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Electrophysiological Evidence for Two Transduction Pathways Within a Bitter-Sensitive Taste Receptor

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Glendinning, John I. and Thomas T. Hills. Electrophysiological evidence for two transduction pathways within a bitter-sensitive taste receptor. *J. Neurophysiol.* 78: 734–745, 1997. Among the sapid stimuli, those that elicit bitter taste are the most abundant and structurally diverse. To accommodate this diversity, animals are thought to use multiple bitter transduction pathways. We examined the role of individual taste receptor cells (TRCs) in this transduction process by focusing on one of the taste organs, or chemosensilla, of a caterpillar (*Manduca sexta*). This chemosensillum (the lateral styloconicum) contains four functionally distinct TRCs: the salt, sugar, inositol, and deterrent TRCs, which are known to respond strongly to, in respective order, salts, sugars, inositol, and compounds humans describe as bitter. Using an extracellular recording technique, we tested three hypotheses for how a structurally diverse array of bitter compounds (salicin, caffeine, and aristolochic acid) could excite the same chemosensillum: several TRCs within the lateral styloconica respond to the bitter compounds; only the deterrent TRC responds to the bitter compounds, through a single transduction pathway; and only the deterrent TRC responds to the bitter compounds, but through multiple transduction pathways. To discriminate among these hypotheses, we tested five predictions. The first addressed how many TRCs within the lateral styloconica responded to the bitter compounds. Subsequent predictions were based on the results of the test of the first prediction and assumed that only the deterrent TRC responded to these compounds. These latter predictions addressed whether the bitter compounds acted through one or multiple transduction pathways. We obtained evidence consistent with the third hypothesis: only the deterrent TRC responded to the bitter compounds; the temporal patterns of firing and concentration-response curves elicited by caffeine and salicin were similar to each other, but different from those elicited by aristolochic acid; the patterns of sensory adaptation and disadaptation elicited by caffeine and salicin were similar to each another, but different from those elicited by aristolochic acid; reciprocal cross-adaptation occurred between caffeine and salicin, but not between aristolochic acid and caffeine or aristolochic acid and salicin; and the responsiveness of individual deterrent TRCs to caffeine and salicin correlated significantly, whereas that to aristolochic acid and caffeine or aristolochic acid and salicin did not. Taken together, these results indicate that the deterrent TRC contains at least two excitatory transduction pathways: one responds to caffeine and salicin and the other to aristolochic acid. To our knowledge, this is the first direct support for the existence of two *bitter* transduction pathways within a single TRC.

INTRODUCTION

One distinctive feature of chemosensory systems is that they respond to a large number of structurally unrelated compounds. They appear to accomplish this feat through a multitude of transduction pathways (e.g., receptor sites, second messenger cascades, and ion channels) in their receptor cells (Ache 1994; Dione and Dubin 1994; Kinnamon and Cummings 1992; Spielman et al. 1992). Although the existence of these different pathways is well documented, their functional organization across populations of taste or olfactory receptor cells within animals is not. For example, it is unclear how many transduction pathways are expressed within individual receptor cells, how extensively different pathways within the same receptor cell overlap in terms of their molecular receptive ranges, and whether the coexistence of multiple transduction pathways within receptor cells increases or decreases an animal's ability to discriminate between different classes of compounds. These issues are critical to our understanding of how chemosensory systems encode natural chemical signals.

In the olfactory system of vertebrates and invertebrates, there is direct evidence for at least two transduction pathways within individual receptor cells (review in Restrepo et al. 1996). Moreover, there is speculation that interactions between these transduction pathways may facilitate olfactory coding and the detection of low concentrations of odorants in complex mixtures (Ache 1994; Restrepo et al. 1996). The situation in the gustatory system, however, is less clear because only a few studies have addressed this issue. The most definitive study reported that sweet-sensitive taste receptor cells (TRCs) in rats can express at least two transduction pathways: one responds to sucrose and the other to nonnutritive sweeteners (Bernhardt et al. 1996). Here, we further explore the functional organization of transduction pathways within the gustatory system but focus on pathways that are activated by compounds humans describe as bitter.

Bitter compounds constitute the largest and most structurally diverse class of gustatory stimuli (Rouseff 1990). That animals accommodate this diversity through a multitude of specific bitter transduction pathways is supported by findings from several experimental paradigms. Psychophysical and electrophysiological studies reveal that attenuating the gustatory response to one bitter compound, through habituation (Glendinning and Gonzalez 1995) or sensory adaptation (McBurney and Bartoshuk 1973; McBurney et al. 1972; Sato and Sugimoto 1995), generalizes to some, but not all, novel bitter compounds. In addition, inbred strains of mice differ greatly in taste sensitivity to bitter compounds, and these interstrain differences are explained most parsimoniously by a model involving multiple transduction pathways (Whitney and Harder 1994). Finally, biochemical and physiological studies of bitter-sensitive TRCs also support the existence of several transduction pathways (Kinnamon and Cummings 1992; Ruiz-Avila et al. 1995; Spielman et al. 1992). Virtually nothing is known, however, about the functional organization of these different transduction pathways across the population of bitter-sensitive TRCs.

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Caterpillars offer several advantages as models for examining how the taste system encodes bitter stimuli. The total population of TRCs is small (between 55 and 65), and is divided into discrete functional units of 3–4 TRCs, which occur in separate taste organs, or chemosensilla (Schoonhoven 1987). In addition, one can study the response properties of individual TRCs in intact preparations through noninvasive recording techniques (Gothilf and Hanson 1994). Because the responses of individual TRCs within a chemosensillum can be discriminated reliably from one another based on their respective temporal patterns of firing, one can study homologous TRCs from different animals (e.g., see Glendinning 1996). Finally, most insect chemosensilla contain one TRC that responds to a structurally diverse range of compounds that humans characterize as bitter, and it is usually called the deterrent TRC (Blaney and Simmonds 1988; Chapman et al. 1991; Dethier 1973); stimulation of this TRC is associated with taste-rejection (Schoonhoven et al. 1992). Despite these experimentally favorable attributes, little is known about how insect chemosensilla respond to bitter compounds.

In this study, we evaluate three alternative hypotheses for how a diverse array of bitter compounds could stimulate the same chemosensillum. Hypothesis A posits that individual chemosensilla contain several deterrent TRCs and that these TRCs each respond to structurally distinct classes of bitter compounds. This hypothesis is based on the observation that most species of caterpillars appear to possess more than one deterrent TRC and that these TRCs often have different response properties (Schoonhoven et al. 1992). However, it should be noted that there have been no reports to date of more than one deterrent TRC within the same chemosensillum.

Hypotheses B and C both postulate that the ability of a chemosensillum to respond to a structurally diverse range of bitter compounds is mediated by a single TRC (i.e., the deterrent TRC). What distinguishes these two hypotheses is the number of transduction pathways thought to be involved. Hypothesis B posits that the deterrent TRC contains a single, relatively nonspecific transduction pathway. Accordingly, the initial events of bitter taste transduction could involve penetration of a bitter compound into the lipid layer of the taste cell membrane, which would subsequently inhibit phosphodiesterase activity in the TRC (Koyama and Kurihara 1972; Kumazawa et al. 1988; Kurihara 1972), or binding of the bitter ligand to one of several membrane-bound receptors, which are all coupled to a common second-messenger system (e.g., Shimada 1975; Shimada et al. 1974).

