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Effects of Aromatic Compounds on Antennal Responses and on the Pheromone-Binding Proteins of the Gypsy Moth (*Lymantria dispar*)

Yongmei Gong and Erika Plettner

Department of Chemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6

Correspondence to be sent to: Erika Plettner, Department of Chemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6. e-mail: plettner@sfu.ca

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Abstract

Female gypsy moths emit a pheromone, (+)-disparlure, which the males follow until they locate the emitter. The male moths' antennae are covered with innervated sensory hairs, specialized in detection of the pheromone. The neurons in these sensory hairs are bathed by a solution rich in pheromone-binding protein (PBP). PBPs are soluble proteins that bind the pheromone and other odorants reversibly with variable thermodynamic and kinetic selectivity and are essential for olfactory responses. Here, we have studied the interaction between 2 gypsy moth PBPs with aromatic compounds that modulate the responses of male moth antennae to (+)-disparlure. The aromatic compounds do not elicit responses by themselves, but when administered together with pheromone, they inhibit, enhance, or prolong the electrophysiological response to the pheromone. Three interactions between the compounds and PBPs were studied: 1) the equilibrium binding of the compounds by themselves to the PBPs, 2) the equilibrium binding of the compounds in the presence of pheromone or a fluorescent reporter ligand, and 3) the effect of the compounds on the conformation of the pheromone-PBP complex. A subset of compounds causes a prolongation of the electroantennogram response, and from this study, we conclude that these compounds follow a structure-activity pattern and stabilize a particular conformer of the PBPs that appears to activate the olfactory response.

Key words: binding affinity, electroantennogram, gypsy moth, insect, olfaction, pheromone-binding protein

Introduction

Insects rely on the sense of smell (olfaction) for foraging, perceiving danger, and finding mates. For example, the gypsy moth, *Lymantria dispar*, a notorious defoliator of deciduous trees, uses pheromone communication for mate finding. The female gypsy moth produces a sex pheromone (*7R,8S*)-epoxy-2-methyloctadecane ((+)-disparlure, (+)-**1**) to attract males during the mating season. The enantiomer, (-)-**1**, itself is neither attractive nor repulsive, but it cancels the upwind flight of the male gypsy moth when it is present with (+)-**1** (Vite et al. 1976). This is an important attribute because 90% of the pheromone blend of a closely related species, *L. monacha*, consists of (-)-**1** (Hansen 1984; Grant et al. 1996). The male moths have developed highly effective and sensitive olfactory system that enables them to find a mate of the same species from far away. Pheromone communication is the key to the reproduction of these insects and, therefore, a powerful tool that can be used to monitor and/or control the pest population.

Insects use a specific set of sensory hairs (sensilla) on the antenna, the long sensilla trichodea, to detect the pheromone molecules. Sensilla trichodea are hollow cuticular cones penetrated by the dendrites of 2–3 olfactory neurons. The dendrites in the sensilla trichodea are bathed by lymph, a solution rich in odorant-binding proteins (OBPs). The information carried by a pheromone molecule is transduced to low-frequency changes in the ionic potential across the neuronal dendrite, detectable as the electroantennogram (EAG). A series of molecular interactions (in the lymph and at the dendritic membrane) have to take place to transduce the signal, involving the OBPs, odorant receptors (ORs), and other proteins (Xu et al. 2005; Benton et al. 2007; Jin et al. 2008; Laughlin et al. 2008; Sato et al. 2008; Wicher et al. 2008).

Pheromone-binding proteins (PBPs), members of the OBP family, specialize in pheromone binding. They can discriminate, albeit slightly, structurally related molecules as indicated by the different binding affinities observed in in vitro assays. For example, one of the 2 PBPs from the gypsy moth, LdisPBP1 binds (-)-**1** more strongly than (+)-**1** with K_ds of 2.2 and 7.1 μM, respectively (Plettner et al. 2000). The PBP from the silk moth (*Antheraea polyphemus*)

ApolPBP has a K_d of 0.64 μM for its pheromone (*E*,*Z*)-6,11-hexadecadienyl-1-acetate and a K_d of 21 μM for binding with the homolog (*E*,*Z*)-4,9-tetradecadienyl-1-acetate (Figure 1) (Du and Prestwich 1995). This difference leads to the conclusion that insect PBPs help to discriminate ligands, and that they preferentially deliver the ligand to the ORs based on the binding strength. However, PBPs bind nonbiological ligands with comparable affinities with the pheromone compounds. For example, the K_d for ApolPBP with 1-aminoanthracene (AMA) is 0.95 μM (Campanacci et al. 2001) and for LdisPBP1 with N-phenyl-1-naphthylamine (NPN) is 1.3 μM (Gong et al. 2010). It is also known that the binding affinities themselves do not correlate with either the EAG or the behavioral response of the insect to the compound (Honson et al. 2003; Laughlin et al. 2008).

PBPs are indispensable for pheromone perception. For example, fly mutants lacking the PBP known as LUSH had a complete loss of sensitivity to the pheromone *cis*-vaccenyl acetate (cVA), consistent with the behavioral insensitivity to this pheromone. Transgenic expression of LUSH restored the function (Xu et al. 2005). It has been proposed that PBPs deliver the ligand to the ORs to trigger an electrophysiological response of the neurons (Vogt et al. 1985; Kaissling 1986; Maida et al. 1995). Based on this hypothesis, we have investigated the relationship between PBP binding and the EAG response induced by a compound. Our group has synthesized 5 classes of compounds derived from or inspired by aromatic (phenolic) odorants from plants (Paduraru et al. 2008). Three classes of compound have been tested here. Our results are consistent with previous literature about other substances (Honson et al. 2003) that there is no obvious connection between the binding affinities of odorants with PBPs and the EAG response those odorants elicit.

