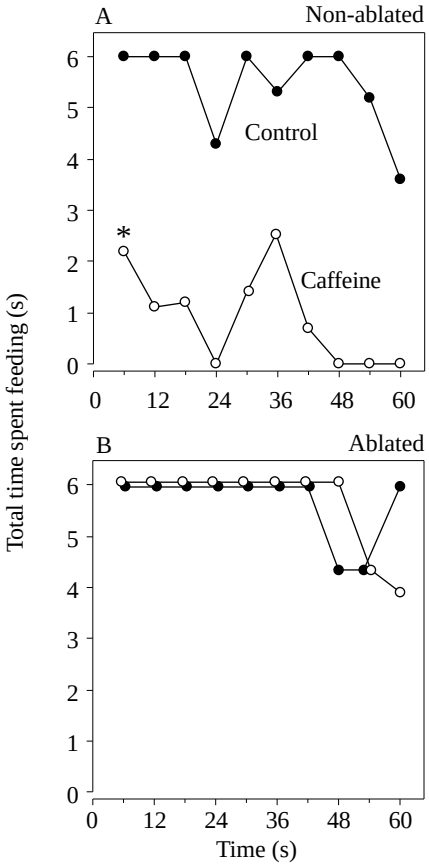

Fig. 8. Total time spent feeding by non-ablated (A) and ablated (B) larvae on the control diet (filled circles) and then the 30.9 mmol kg^{-1} caffeine diet (open circles) during 10 successive 6 s time intervals. These time intervals correspond to the initial 60 s of the 2 min feeding tests. All other details as in Fig. 7. N=25 larvae per panel.

unpublished data). Once in the haemolymph, nicotine could have produced several effects. The most probable is that it penetrated the blood–brain barrier of the nearby suboesophageal ganglion and activated the cholinergic neuropile (*sensu* Morris, 1984); given that this ganglion plays a major role in the generation of chewing motor output (Griss *et al.* 1991), such activation could have elicited feeding rejection. Alternatively, the absorbed nicotine could have stimulated internal chemoreceptors that had inhibitory afferents projecting into the feeding control centre. That such chemoreceptors could exist is supported by a report of internal chemoreceptors in *Mamestra brassicae* which detect histidine and trehalose in the haemolymph (Okajima *et al.* 1989).

It is difficult to determine how general these findings are to other insects because so few other investigators have attempted to distinguish the relative involvement of pre- *versus* post-ingestive mechanisms in rejection responses (but see Cottee *et al.* 1988; Usher *et al.* 1989). Most studies of feeding responses to toxic plant compounds have employed endpoint assays, which determine total consumption over a fixed period (usually several hours to days; Lewis and van Emden, 1986). This type of assay offers limited insight into underlying mechanisms because it ignores the actual behavioural effects of the test compounds and their latency of onset. Indeed, this limitation of endpoint assays may explain why previous studies of *M. sexta* responses to ingested nicotine (e.g. Self *et al.* 1964; Parr and Thurston, 1972) failed to detect the rapid rejection and toxic responses described herein.

Significance of the toxic response to nicotine

It is almost certain that the toxic responses were elicited by a post-ingestive response mechanism given (1) that they persisted after ablation of all mouthpart chemoreceptors, (2) that injection of nicotine directly into the haemolymph of nicotine-naive, fifth-instar *M. sexta* elicited the same toxic responses (Snyder *et al.* 1993), and (3) that twitching and writhing (i.e. the toxic responses) are well-documented effects of nicotine on the central nervous system of insects (Negherbon, 1959). Thus, the toxic responses establish that ingested nicotine could have passed through the gut wall and activated the central nervous system in the same time as the feeding rejection responses.