In contrast, hypothesis C posits that the deterrent TRC contains several transduction pathways, each with different molecular receptive ranges. This hypothesis is derived from the observation that individual sweet-sensitive TRCs can express two transduction pathways: one responds to sucrose with an increase in adenosine 3′,5′-cyclic monophosphate (cAMP) and Ca^{2+} uptake and the other to nonnutritive sweeteners with an increase in IP_3 and Ca^{2+}-release (Bernhardt et al. 1996). Even though there is evidence for similar transduction pathways within bitter-sensitive TRCs of mammals [one involving an increase in IP_3 and Ca^{2+}-release (Akabas et al. 1988; Spielman et al. 1996) and the other a decrease in cAMP and Ca^{2+} uptake (Kolesnikov and Margolskee 1995)], no investigator, to our knowledge, has yet determined whether both of these pathways can coexist within the same bitter-sensitive TRC.

To discriminate between these three hypotheses, we used the tobacco hornworm caterpillar (*Manduca sexta*) as our model system and focused on a bilateral pair of chemosensilla (the lateral styloconica), which respond to a diverse range of compounds that humans describe as bitter. Our approach involved testing five predictions of the hypotheses (Table 1). The first prediction addresses how many TRCs within the lateral styloconica are stimulated by bitter compounds. The remaining four predictions assume that only one TRC (i.e., the deterrent TRC) is activated by the bitter compounds and address whether the bitter compounds stimulate this TRC through one or multiple transduction pathways.

Part of this work was published previously as an abstract (Glendinning 1995).

METHODS

Insects, diets, and taste stimuli

We obtained caterpillars 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 L:8D photoperiod (Bell and Joachim 1976). We used caterpillars 2 days after completing their molt to the fifth stadium. All caterpillars were naive to the test compounds before testing. To control for any potential differences among caterpillars from different egg batches, we interspersed individuals from each batch across experimental treatments.

We used three structurally distinct taste stimuli: caffeine (a methylxanthine), salicin (a phenolic glycoside), and aristolochic acid (an aporphinoid). These compounds (from Sigma Chemical) all strongly stimulate TRCs within the lateral styloconica (Schoonhoven 1972; Glendinning, unpublished data), elicit rapid taste-rejection in *M. sexta* (de Boer et al. 1977; Wrubel and Bernays 1990; Glendinning, unpublished data), and taste bitter to humans (Glendinning 1994). Even though neither of these compounds occurs in normal food plants of *M. sexta* (i.e., plants within the Solanaceae) (Harborne and Baxter 1993), they nevertheless occur in plants within the geographic range of *M. sexta* and thus might be encountered by free ranging individuals.

Electrophysiological recording procedure

Like most caterpillars, *M. sexta* has eight bilateral pairs of gustatory chemosensilla associated with its mouth parts, and they all occur outside its cibarial cavity (i.e., mouth). As compared with vertebrate taste buds, these insect ''taste buds'' have a simple structure: each contains three to four dendritic processes, which arise from cell bodies located at the base of the chemosensillum. Tastants gain access to these processes by diffusing through a tiny pore at the tip of the chemosensillum. When tastants reach the receptor membrane at the distal end of the dendritic processes, they are thought to induce inward current across the membrane and thereby elicit spiking near the cell body (Morita 1959). These bipolar sensory neurons extend directly to the subesophageal ganglion in the CNS.

We focused on one chemosensillum: the lateral styloconica. Each chemosensillum occurs bilaterally and contains four functionally differentiated TRCs. They are called the inositol, sugar, salt, and deterrent TRCs, and they respond strongly to inositol, nutritive sugars, salts, and bitter compounds, respectively (Schoonhoven 1972; Schoonhoven et al. 1992). Each TRC responds to its best stimuli with a characteristic temporal pattern of firing: that from the salt TRC is temporally irregular, that from the inositol TRC is

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TABLE 1. *Predictions taken from alternative hypotheses about how several structurally distinct bitter compounds could elicit spiking within the same chemosensillum*

Predictions	Alternative Hypotheses		
	Bitter compounds stimulate different TRCs	Bitter compounds stimulate a single TRC	
		Several transduction pathways	Single transduction pathway
1. Binary mixtures of two bitter compounds will stimulate a single TRC	−	+	+
2. Bitter compounds will elicit different concentration-response curves and temporal patterns of firing	+	+	−
3. Bitter compounds will produce different patterns of adaptation and disadaptation	+	+	−
4. Sensory adaptation to one bitter compound will not cross-adapt to the others	+	+	−
5. Responsiveness of a TRC to one bitter compound will not covary with responsiveness to the others	+	+	−

−, not predicted by the hypothesis; +, predicted by the hypothesis. TRC, taste receptor cell.

strongly phasic-tonic; that from the sugar TRC is less strongly phasic-tonic; and that from the deterrent TRC is predominantly tonic, with a variable latency of onset (Fig. 1, *A–D*). Owing to the distinctive nature of each TRC's temporal pattern of firing, we were able to discriminate them readily.

We recorded action potentials from TRCs within the lateral styloconica with a noninvasive extracellular recording technique (Gothilf and Hanson 1994). In brief, this method involved anesthetizing the caterpillar by sealing it within a grounded vial containing 0.1 M KCl (with its head protruding) and then placing a recording/stimulating electrode over the tip of one of its lateral styloconica. Because the recording electrode contained the tastant solution, we could stimulate and record from the deterrent TRC simultaneously.

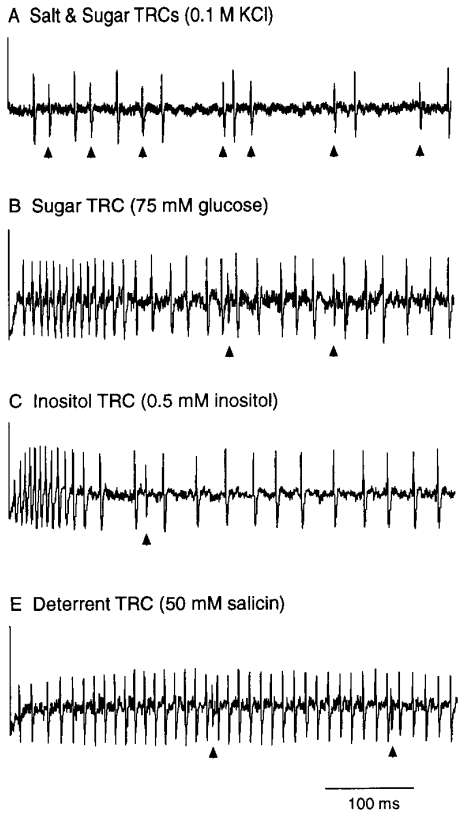

FIG. 1. Typical neural records from 4 taste receptor cells (TRC) within lateral styloconic sensilla. Traces illustrate temporal pattern of firing by salt and sugar TRCs in response to 100 mM KCl (*A*), sugar TRC to 75 mM glucose (*B*), inositol TRC to 0.5 mM inositol (*C*), and deterrent TRC to 50 mM salicin (*D*). Because 100 mM KCl was present in all solutions (for conductivity), all neural records contain variable number of spikes from salt TRC; these spikes are indicated (▲).

We processed neural records using a high-impedance preamplifier with a baseline-restoring circuit (George Johnson, Baltimore, MD) (see Frazier and Hanson 1986) and an AC-coupled amplifier-filter system with a band-pass set at 130–1,200 Hz. We digitized and stored neural records directly onto a computer with a software program called SAPID Tools (Smith et al. 1990).

For each caterpillar, we randomly selected one lateral styloconic sensillum and subjected it to various stimulation protocols (see below), always pausing ≥3 min between successive stimulations of the same sensillum. In all cases, we quantified the number of action potentials generated from 10 ms after contact with the sensillum; the actual length of recording varied among recordings. To minimize solvent evaporation at the tip of the recording/stimulating electrode, we drew fluid from the tip with a piece of filter paper <7 s before each stimulation.

All bitter compounds were dissolved in a 10% ethanol solution containing 100 mM KCl; the ethanol was necessary for complete dissolution of the relatively hydrophobic aristolochic acid, and the KCl for electrical conductivity. The 10% ethanol concentration itself does not elicit or inhibit firing in any of the TRCs and is not deleterious to the TRCs (Peterson et al. 1993; Glendinning, unpublished data).