Recent literature has shown that the D118A LUSH mutant, of which the C-terminal segment has been locked into an active conformation by substitution of Asp118 to Ala, induced electrophysiological activity of the corresponding neurons, in the absence of cVA (Laughlin et al. 2008). Inspired by this finding, we have attempted to correlate the effect of the synthetic compounds on PBP conformation to their effect on EAG recordings. Previously, we have described the effect of the synthetic compounds on the depth of the EAG depolarizations of mixed and pure pheromone plumes (Plettner and Gries 2010). For a subset of the compounds and compound sets tested (Figure 2), we noticed a prolongation of the EAG signal of a pheromone + compound mixture, due to a slower rate of recovery after the stimulus. Here, we describe 2 aspects of the aromatic compounds: 1) the interaction of the compounds with gypsy moth PBPs and 2) the structure-activity relationship (SAR) of our synthetic compounds with respect to the prolongation of EAG signals induced by mixed compound/pheromone stimuli. We have also measured the effect of signal-prolongation compounds on the conformation of the 2 PBPs, and we interpret the EAG results in terms of the effect the active compounds have on the PBP conformation.

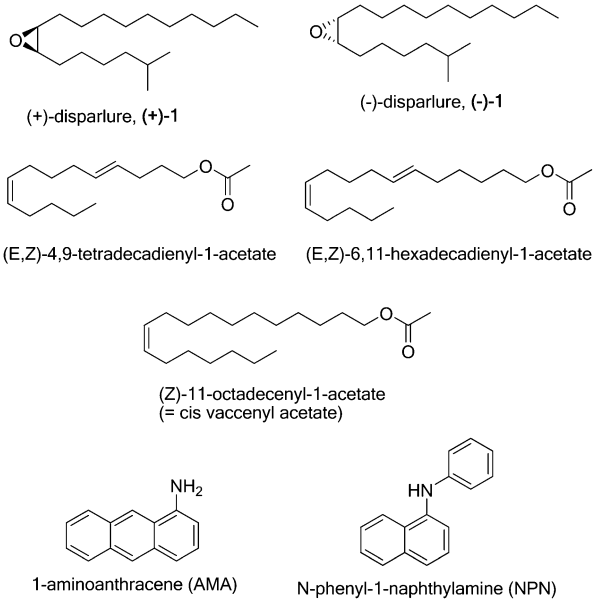

Figure 1 Structures of pheromones and fluorescent dyes mentioned in the introduction of this paper.

Materials and methods

Chemicals

Synthesis of the aromatic compounds tested has been described previously (Paduraru et al. 2008). Structures and corresponding nomenclature are shown in Figure 2 and these follow Paduraru et al. (2008). N-Phenyl-1-naphthylamine

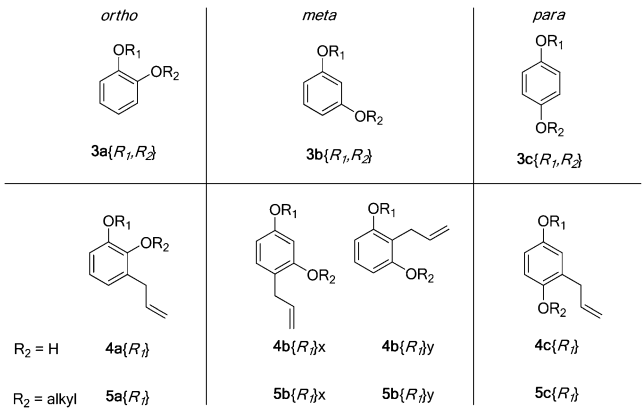

Figure 2 Structures of the aromatic compounds tested. R_1 or R_2 = CH_3 (Me) *1*, CH_2CH_3 (Et) *2*, $(CH_2)_2CH_3$ (Pr) *3*, $(CH_2)_3CH_3$ (Bu) *4*, $(CH_2)_2CH(CH_3)_2$ (iPent) *5*, and allyl *6* group. For example, **3c**{*2,3*} is 1-ethoxy-4-propoxybenzene, whereas **5b**{*1,1*}x is 1,3-dimethoxy-4-allylbenzene. Small sets of compounds were tested as part of previous studies (e.g., Plettner and Gries 2010). For example, **3a**{*3,1-5*} is a set of 1-propoxy-2-(variable alkoxy)benzenes. This coding scheme has been taken from Paduraru et al. (2008).

(NPN, 98%) and (+)-disparlure (95%) were purchased from Aldrich Chemical Co.

EAG experiments

The detailed procedures of EAG recordings were described in Paduraru et al. (2008) and Plettner and Gries (2010). Briefly, recordings were made with Syntech equipment, using Ag/AgCl electrodes in glass capillaries, bathed in insect ringer solution (Plettner and Gries 2010). Male gypsy moth pupae were obtained through the Canadian Forest Service (Insect Rearing Facility) and were emerged at Simon Fraser University in individual Ziploc containers with a moist filter paper, in an incubator at 18–20 °C during light (15 h) and at 16–18 °C during dark (9 h) periods.

For EAG, a moth antenna was dislodged, connected to the reference electrode at the base and to the recording electrode at the cut tip. A constant stream of purified air (charcoal filter) was passed over the antenna (300 mL/min) through a delivery tube placed ~1 cm from the antenna. Stimuli were placed on filter paper discs (Whatman # 1), the solvent was evaporated, and the discs were placed in Pasteur pipettes. These cartridges could be prepared ahead of time, wrapped individually, and kept at –80 °C until needed. A stimulus cartridge was placed in the side arm of the air delivery tube and connected to the Syntech CS-O5 stimulus controller. Puffs were delivered through the cartridge at 600 mL/min.