The high frequency of toxic responses to naturally occurring concentrations of nicotine was unexpected in a 'nicotine-tolerant' insect such as *M. sexta*. However, two observations suggest that the toxic responses were relatively innocuous. First, no caterpillars died after exhibiting such a response; in fact, a large percentage of them recommenced feeding within seconds. This transient toxic response contrasts with the report described above involving two species of noctuid caterpillar (Glendinning and Slansky, 1994), where ingestion of a 20 mmol kg^{-1} nicotine diet produced twitching, regurgitation and subsequent death in nearly 100 % of the individuals tested. Second, I never observed an individual *M. sexta* exhibiting the toxic response more than once, either in the 2 min feeding tests described herein or in 30 min feeding tests with 46.2 mmol kg^{-1} nicotine diets described elsewhere (Snyder and Glendinning, 1996). This indicates that the mechanism underlying the toxic response became inactive after a single triggering, at least at the concentrations tested (i.e. $\leqslant$46.2 mmol kg^{-1}).

Chemosensory effects of nicotine

Numerous investigators have reported instances where feeding inhibitors reduce the responsiveness of sugar taste receptors (see reviews by Frazier, 1992; Schoonhoven *et al.* 1992). However, I am not aware of any report of the reverse phenomenon: that is, feeding inhibitors stimulating a sugar receptor. Thus, the finding that 0.1 mmol l^{-1} nicotine stimulated the lateral sugar receptor in *M. sexta* appears to be novel. Further work is needed to explain the underlying mechanisms and functional significance of this perplexing finding. With regard to the underlying mechanisms, it would be useful to determine (1) whether nicotine stimulated the sugar receptor directly or enhanced the response of the sugar receptor to KCl; and (2) why the stimulatory effect of nicotine occurred only on the lateral sugar receptor and disappeared at concentrations greater than 0.1 mmol l^{-1}. With regard to the functional significance, it would be useful to explore why a toxic compound would stimulate a taste receptor normally associated with phagostimulation. One possible explanation stems from the ecological importance of nicotine to *M. sexta*: it is the dominant alkaloid in most tobacco species (Saitoh *et al.* 1985) and tobacco is a major host plant for this insect (Madden and Chamberlin, 1945). Accordingly, *M. sexta* may have evolved a novel gustatory mechanism (i.e. adding a nicotine acceptor site to its lateral sugar receptor) that stimulates consumption of this important food resource when it contains low levels of nicotine (equivalent to the 0.1 mmol l^{-1} stimulating solution); tobacco tissues with less or more than this concentration would be less phagostimulatory. This explanation is based on an innate taste model, which hypothesises that the taste system of insects has evolved response properties which help them locate optimal food resources (Simpson, 1994).

There are previous reports of a feeding inhibitor (warburganol) suppressing the responsiveness of inositol receptors in both *M. sexta* (Frazier, 1986) and *Spodoptera exempta* (Ma, 1977), but this effect did not manifest itself until after the receptors had been stimulated continuously with warburganol for several minutes. I found that nicotine had an immediate inhibitory effect on the lateral inositol receptor of *M. sexta* and that the suppression occurred principally during the phasic portion of the sensory response at a concentration that elicited approximately 50 % (but not 75 %) of the maximal response. Although Ma (1977) did not determine whether warburganol suppressed the phasic portion of the inositol response disproportionately, visual inspection of sensory responses in his paper suggests that it did.

In conclusion, while these chemosensory effects shed further light on the complex and subtle ways that plant secondary compounds can modulate an insect's gustatory response to

nutrients, they fail to provide a plausible chemosensory mechanism for the nicotine rejection response. The irrelevance of chemosensory input to this response is further revealed by the chemosensilla ablation experiment. A more parsimonious explanation is that the nicotine rejection response was mediated by a post-ingestive mechanism that was activated within 30 s of initiating feeding. That nicotine could have been absorbed within 30 s is supported by the close temporal coincidence between the onset of the rejection response and the toxic response mediated by the central nervous system.

I thank Reg Chapman and Mark Snyder for timely advice throughout this study, Liz Bernays, Reg Chapman, Spence Behmer and two anonymous reviewers for valuable editorial comments, and Michelle Leggett and Maria Truong for help collecting the behavioural and electrophysiological data, respectively. This project was supported by research grant numbers 5 F32 DC 00082-02 and 5 R29 DC 02416-02 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health.

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