How many TRCs are activated by the bitter compounds? (test of first prediction)

If all three bitter compounds stimulate the same TRC within the lateral styloconica (i.e., the deterrent TRC), then binary mixtures of the bitter compounds should activate only the deterrent TRC and cause it to fire at a higher rate than either compound individually (e.g., van Loon and van Eeuwijk 1989) (see Table 1). If the bitter compounds stimulate different TRCs, then binary mixtures of the bitter compounds should activate more than one TRC.

To evaluate these predictions, we used concentrations that caused intermediate levels of stimulation (i.e., 0.5 mM caffeine, 3 mM salicin, or 0.001 mM aristolochic acid) and tested a total of 12 lateral styloconic sensilla (each from different caterpillars). We recorded the initial 500 ms of response to 0.5 mM caffeine alone, 0.001 mM aristolochic acid alone, and then the mixture of both or 0.5 mM caffeine alone, 3 mM salicin alone, and then the mixture of both. We did not test binary mixtures of salicin and aristolochic

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acid because we felt it was unnecessary; if binary mixtures of caffeine and aristolochic acid stimulate the deterrent TRC exclusively, and binary mixtures of caffeine and salicin do the same, then it follows logically that binary mixtures of salicin and aristolochic acid also would stimulate the deterrent TRC exclusively.

To determine whether the binary mixture caused the deterrent TRC to fire at a significantly greater rate than either compound alone, we made paired (one-tailed, *t*-test) comparisons between the sensory response to each component and that to the mixture. In these and all subsequent comparisons, we performed a Bonferroni correction on the alpha level to control for the use of multiple two-way comparisons on the same data set [i.e., divided the alpha level by the number of comparisons (alpha = 0.05/2)].

As a control, we felt it was necessary to confirm that a binary mixture actually could activate two TRCs simultaneously. To this end, we tested an additional prediction: binary mixtures of two compounds, which are thought to activate different TRCs within the lateral styloconica, should stimulate more than one TRC. We tested binary mixtures of inositol (or glucose) and either of the bitter compounds. We again used concentrations that caused moderate levels of stimulation. We stimulated a lateral styloconic sensilla with either 0.5 mM inositol alone, each of the bitter compounds alone (same concentrations as above), and then the mixture of both, or 75 mM glucose alone, each of the bitter compounds alone (same concentrations as above), and then the mixture of both. The spikes from different TRCs were discriminated as described above. For each test, we stimulated a total of 12 sensilla (each from different caterpillars).

Responses of the deterrent TRC to different concentrations of the bitter compounds (test of second prediction)

If each of the three bitter compounds stimulates the deterrent TRC through different transduction pathways, then we predicted that each should elicit different concentration-response curves and temporal patterns of firing (see Table 1). For example, bitter compounds that act directly on ion-gated channels in TRC membranes (e.g., quinine in salamanders) (Kinnamon 1992) might be expected to elicit a response more rapidly than those that act through second messenger systems (e.g., IP_3 in mice) (Spielman et al. 1996). If the bitter compounds act on the same transduction pathway, then the temporal patterns of firing that they elicit, and their concentration-response curves should be similar.

To test these predictions, we used five to six concentrations of each bitter compound (aristolochic acid: 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.1 mM; caffeine: 0.05, 0.1, 0.5, 1, 5, 10 mM; and salicin: 0.5, 1, 5, 10, 50 mM) and recorded how increasing concentrations of each compound influenced both the total number of action potentials produced by the deterrent TRC across the first 1,000 ms of stimulation and the temporal distribution of action potentials across the same period of stimulation (i.e., number of spikes during each successive 100-ms time bin). For each stimulus, we tested a total of 12–15 lateral styloconic sensilla (each from different caterpillars).

Patterns of adaptation and disadaptation to the bitter compounds in the deterrent TRC (test of third prediction)

If each of the three bitter compounds stimulates the deterrent TRC through different transduction pathways, then we predicted they would produce different patterns of adaptation and disadaptation (see Table 1). This prediction derives from the observation that specific transduction pathways often exhibit characteristic patterns of sensory adaptation (e.g., Ozaki and Amakawa 1992). If all bitter compounds stimulate the same transduction pathway, then we predicted that each compound would elicit similar patterns of adaptation and disadaptation.

To evaluate these predictions, we selected a concentration of each compound that elicited the maximal firing rate (5 mM caffeine, 50 mM salicin, and 0.1 mM aristolochic acid) and tested each TRC with all three of these solutions. The testing procedure for a given compound involved the following three steps: record response of TRC to the test compound for 15 s to determine the pattern of adaptation (henceforth, *stimulation 1*); cease stimulating TRC for 30 s to permit some level of disadaptation; and restimulate TRC with the same test compound for 15 s to assess the extent of disadaptation that occurred (henceforth, *stimulation 2*). We paused 5 min between different compounds so as to permit complete disadaptation.

We tested a total of 15 deterrent TRCs (each from different caterpillars) for each adaptation/disadaptation test. Sensory adaptation was indicated by a marked decline in firing rate over time during *stimulation 1*. To determine whether complete disadaptation occurred during the 15-s period between *stimulations 1* and *2*, we ran a two-way repeated measure analysis of variance (ANOVA) with time and sequential stimulations as within factors. In both tests, the response variable was the temporal pattern of firing during each 15-s period of stimulation (i.e., number of spikes per 500 ms; alpha $\leq$ 0.05).

Patterns of cross-adaptation among the bitter compounds in the deterrent (test of fourth prediction)

If each of the three bitter compounds stimulated the deterrent TRC through different transduction pathways, then we predicted that sensory adaptation to one bitter compound would not cross-adapt to the others (see Table 1). Cross-adaptation between two of the bitter compounds would indicate that both activate a common pathway, whereas a significant lack of cross-adaptation would indicate that both activate independent pathways. Cross-adaptation is an accepted and effective technique for evaluating the independence of transduction processes or binding sites within chemosensory cells (e.g., Caprio and Byrd 1984; Daniel et al. 1994; Hazelbauer et al. 1987; Rehnberg et al. 1989; Sato and Sugimoto 1995; Shimada 1987).

The test solutions were the same as those used in the previous experiment. Our cross-adaptation protocol was as follows: record initial response of the deterrent TRC to the test compound for 15 s; cease stimulating the TRC for 5 min to permit complete disadaptation; stimulate the same TRC with the adapting compound for 15 s; cease stimulating the deterrent TRC for 30 s; and restimulate the same TRC with the test compound for 15 s to assess the extent of cross-adaptation. The long (i.e., 30 s) period for disadaptation in the fourth step was unavoidable given practical difficulties associated with switching electrodes between the third and fifth steps.

We tested 12–16 deterrent TRCs (each from different caterpillars) for a given cross-adaptation test. To determine whether cross-adaptation occurred, we ran a two-way repeated measure ANOVA with time and sequential stimulations (during the first and fifth steps) as within factors. The temporal pattern of firing during each 15-s period of stimulation (i.e., number of spikes per 500 ms) was the response variable. Cross-adaptation would be indicated by a significant effect of repeated stimulation and/or the interaction of repeated stimulation and time.

Does the response of individual deterrent TRCs to the bitter compounds covary? (test of fifth prediction)

If each of the three bitter compounds stimulated the deterrent TRC through different transduction pathways, then we predicted that the responsiveness of the deterrent TRC to one bitter compound would not covary with its responsiveness to the others (see Table 1). On the contrary, significant covariance would indicate that the compounds act through a common transduction pathway.