Several stimuli were puffed over the antenna sequentially: i) clean air, ii) pure (+)-disparlure (100 ng), iii) (+)-disparlure (100 ng) + test compound (1 μg), iv) (+)-disparlure (100 ng) + test compound (10 μg), v) (+)-disparlure (100 ng) + test compound (100 μg), vi) pure (+)-disparlure (100 ng) (Figure 3). There were 3 different effects the test compounds had on the EAG waves of the mixed puffs (iii-v) or on the last pure (+)-disparlure puff (vi) relative to the first one (ii). First, the depth of depolarization of the mixed puffs could be either enhanced or inhibited. We termed this short-term inhibition (STI). Second, the total time (s) of the EAG responses to mixed stimuli could be increased by certain compounds, mainly due to a slowing of the recovery. We termed this EAG peak broadening (PB). Third, the depth of the depolarization of the last pure (+)-disparlure puff (vi) relative to the first (+)-disparlure puff (ii) could be diminished. We termed this long-term inhibition (LTI). Each compound was replicated at least 6 times; details of replication can be found in the supplemental information of Plettner and Gries (2010).

PBP binding experiments

NPN competition binding assays

The binding affinities of the aromatic compounds were measured first with a fluorescence-based displacement experiment, conducted on 96-well plates. All the compounds

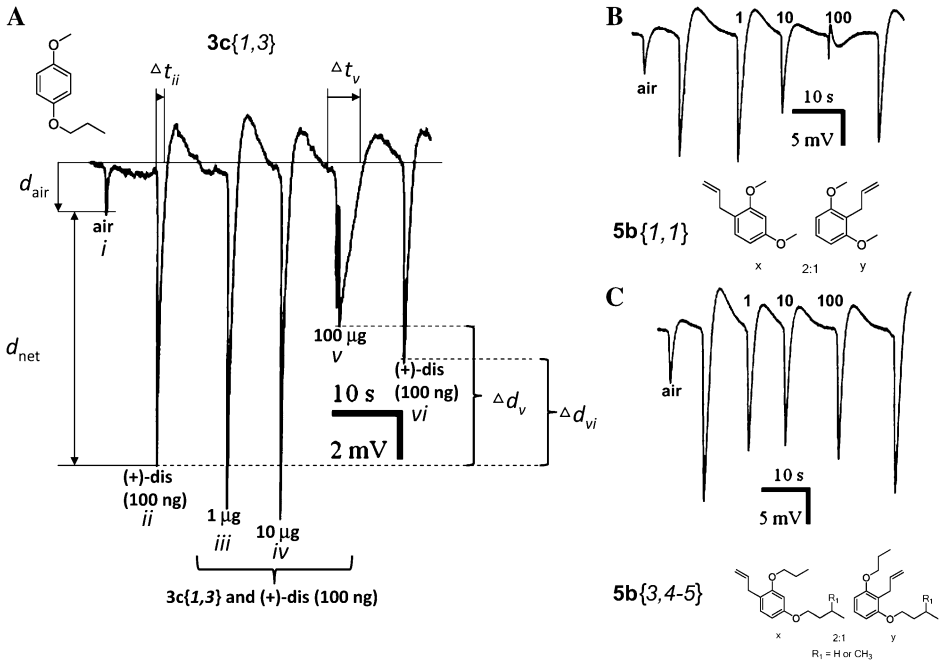

Figure 3 (A) Example of an EAG trace, illustrating the experimental procedure. Six puffs were recorded: clean air (i), pure (+)-disparlure (100 ng on the cartridge) (ii), mixture of 1 μg of the compound with 100 ng of the pheromone (+)-disparlure (iii), mixture of 10 μg of the compound with 100 ng of the pheromone (iv), mixture of 100 μg of the compound with the pheromone (v), and pure (+)-disparlure (100 ng) (vi). See text for other experimental details. (B) Example of a trace obtained in this experimental series with compound **5b**{*1,1*}, a 2:1 mixture of isomers x and y. (C) Example of a trace obtained with compound set **5b**{*3,4-5*}, a set that did not cause any significant activity with regard to depolarization, signal length, or long-term effects.

were dissolved in distilled MeOH to a final concentration of 2 mg/mL and diluted further to 0.14 mg/mL. On a preloading plate, the concentrations of each compound were 0, 2.3, 4.7, 7, 9.3, 12.8, 17.5, and 35 μg/mL from row A to H. N-phenylnaphthylamine, NPN, the fluorescent reporter, was prepared as a 2 mM stock in MeOH. PBPs (4 μM) were incubated with excess NPN (12 μM for PBP1 and 40 μM for PBP2) in 50 mM phosphate buffer, pH 7.0, for 30 min. The PBP/NPN mixtures were distributed into each well, 200 μL/well. Next, 4 μL of the compound solutions from the preloading plate were pipetted into the corresponding well, using a multichannel pipettor. Each PBP.ligand combination was run in 4 replicates. Plates were then scanned at 385 nm with the excitation wavelength of 337 nm on a Cary Eclipse Fluorimeter. Each compound was run in 3 replicates.

GC assays

In this study, GC assays were conducted to test the binding affinities of (+)-disparlure in the presence of the aromatic compounds and the binding affinities of the aromatic compounds alone. The GC assays followed the P2 gel filtration method. The detailed procedure can be found in Paduraru et al. (2008).