To test this prediction, we stimulated a total of 40 lateral deter-

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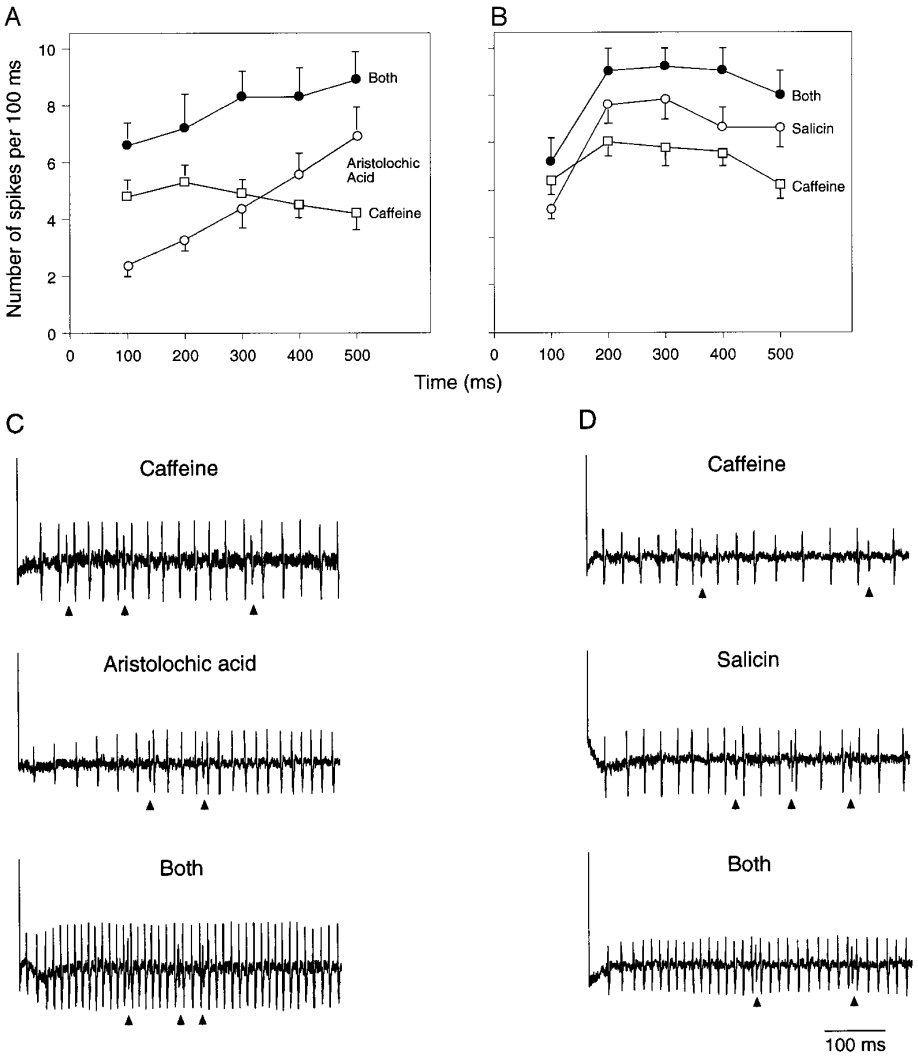

FIG. 2. Sensory responses of deterrent TRC to binary mixtures of caffeine and aristolochic acid or caffeine and salicin. We present mean ± SE temporal pattern of firing in response to 0.001 mM aristolochic acid alone, 0.5 mM caffeine alone, and the mixture of both (*A*), or 3 mM salicin alone, 0.5 mM caffeine alone, and the mixture of both (*B*). Representative neural records from data in *A* and *B* are provided in *C* and *D*, respectively (all traces in *C* and *D* are from the same chemosensillum). Spikes from salt TRC are indicated with arrowheads. Results in *A* and *B* are derived from 12 deterrent TRCs, each from different caterpillars. See Table 2 for statistical analysis of these data.

rent receptors (each on a different caterpillar) with 5 mM caffeine, 50 mM salicin, and 0.1 mM aristolochic acid. Then we tested for a significant correlation between the response to all three compounds, using three separate Pearson product-moment correlations (alpha = 0.05/3). The response variable was total number of spikes across the first second of stimulation.

RESULTS

How many TRCs are activated by the bitter compounds?

Binary mixtures of the bitter compounds strongly activated only one TRC in all of the lateral styloconica studied. Based on the distinctive temporal pattern of firing, we inferred that it was the deterrent TRC that responded (Fig. 2). Visual inspection of the traces (e.g., Fig. 2, *C* and *D*) reveals that the salt TRC also fired infrequently, presumably in response to the 0.1 M KCl present in the stimulating solution. That the salt TRC was not responding to the bitter compounds is demonstrated by the fact that its response (i.e., firing rate) to the binary mixtures was indistinguishable from that to solutions containing only one bitter compound (Fig. 2, *C* and *D*). Finally, the firing rates of the deterrent TRC (during the initial 500 ms of stimulation) were significantly higher in response to the binary mixture of aristolochic acid and caffeine than to either compound alone and to the binary mixture of salicin and caffeine than to either compound alone (Fig. 2, *A* and *B*; Table 2).

In contrast, binary mixtures of caffeine and inositol stimulated the deterrent and inositol TRCs; likewise, binary mixtures of caffeine and glucose stimulated the deterrent and sugar TRCs (Fig. 3, *A–C*). To illustrate these results, we provided neural records from a single lateral styloconica

TABLE 2. *Sensory response of the deterrent TRC to various stimulants*

Test	Tastant Solution	Total Spikes
A	Aristolochic acid	22.5 ± 2.9*
	Caffeine	23.7 ± 2.5*
	Mixture	40.2 ± 4.9
B	Salicin	21.0 ± 1.7*
	Caffeine	18.0 ± 1.3*
	Mixture	26.3 ± 2.2

We present total spikes over the initial 500 ms of stimulation (means ± SE) by the deterrent TRC. We used paired *t*-test comparisons (one-tailed) to determine whether the number of spikes elicited by either component alone was significantly less than the mixture of both (* $P \leq 0.05/2$). See Fig. 2 for more details. In test A, we used 0.001 mM aristolochic acid, 0.1 mM caffeine, and the mixture of both, and in test B, we used 3 mM salicin, 0.1 mM caffeine, and the mixture of both.

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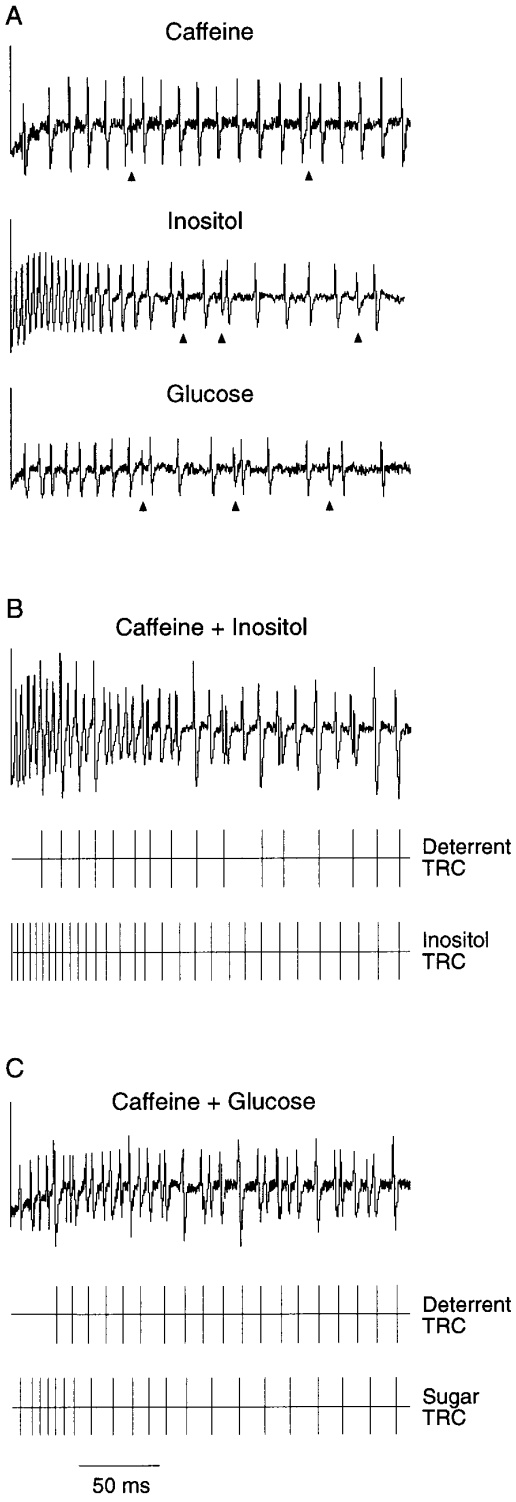
responding to caffeine, inositol, glucose, and the two binary mixtures. Owing to the complex nature of the neural responses to the binary mixtures, we also indicated the inferred location of spikes from each TRC. It is apparent that two TRCs responded vigorously to each binary mixture, but only one responded vigorously to caffeine, inositol or glucose alone.