First, we measured the blend effect, the pheromone binding to the protein in the presence of a second compound. Blend effects have been detected previously, for example, racemic disparlure bound less strongly than either enantiomer (Plettner et al. 2000), and the (-)-disparlure/(*Z*)-2-methyloctadec-7-ene mixture bound more strongly than either component (Honson et al. 2003). We added 5 μM aromatic compound to a mixture of 4 μM PBP and 5 μM (+)-disparlure, in 20 mM Tris buffer, pH 7.4. The mixture was incubated and analyzed as described previously (Paduraru et al. 2008). The blend effect is represented by ΔK_d, which is the difference between the K_d values of (+)-disparlure in the absence and in the presence of the aromatic compound ($K_{d(\text{absence})} - K_{d(\text{presence})}$). A positive blend effect has a positive ΔK_d value, meaning that the aromatic compound has enhanced the binding affinity of PBP for (+)-disparlure. Each compound was tested in 4 replicates.

Stern–Volmer constant measurements

The ratio of Stern–Volmer constants reflects the accessibility of the tryptophan residue being quenched in the experiment to 2 different quenchers (iodide and acrylamide in this case) (Gong et al. 2010). Portions of 5 mM quencher (potassium iodide or acrylamide) stock solutions were added consecutively to 500 μL 2 μM protein samples either without or with ligand. To estimate the exposure of the Trp residue in the PBP.ligand complex, 10 μM of **3c** series compounds were used. The PBP and ligand mixtures were incubated for at least 2 h before each test. Three independent samples under each condition were used. Samples were excited at 295 nm, and data were collected from 310 to 350 nm on a PTI fluorimeter equipped with 814 photomultiplier detection system at 20 °C. Each compound was tested in 3 replicates.

Calculations of the SAR

Compounds that caused the strongest PB in this study (**3c**{*1,3*} and **5b**{*1,1*}) were assumed to be active at or near their global energy minima. Set **5b**{*1,1*} consists of 2 isomers (x and y, Figures 2 and 3; Paduraru et al. 2008), and the x isomer was assumed to be the active one because it can be superimposed easily on compound **3c**{*1,3*}. Two other compounds that caused strong PB in another study (Chen et al. 2010) were also chosen as reference compounds: (+) 1-propoxy-5-(2′-propoxyethyl)cyclopent-2-ene, "(+)-dipropyl," and the (-) enantiomer, "(-)-dipropyl." Upon inspection of the minimum conformations of these compounds, 6 points were noticed that could be superimposed. The distances between pairs of these points were measured for all the reference compounds at energy minima. The other individual compounds were then minimized first, and the heat of formation, H_{min}, was recorded. The compounds were then placed in a conformation, in which the 6 reference points fit to the distance ranges shown in Figure 8C. The heat of formation for this "active conformation," H_{act} was calculated, and the difference between the 2 heats of formation, $\Delta H = H_{\text{act}} - H_{\text{min}}$, was calculated. This was taken as the enthalpy penalty for a particular compound to fit into the active conformation (supplementary Figure 3S).

All molecular simulations were done using a semiempirical method in the MOPAC interface in CHEM 3D Ultra 11.0 (CambridgeSoft) and using the PM3 method. The wave function used was closed-shell (restricted), the optimizer was EF and the solvent was water. Compounds were drawn with ChemDraw, then copied into CHEM 3D, and minimized with the molecular mechanics program MM2. These minimized structures were then minimized again using MOPAC, as described above.

Physiological data were evaluated from the EAG traces (e.g., Figure 3), by measurement of the depolarizations (d) in mV and the EAG signal length (from stimulation to the return of the potential to baseline) (*t*) in s. STI refers to the decrease of the depolarization of a mixed antagonist/pheromone plume, relative to the depolarization of a normal pure pheromone stimulus (eq. 1). LTI refers to the decrease in the depolarization of a pure pheromone stimulus following a mixed antagonist/pheromone plume, relative to a normal pure pheromone stimulus (eq. 2). PB refers to the prolongation of the EAG signal corresponding to a mixed pheromone/compound stimulus, relative to a normal pure pheromone stimulus (eq. 3).

$$\text{STI} = 100 \times \Delta d_v / d_{\text{net}}. \quad (1)$$

$$\text{LTI} = 100 \times \Delta d_{\text{vi}} / d_{\text{net}}. \quad (2)$$

$$PB = 100 \times (\Delta t_v - \Delta t_{ii})/\Delta t_{ii}, \quad (3)$$

Where d are depolarizations in mV and t are times in s, and the subscripts refer to either a puff number (see Materials and Methods above) or a net value (Figure 3).

Data analysis

Dissociation constants of the compounds measured by the GC assay were calculated according to equation (4) (Plettner et al. 2000; Paduraru et al. 2008; Gong et al. 2009).

$$K_d = \frac{P_{free} \times L_{free}}{P \cdot L}. \quad (4)$$

Dissociation constants of the inhibitors (K_i) from the fluorescence competitive binding assay were calculated from the corresponding EC_{50} values, using equation (5).

$$K_i = \frac{EC_{50}}{1 + \frac{NPN_{free}}{K_{d,NPN}}}. \quad (5)$$

EC_{50} is the concentration of the compound that competes for half the specific binding. NPN_{free} is the free concentration of NPN, and $K_{d,NPN}$ is the dissociation constant of the complex PBP.NPN. The EC_{50} value was evaluated by plotting the percentage of binding (B) against the logarithm of the concentration of competitor and fitting the curve to the following equation.

$$B = C_T + \frac{C_T - C_B}{1 + 10^{x - \log EC_{50}}}. \quad (6)$$

C_T and C_B are the top and the bottom values from the curve, respectively. Data were analyzed with the Origin program.

Results

EAG responses altered by a compound

Figure 3 shows a typical EAG experimental trace. The following 3 parameters were measured in this experiment, as shown in the figure: STI, LTI, and PB (eqs. 1–3).

$$STI = 100 \times \Delta d_v / d_{net}. \quad (7)$$

$$LTI = 100 \times \Delta d_{vi} / d_{net}. \quad (8)$$

$$PB = 100 \times (\Delta t_v - \Delta t_{ii})/\Delta t_{ii}. \quad (9)$$

Where d are depolarizations in mV and t are times in s, and the subscripts refer to either a puff number (see Materials and Methods above) or a net value (Figure 3). STI represents the effect of the compound on the pheromone response when it is applied simultaneously with pheromone. LTI represents the long-term effect of the compound and pheromone mixture on the antennal response to pure pheromone. Both STI and LTI can depict either an antagonist (positive value) or an agonist (negative value) effect. PB manifests itself in a slower recovery phase of the EAG signal, which results in a broader EAG wave. A good example of broadening can be seen for puff v in Figure 3. The SAR for PB of the EAG signal showed clearly the size requirements of the compounds that caused PB (Figure 4). For the **3c** compounds, **3c**{*1*,*3*}, 1-methoxy-4-propoxybenzene, induced the strongest broadening (Figure 4C). SAR for both STI and LTI activities were discussed in a separate paper (Plettner and Gries 2010), but no analysis was made there with respect to the interaction between the PBPs and the compounds. Here, we focus on the interaction of the compounds with the 2 known gypsy moth PBPs and on EAG signal prolongation, PB. Data obtained for the compounds are tabulated in the supplementary information.

PBP ligand binding and EAG

There is no straightforward connection between the affinity of the compounds for PBP and EAG responses recorded for the entire collection of compounds. We only discuss those groups of compounds which show some correlation here.

Negative correlation between K_i and K_d

We used 2 methods to assess ligand binding to the PBPs: 1) a widely used fluorescence displacement assay and 2) a direct equilibration of the ligand with the PBP and separation of protein-bound from nonbound ligand by gel filtration. The fluorescence displacement assay gives K_i, the fluorescence displacement constant, whereas the equilibrium-binding assay gives a dissociation constant, K_d. The K_i value for each compound or set was obtained by fitting the data to a one-site competitive binding equation. When comparing compounds, a smaller K_i or K_d value is normally interpreted as stronger binding of the compound to the PBP. Paradoxically, the correlation between the available K_i and K_d values measured for our PBPs showed a negative slope (Figure 5). This means that those compounds that displaced NPN from PBP more effectively are those which by themselves did not bind PBP very strongly in the GC assay. This raises an important caveat for previous studies, in which only fluorophore displacement K_i values were used to make inferences about PBP-binding selectivities (Campanacci et al. 2001; Zhou et al. 2004), namely, that blend effects between NPN and the compound of interest could affect the relative binding affinity of a series of compounds. Therefore, the NPN displacement assay provides information about the relative ability of a series of compounds to displace the fluorescent reporter from the binding site of the PBP but not about absolute affinity of the PBP for the compounds being compared.