We obtained virtually identical results when we stimulated the lateral styloconica with binary mixtures of salicin and inositol (or glucose) or aristolochic acid and inositol (or glucose). Thus these results demonstrate that bitter compounds activate a different TRC than do glucose and inositol. In addition, they confirm that binary mixtures of compounds can strongly activate at least two TRCs within the lateral styloconica at the same time.



Responses of the deterrent TRC to different concentrations of the bitter compounds

Aristolochic acid elicited a qualitatively different temporal pattern of firing in the deterrent TRC than did caffeine or salicin. For all concentrations of aristolochic acid tested, the number of spikes during the first 100 ms was low but increased markedly over the next 900 ms (Fig. 4*A*). Further, the shape of the time-response curve changed with concentration: it was linear at concentrations ≤ 1 μM and asymptotic at higher concentrations. The time-response curves for caffeine and salicin also exhibited delayed onset, but they all reached their maximal firing rate 100–200 ms after stimulus contact and then decreased gradually and linearly during the subsequent 800 ms (Fig. 4, *B* and *C*).

The concentration-response (C-R) curves (as indicated by total spikes during the first second of stimulation) also differed among the three bitter compounds (Fig. 5). As compared with the C-R curves for caffeine and salicin, that for aristolochic acid had a narrower dynamic range, reached its maximal firing rate at a concentration 2.5–3.0 log units lower, and attained a maximal firing rate that was approximately two times greater. The differences in shape of the C-R curves for caffeine and salicin were much more subtle: that for caffeine was hyperbolic whereas that for salicin was logistic. Further, the C-R curve for caffeine reached its maximal firing rate at a concentration 0.5 log unit lower than that for salicin.

Visual inspection of sensory records revealed another robust difference between the responses to the three bitter compounds. Whereas caffeine and salicin elicited spike amplitudes that were highly regular, aristolochic acid elicited spike amplitudes that waxed with time (e.g., see Fig. 7).

Patterns of adaptation and disadaptation to the bitter compounds in the deterrent TRC

All three compounds elicited patterns consistent with sensory adaptation (Fig. 6, *A–C*, ○). The initial response to caffeine and salicin (i.e., *stimulation 1*) began vigorously but then decreased with time to a level that was ~50% of the maximal firing rate. Even though the initial response to

FIG. 3. Sensory responses of different TRC to solutions containing single component stimuli (*A*; 0.5 mM caffeine, 0.5 mM inositol, or 75 mM glucose), a binary mixture (*B*) of 0.5 mM caffeine and 0.5 mM inositol, and a binary mixture (*C*) of 0.5 mM caffeine and 75 mM glucose. In *A*, only 1 TRC fired regularly and rapidly: deterrent, inositol, and glucose TRC, respectively. However, in traces in *B* and *C*, 2 TRCs are firing regularly and rapidly, creating a complex temporal pattern of spiking. Unusually large spikes in these latter traces correspond to instances where 2 TRCs fired synchronously. To facilitate interpretation of these complex neural records, we have provided neural record at *top* and inferred location of spikes from different TRCs *below*. Note similarity in temporal pattern of firing of each TRC between traces containing single component stimuli and those containing binary mixtures. Spikes from salt TRC were observed only in traces within *A*, and they are indicated (▲).

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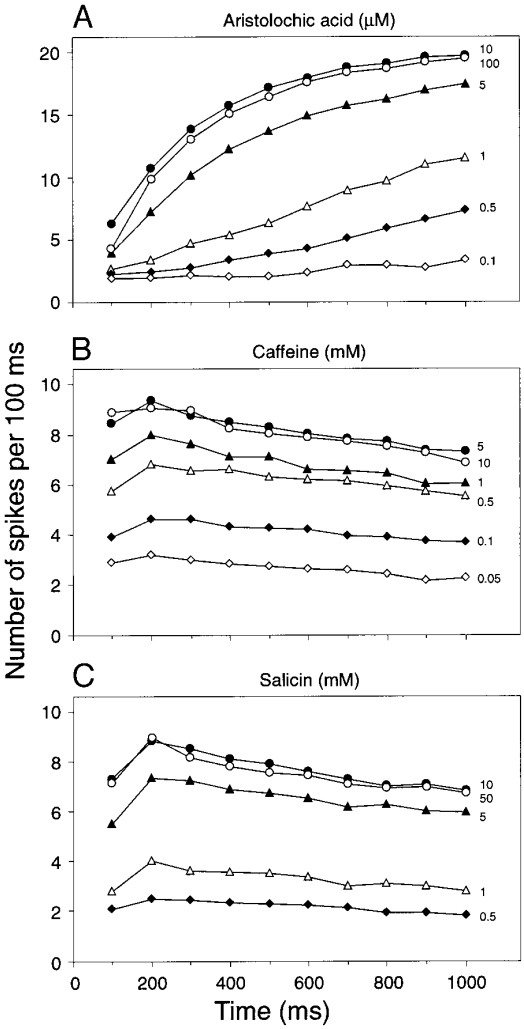

FIG. 4. Temporal pattern of firing (mean number of spikes per 100 ms) by deterrent TRC in response to a range of concentrations of aristolochic acid (*A*), caffeine (*B*), and salicin (*C*). Each line represents a different concentration (actual values are provided to *right* of each line). Results are derived from 12 deterrent TRCs (each from a different caterpillar), and each TRC was stimulated with full range of concentrations. Note that *y*-axis scale for *A* differs from that for *B* and *C*.

aristolochic acid took several seconds to reach its maximal firing rate, the firing rate subsequently decreased to a level ~50% of the maximum. In addition, the deterrent TRC failed to disadapt to all three bitter compounds during the 30 s gap between *stimulations 1* and *2*; this is revealed by a comparison of lines containing ○ versus ● (Fig. 6, *A–C*).

These observations are supported by results from the two-way ANOVA (Table 3). First, there was a significant effect of repeated stimulation on the firing rate for all three compounds, demonstrating that the deterrent TRC failed to completely disadapt to any of the compounds. However, it should be noted that the response to caffeine and salicin disadapted to a much greater extent during the 30-s pause between *stimulations 1* and *2* than did that to aristolochic acid (see Figs. 6 and 7). Second, there was a significant effect of time for each compound, confirming that sensory adaptation occurred during both *stimulation 1* and *2*. Finally, there was a significant interaction between repeated stimulation and time, revealing that the shape of the adaptation curves differed consistently between *stimulations 1* and *2*. For caffeine and salicin, the adaptation curve from *stimulation 2* adapted more quickly than did that from *stimulation 1*. For aristolochic acid, the pattern of adaptation apparent during *stimulation 1* was virtually absent in *stimulation 2*; in addition, the initial firing rate was lower during *stimulation 2*.

Patterns of cross-adaptation among the bitter compounds in the deterrent TRC

We obtained evidence of cross-adaptation between some but not all of the compounds. For example, the normal response of the deterrent TRC to aristolochic acid was not affected by adaptation to salicin or caffeine (Fig. 8, *A* and *B*). Likewise, the normal response of the deterrent TRC to salicin or caffeine was not affected by adaptation to aristolochic acid (Fig. 8, *C* and *E*). In all of these tests, the two-way ANOVA revealed no significant effect of repeated stimulation, or interaction between repeated stimulation and time, on the firing rate (Table 4). There was a significant effect of time in all comparisons, however, which confirms the result of the previous experiment: that aristolochic acid, caffeine, and salicin reliably elicit sensory adaptation in the deterrent TRC.

In contrast, the normal response of the deterrent TRC to salicin was attenuated after adaptation to caffeine (Fig. 8*D*). This observation is supported by a significant effect of repeated stimulation, and the interaction between repeated stimulation and time, on the firing rate (Table 4). In the reciprocal experiment as well, the normal response of the deterrent TRC to caffeine was attenuated after adaptation to salicin (Fig. 8*F*). In this case, there was a significant interaction between repeated stimulation and time, but not of repeated stimulation alone (Table 4). The significant interaction term illustrates that the shapes of the curves in Fig. 8*F* differed significantly from one another. Even though the effect of repeated stimulation alone was not significant, the trend was in the expected direction.

These results demonstrate that reciprocal cross-adaptation occurs between caffeine and salicin but not between caffeine and aristolochic acid or between salicin and aristolochic acid. It should be noted that the extent of cross-adaptation between salicin and caffeine was not symmetrical: caffeine had a greater impact on the salicin response.

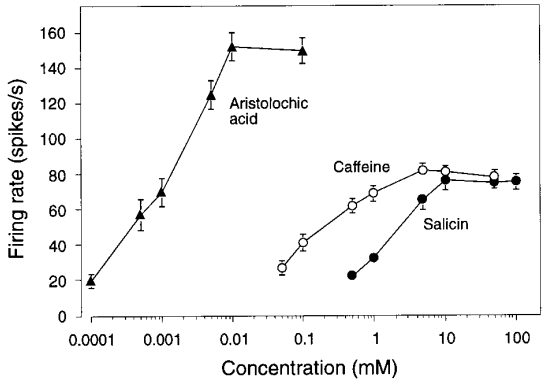

FIG. 5. Concentration-response curves (number of spikes/s) for deterrent TRC in response to aristolochic acid, caffeine and salicin (means ± SE). For more details, see Fig. 4.

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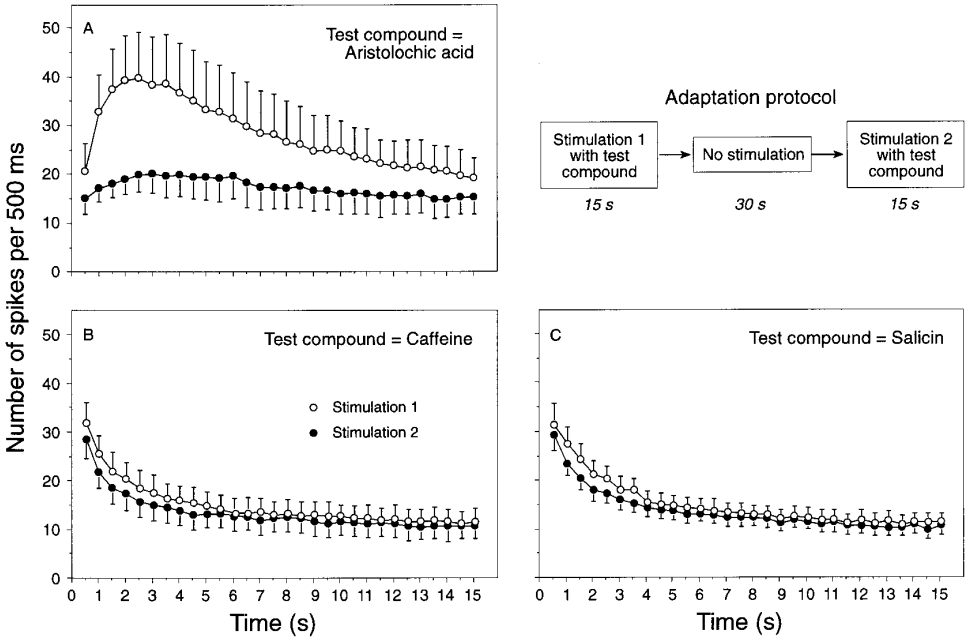

FIG. 6. Test for sensory adaptation and disadaptation of deterrent TRC to 3 bitter compounds: 0.1 mM aristolochic acid (*A*), 5 mM caffeine (*B*), and 50 mM salicin (*C*). We indicate adaptation protocol in *top right* portion of figure. In each panel, we provide mean ± SE number of spikes per 500-ms bin during *stimulation 1* (○) and *stimulation 2* (●). These data are based on response of 15 deterrent TRCs, each from different caterpillars. We inferred adaptation if firing rate decreased significantly with time during *stimulation 1* and disadaptation if temporal pattern of firing during *stimulation 2* did not differ significantly from that during *stimulation 1*. See Table 3 for a statistical analysis of these results.

Does the response of individual deterrent TRCs to the bitter compounds covary?

The sensory responses of the deterrent TRC to caffeine and salicin were significantly correlated ($r = 0.71$, df = 38, $P \leq 0.05/3$), whereas those to caffeine and aristolochic acid ($r = 0.34$, df = 38, $P > 0.05/3$) or salicin and aristolochic acid ($r = 0.33$, df = 38, $P > 0.05/3$) were not (Fig. 9, *A–C*). Thus these results demonstrate that the responsiveness of the deterrent TRC to caffeine and salicin covaries, but that to aristolochic acid and the other compounds does not.

DISCUSSION

We initially proposed three alternative hypotheses to explain how the lateral styloconica could respond to three compounds that are as structurally diverse as caffeine, salicin, and aristolochic acid. The first hypothesis was that several TRCs within the lateral styloconica mediated the response to these compounds. If this was the case, then we predicted that binary mixtures of the bitter compounds would activate more than one TRC. However, this was not the case; only the deterrent TRC responded to the binary mixtures, and it did so at a significantly higher firing rate than was elicited by either of the bitter compounds alone. A key assumption of this prediction was that more than one TRC within the lateral styloconica could fire vigorously at the same time. To evaluate this assumption, we tested binary mixtures of each bitter compound with known ligands of the inositol and sugar TRCs. In this latter experiment, at least two TRCs responded vigorously to the binary mixtures. Taken together, these results lead us to conclude that the three bitter compounds stimulate the deterrent TRC exclusively.

TABLE 3. *Analysis of the tests for adaptation and disadaptation in Fig. 6, A–C*

Test Compound	Source of Variation	df	*F*
Aristolochic acid	Repeated stimulation	1, 11	20.2*
	Time	29, 319	14.7*
	Interaction	29, 319	14.3*
Caffeine	Repeated stimulation	1, 14	15.2*
	Time	29, 406	108.2*
	Interaction	29, 406	3.0*
Salicin	Repeated stimulation	1, 14	9.5*
	Time	29, 406	147.6*
	Interaction	29, 406	3.3*

We subjected the data in each panel to a two-way repeated measure analysis of variance (ANOVA), with repeated stimulation (i.e., response to the test compound both during and after adaptation) and time (30 consecutive 500-ms bins) as the independent variables and discharge rate during each 500-ms interval as the response variable (* $P \leq 0.05$). See Fig. 6 for more details.