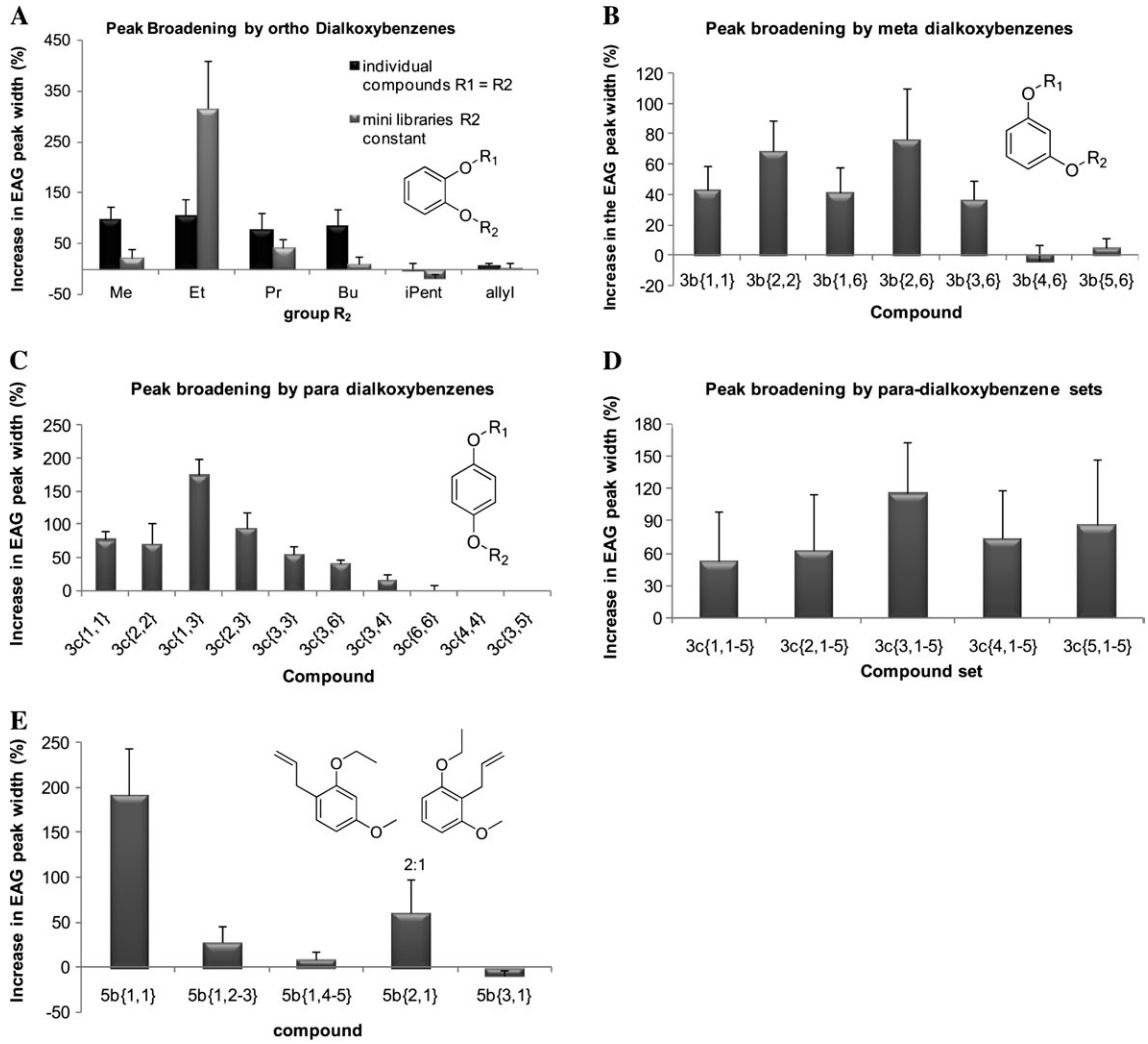

Figure 4 SAR for PB activity, for puff *v* (mixture of 100 ng (+)-disparlure with 100 µg of the compound or set). These values reflect the decrease (inhibition, positive values) or increase (enhancement, negative values) relative to the first pure disparlure puff (ii). (A) Ortho-dialkoxybenzene compounds or libraries. Me, methyl; Et, ethyl; Pr, n-propyl; Bu, n-butyl; iPent, isopentyl (3-methylbutyl). (B) and (C) Meta- and para-dialkoxybenzenes, respectively. (D) Para-dialkoxybenzene sets, with approximately equimolar mixtures of R_2 = Me, Et, Pr, Bu, and iPent and R_1 constant. (E) Allyl dialkoxybenzene sets.

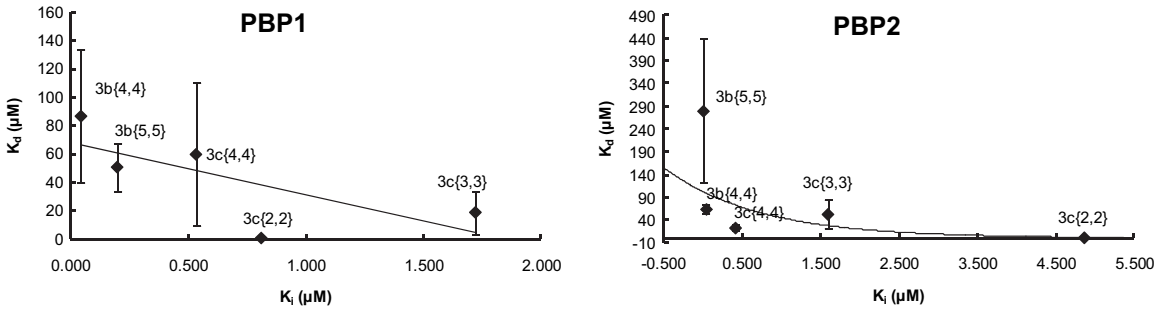

Figure 5 The dissociation constant of **3c** series compounds measured with the GC assay (K_d) correlates negatively with that obtained from the NPN displacement experiment (K_i) for PBP1 and PBP2.

Correlation between K_i and LTI

K_i values from all tested compounds were plotted against corresponding LTI values in Figure 6. The correlation was weak but overall, the strongest LTI effect came from the compounds with smaller K_i values with both PBPs. Overall, a small K_i is necessary but not the only condition that

leads to strong LTI of the pheromone signal. If plots of K_i versus LTI for each individual series of compounds are compared, it is impossible to reach a conclusion (supplementary). For some series of compounds, for example, the para-substituted **3c** library series, K_i values correlated negatively with the LTI, whereas for other series of compounds, the plots showed a positive correlation (e.g., the **5a** library series). From these analyses, we cannot predict the LTI effect of a compound from its ability to displace NPN from the binding pocket of PBPs.

The blend effect and EAG

Correlation between ΔK_d and peak broadening

To correlate the blend effect and the LTI effect, we chose compounds that induced a strong LTI and tested them for a blend effect with (+)-disparlure, using a GC-based assay. No connection was found between the LTI and the blend effect. Instead, we have found that for PBP1, the blend effect (ΔK_d) was related to PB. For PBP1, the compounds tested could be categorized into 2 series: series 1 induced positive blend effects and series 2 induced negative blend effects. The strongest PB came from the compounds with either the strongest positive blend effect (series 1, **3c**{*5,1-5*} and **3c**{*4,1-5*}) or the weakest negative blend effect (series 2, **5b**{*1,1*}) (Figure 7). This indicated that in both cases, the strongest possible binding of (+)-disparlure with PBP1 in the presence of that series of compounds would induce the strongest PB.

PBP conformation and peak broadening

Correlation between the PBP conformation (measured by the ratio of Stern–Volmer constants) and peak broadening

The ratios of Stern–Volmer (SV) constants reflect the relative accessibility of a tryptophan residue to 2 quenchers, in our case: iodide (a hydrophilic, hindered quencher) and acrylamide (a hydrophobic, nonhindered quencher). A change in the ratio of SV constants reflects a change in the accessibility of the tryptophan residue to the quenchers. This, in turn, reflects a change in the conformation of the protein. (Gong et al. 2010)

We have obtained the ratio of the Stern–Volmer constants for the **3c** compounds (**3c**{*1,1*}, **3c**{*2,2*}, **3c**{*1,3*}, **3c**{*3,3*}, and **3c**{*3,5*}), with both PBPs. Overall, compounds with a smaller volume gave higher ratios (Figure 8A). A very important result is shown in Figure 8B: compounds that gave the same ratio of the Stern–Volmer constants as (+)-disparlure showed a larger PB effect than compounds that give significantly larger or smaller ratios than disparlure. Thus, the compounds we have studied here induce different conformations when added to the PBPs together with the cognate ligand of the PBPs, (+)-disparlure. The observation that compounds, which give similar SV ratios to pure disparlure when bound to PBPs, cause EAG signal prolongation is consistent with our hypothesis that the PB might be related to the stabilization of the active conformation of the PBPs induced by the cognate pheromone ligand.

SAR for the prolongation of EAG signals

Superposition of energy minimized conformations of the most active EAG peak-broadening compounds, as well as of the most active compounds from another study (Chen et al. 2010), suggests that certain structural features are required to cause EAG signal prolongation. The compounds fit into a site with at least 4 major recognition points (Figure 8) and 2 more flexible less important recognition points. The compounds of lower activity studied here require the adoption of an unstable conformation to fit into the site (supplementary information, Figure 3S). Furthermore, we observed larger energy penalties required for the less active compounds, which validates the structure-activity model shown in Figure 9.

The location of the "site" for prolongation activity is not known but given that the PBPs appear to be involved in PB activity (see above), the PBP is a likely candidate for the binding site that mediates this activity. The site, however, could also involve the OR or a protein–protein interface that forms when the cognate odorant (the pheromone in our case) interacts with the dendritic membrane in the sensillum. It is

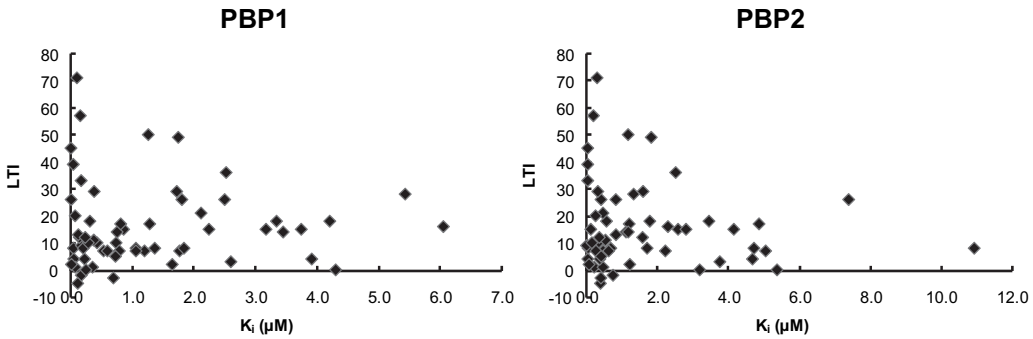

Figure 6 Lack of correlation between PBP binding and LTI of the EAG signal (puff *vi* in Figure 2). LTI = 100 × $\Delta d_{vi}/d_{net}$ (Figure 3). Shown are the combined data from all the aromatic compounds and sets of compounds tested.

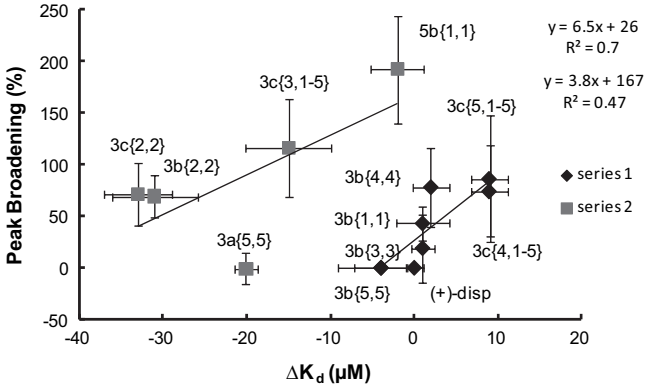

Figure 7 Correlation between EAG signal prolongation (PB) activity and the blend effect of aromatic compounds on the binding of PBP1 with (+)-disparlure. PB = 100 × ($\Delta t_v - \Delta t_{ii}$)/$\Delta t_{ii}$ (Figure 3). The blend effect was measured as the dissociation constant of (+)-disparlure in the absence and the presence of the aromatic compound or set of compounds (ΔK_d = $K_{d(\text{absence})} - K_{d(\text{presence})}$). Positive ΔK_d values indicate an enhancement of binding affinity between PBP and pheromone, by the added compound.

important to note that the aromatic compounds studied here and in Plettner and Gries (2010) exhibited their activities only when administered together with (+)-disparlure or another cognate ligand of the system. In contrast, the disparlure mimics studied in Chen et al. (2010) were weakly active by themselves and gave broad EAG signals. When administered together with (+)-disparlure, those compounds appeared to compete slightly with (+)-disparlure but still gave broadened EAG signals (Chen et al. 2010). The most active PB compounds from that study (the dipropyl congeners) also fit into the SAR model from this study (Figure 9).

Discussion

Communication with species-specific signal chemicals (pheromones) plays an important role in insect reproduction. For example, in the case of moths, the female releases the pheromone, and the males detect and follow the pheromone plume upwind to mate. Among the components of the olfactory system, PBPs are the most abundant extracellular proteins in the sensory hairs of insects and these proteins are important for pheromone detection. The aim of this project was to develop an effective screening assay for the pheromone olfaction inhibitor, based on the target olfactory system from the gypsy moth. We aimed to build a connection between the PBP-binding data and the EAG recordings database for aromatic compounds (Plettner and Gries 2010).