The two remaining hypotheses are consistent with this conclusion. They differ only in terms of how the bitter compounds are purported to activate the deterrent TRC. One hypothesis posits that they do so through a single, relatively nonspecific transduction pathway, whereas the other posits that they do so through several transduction pathways. We tested four predictions as a way of discriminating between these two hypotheses (see predictions 2–5 in Table 1). The results were as follows: the temporal patterns of firing and concentration-response curves elicited by caffeine and salicin were similar to each other but qualitatively different from those elicited by aristolochic acid; the patterns of sensory adaptation and disadaptation elicited by caffeine and salicin were similar to each another but different from those elicited by aristolochic acid; reciprocal cross-adaptation was observed between caffeine and salicin but not between aristolochic acid and caffeine or aristolochic acid and salicin; and the responsiveness of individual deterrent TRCs to caffeine and salicin correlated significantly, whereas that to aristolochic acid and caffeine or aristolochic acid and salicin did not. Finally, it is notable that the sensory responses elicited by caffeine and salicin had relatively constant spike amplitudes over time, whereas those elicited by aristolochic acid had spike amplitudes that waxed and then waned with time.

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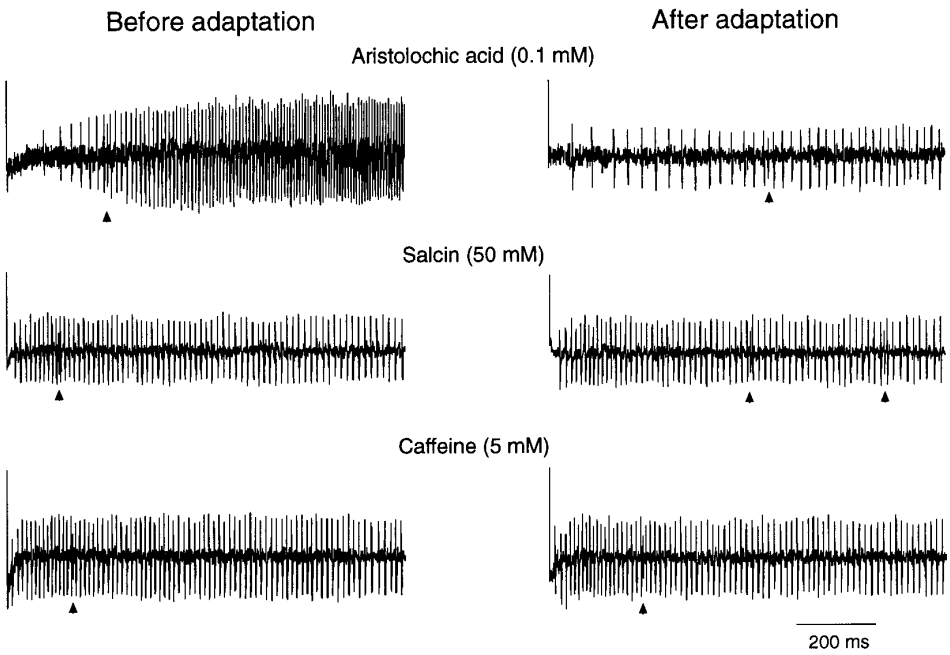

FIG. 7. Representative neural records from sensory adaptation and disadaptation experiment. For more details, see Fig. 6. Spikes from the salt TRC are indicated with arrowheads.

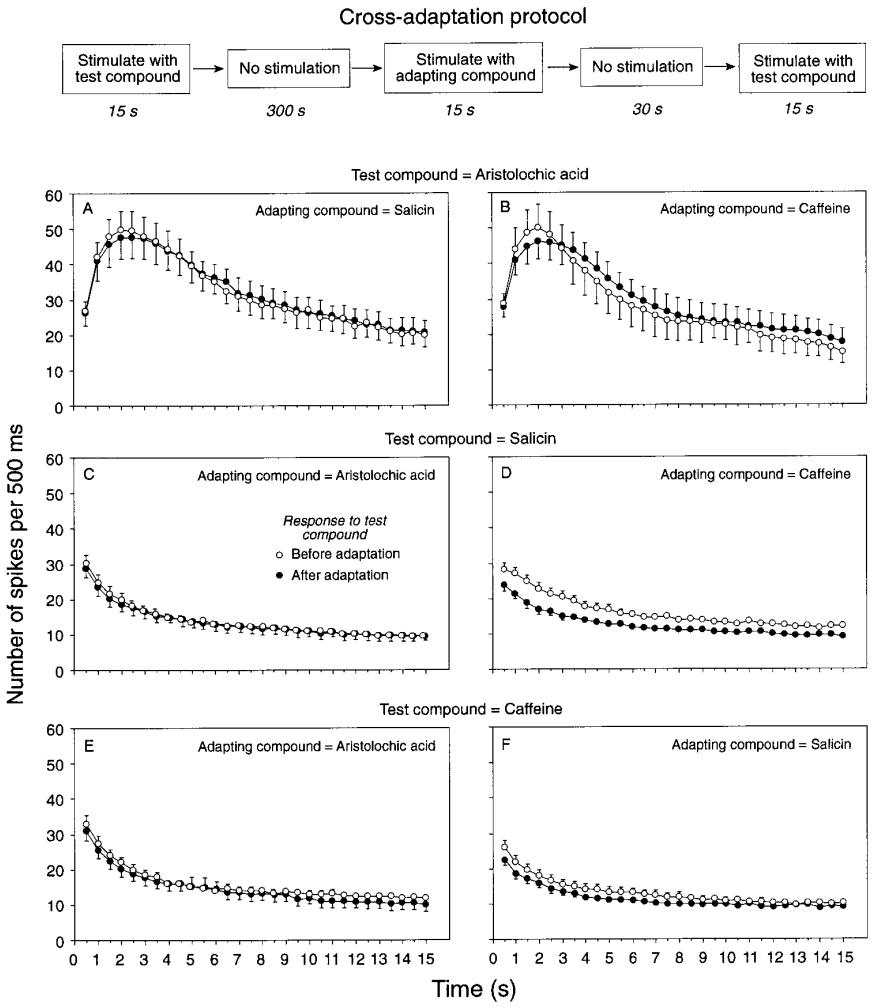

FIG. 8. Test for cross-adaptation between 0.1 mM aristolochic acid, 5 mM caffeine, and 50 mM salicin in deterrent TRC. We illustrate cross-adaptation protocol at *top* of figure. *A* and *B*: adaptation to salicin and caffeine affected normal response to aristolochic acid. *C* and *D*: adaptation to aristolochic acid and caffeine affected normal response to salicin. *E* and *F*: adaptation to aristolochic acid and salicin affected normal response to caffeine. Response variable is mean ± SE number of spikes per 500-ms bin. We inferred cross-adaptation when response to test compound before adaptation differed significantly from that after adaptation. See Table 4 for a statistical analysis of these results. Each panel is derived from 12 to 16 deterrent TRCs, each from different caterpillars.

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TABLE 4. *Analysis of the cross-adaptation tests in Fig. 8, A–F*

Test Compound	Adapting Compound	Source of Variation	df	*F*
Aristolochic acid	Caffeine	Repeated stimulation	1, 11	0.26 NS
		Time	29, 319	13.25*
		Interaction	29, 319	1.29 NS
Aristolochic acid	Salicin	Repeated stimulation	1, 11	0.03 NS
		Time	29, 319	11.37*
		Interaction	29, 319	0.47 NS
Caffeine	Aristolochic acid	Repeated stimulation	1, 11	0.71 NS
		Time	29, 319	99.80*
		Interaction	29, 319	1.21 NS
Salicin	Aristolochic acid	Repeated stimulation	1, 11	0.05 NS
		Time	29, 319	86.50*
		Interaction	29, 319	0.53 NS
Caffeine	Salicin	Repeated stimulation	1, 15	2.76 NS
		Time	29, 435	112.90*
		Interaction	29, 435	1.50*
Salicin	Caffeine	Repeated stimulation	1, 15	23.86*
		Time	29, 435	106.14*
		Interaction	29, 435	6.08*

We subjected the data in each panel to a two-way repeated measure ANOVA, with repeated stimulation (i.e., response to test compound before and after exposure to the adapting stimulus) and time (30 consecutive 500-ms bins) as the independent variables, and discharge rate during each 500-ms interval as the response variable (NS, $P > 0.05$; * $P \leq 0.05$). See Fig. 8 for more details.

Taken together, these findings provide unambiguous support for the conclusion that the deterrent TRC contains at least two excitatory transduction pathways: one responds to caffeine and salicin and the other to aristolochic acid. To our knowledge, this is the first direct support for the existence of two bitter transduction pathways within a single TRC.

This conclusion is supported by further analysis of the mixture data, using an index of response called the independent component index (ICI). The ICI is purported to indicate whether the components of a binary mixture activate a sensory receptor cell through independent pathways (Caprio et al. 1989; Cromarty and Derby 1997; Hyman and Frank 1980). It is calculated as $R_{ab}/(R_a + R_b)$, where a and b represent the two chosen tastants at response-matched concentrations, R_a and R_b represent the response magnitudes (i.e., total spikes during 500 ms) to a and b, respectively, and R_{ab} represents the response magnitude to the binary mixture of a and b. Accordingly, if the two components stimulate a receptor cell through independent pathways, then the response to the mixture should equal the sum of the response to the single components (i.e., the ICI would be statistically indistinguishable from 1). If the two components activate the same transduction pathway, the response to the mixture should be greater or less than the sum of the single components (i.e., the ICI would be significantly greater or <1).

Using the results in Table 2, we calculated that the mean ± SE ICI value for binary mixtures of caffeine and aristolochic acid was 0.87 ± 0.08, and for binary mixtures of caffeine and salicin was 0.68 ± 0.04. Next, using the one-sample *t*-test (two-tailed; alpha ≤ 0.05), we determined whether either of these means differed significantly from 1. Whereas the mean ICI for the mixture of caffeine and salicin was significantly <1 ($t_{(9)} = 7.96$), that for the mixture of caffeine and aristolochic acid did not differ from 1 ($t_{(9)} = 1.60$). Thus these findings reinforce the conclusion that the deterrent TRC contains at least two independent transduction pathways.

Functional significance

We can envision several ways that herbivores like *M. sexta* could benefit from having multiple bitter transduction pathways within the same TRC. One stems from the finding that the mixture of caffeine and aristolochic acid elicited about 1.7 times as many spikes/second as either compound alone, whereas the mixture of caffeine and salicin elicited only ~1.3 times as many spikes/second as either component alone (Table 2, Fig. 2). Given that caffeine and aristolochic acid stimulate different transduction pathways, our findings indicate that simultaneous activation of two pathways within the same deterrent TRC increases the chances of *M. sexta* detecting mixtures of bitter and potentially toxic compounds. The ecological relevance of this hypothesis is demonstrated by the facts that plant tissues often contain complex mixtures of bitter compounds (Rouseff 1990) and that many of these compounds are toxic at low concentrations (Holyoke and Reese 1987).

Another benefit to having multiple bitter transduction pathways within the same TRC is that compared with many other insects and vertebrates, caterpillars possess a limited number of TRCs (Bernays and Chapman 1994). Thus given that the molecular receptive range of any given transduction pathway is limited, the expression of multiple transduction pathways within each deterrent TRC may be the most parsimonious way to expand the range of bitter and potentially toxic compounds to which a caterpillar's gustatory system can respond.

There may also be costs associated with having multiple transduction pathways within the same TRC. If the CNS of *M. sexta* cannot discriminate between spikes from two transduction pathways within the same deterrent TRC, then its ability to discriminate between different bitter and potentially toxic compounds may be compromised. That herbivores like *M. sexta* might benefit from such a discriminatory ability is likely given that many compounds that taste bitter and/or elicit taste-rejection are nontoxic (Bernays 1991; Bernays and Cornelius 1992; Glendinning 1994; Harley and Thorsteinson 1967). Rejection of all foods that strongly stimulate the deterrent TRC may cause insects to taste-reject many harmless and potentially nutritious foods. However, if two transduction pathways within a TRC produce different

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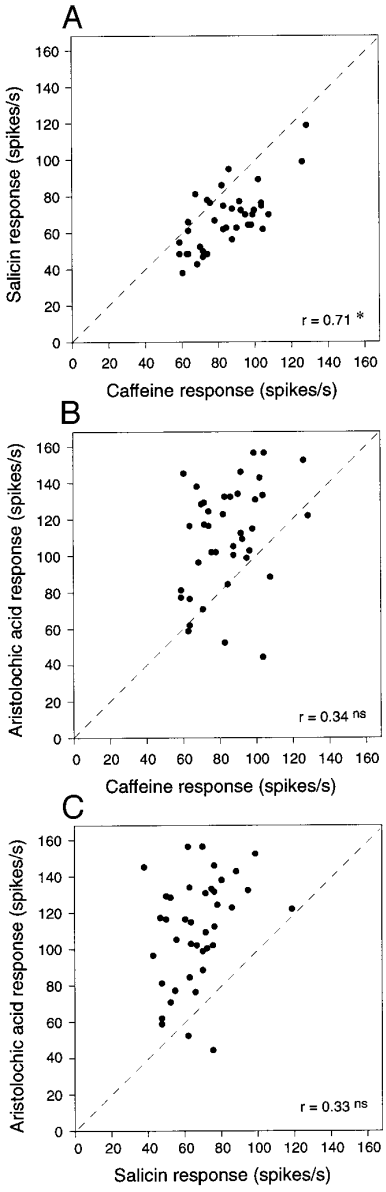

FIG. 9. Correlations of sensory response of individual deterrent TRCs to caffeine (5 mM), salicin (50 mM), and aristolochic acid (0.1 mM). Correlation in *A* is significant ($P \leq 0.05/3$), but those in *B* and *C* are not ($P > 0.05/3$). Line of equality (– – –) and Pearson product-moment correlation coefficient (r) are provided in each panel. All correlations involved the same 40 deterrent TRCs (each from different caterpillars).

temporal patterns of firing, and if the CNS can discriminate between these two patterns of firing, then the CNS may still be able to distinguish between spikes produced by the different transduction pathways. The results of this study present an ideal situation for evaluating this idea with an associative learning test: whereas caffeine and salicin elicited rapid spiking almost immediately after stimulation (i.e., within 50 ms), aristolochic acid elicited a pattern of spiking that gradually increased with time and did not plateau until several seconds after stimulation.

Several studies with vertebrates have reported that structurally different compounds can elicit different temporal patterns of spiking in peripheral taste nerves (Ogawa et al. 1973; Pietra et al. 1972) and at higher levels in the gustatory system (Di Lorenzo and Schwartzbaum 1982). However, the functional significance of these findings is unclear because no one has demonstrated that the vertebrate CNS can utilize this temporal information as a basis for discriminating between compounds.

In conclusion, we have provided direct electrophysiological support for the existence of two excitatory transduction pathways within a bitter-sensitive TRC in *M. sexta*. These pathways appear to have nonoverlapping molecular receptive ranges, and their responsiveness to their respective ligands is modulated independently. Studies are currently underway to determine the mechanistic basis and functional significance of these transduction pathways.

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