It was difficult to link the PBP binding directly to EAG responses. As indicated in the results, the apparent binding affinities of a compound to the PBPs did not correlate to any EAG responses (Figure 6 and data not shown). Part of this problem is due to the complexity of EAG signals. An EAG peak represents the overall electrophysiological behavior of the whole moth antenna. PBP binding is only the first step of

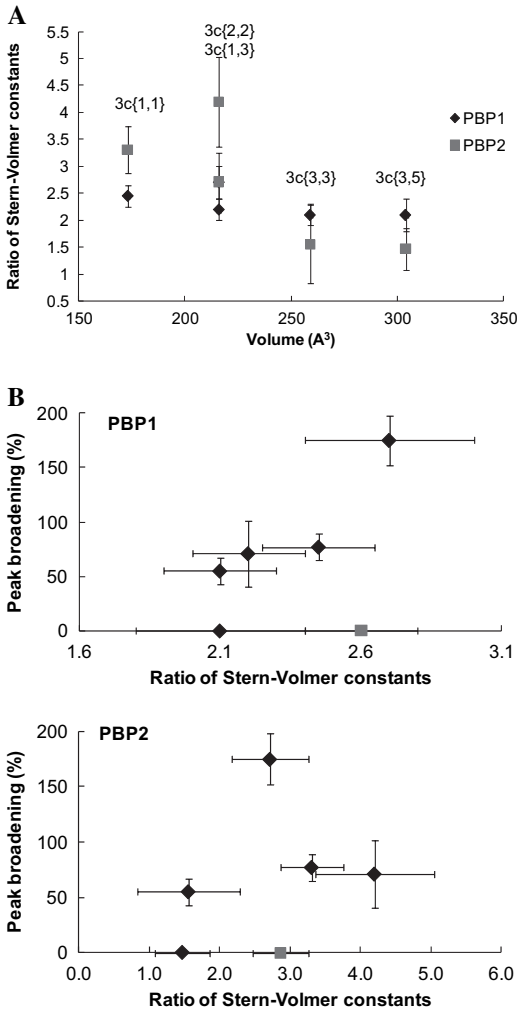

Figure 8 (A) SAR for the ratio of the Stern–Volmer (SV) constants for both PBPs. The ratio of SV constants is sensitive to the conformation of the protein (see text). (B) Correlation between EAG signal prolongation (PB) and the ratio of Stern–Volmer constants for compounds **3c**{*1,3*}, **3c**{*1,1*}, **3c**{*2,2*}, **3c**{*3,3*}, **3c**{*3,5*} (in order of decreasing SV ratio). Black diamonds: **3c** compounds; gray square: (+)-disparlure. Bars indicated the standard error for PB and the error propagation from the fitting errors for the ratio of the Stern–Volmer constants.

the signal transduction process in portions of sensory hairs, and this is followed by many other peripheral and neuronal events (reviewed in: Plettner and Gries 2010). However, we did observe some correlation between PBP binding and the prolongation (PB) of an EAG signal. Compounds with PB activity altered the binding affinities of (+)-disparlure with PBP1, and this behavior correlated to PB (Figure 7). We also found, among the para (**3c**) compounds, a connection between PB and the ratio of the Stern–Volmer constants (Figure 8B). The ratio of the Stern–Volmer constants reflects the local conformation of a tryptophan residue in PBPs (Gong et al. 2010). Both of these connections suggest that compounds, which stabilize an active conformation of the

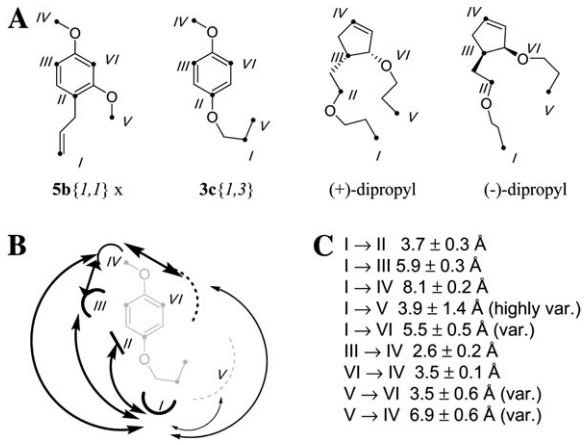

Figure 9 (A) Most active EAG signal prolongation-causing compounds. The "dipropyl" compounds are from Chen et al. 2010, the other 2 compounds are presented in this paper. The 6 recognition points for the SAR are shown on the structures and numbered with roman numerals. (B) Recognition features (odotopes) for EAG signal prolongation ("PB") activity. Points and distances that are required and not significantly variable are shown in bold, continuous lines, whereas variable features are shown with thin, broken lines. (C) Average distances between recognition points for the 4 most active compounds at energy minima.

PBP-pheromone complex, cause prolongation of the EAG signal.

We have proposed before that PBPs interact with ligands in a stepwise mechanism (Gong et al. 2009). Furthermore, some of the steps in the mechanism reflect conformational changes induced by a ligand bound to the PBP (Gong et al. 2010). Other researchers have shown that a conformer of PBP-pheromone complex activates the relevant OR, perhaps by a direct OR–PBP–pheromone interaction (Laughlin et al. 2008). It is therefore reasonable to hypothesize that a PBP may also activate the ORs if an artificial compound happens to trigger a similar conformational change in the PBP than the pheromone, or if the active conformer of the PBP-pheromone complex is stabilized by an added compound. Our hypothesis is that the EAG prolongation (or PB) is the prolonged activation of the ORs triggered by the active complex of PBP and cognate ligand. Furthermore, the active conformation of that complex appears to be longer lived than normal, either through stabilization of the active form or destabilization of the inactive form of the PBP-ligand complex (Figure 8B). In both cases, the OR activation would be prolonged. Given that stronger pheromone binding correlated with PB activity (Figure 6), the former possibility (stabilization of the active conformer, relative to inactive conformers) is more likely.

Another important observation is that K_i and K_d values, obtained by fluorescence displacement assays and GC assays of PBPs, respectively, correlated negatively. Previous work with gypsy moth PBPs suggested that the K_d values obtained in GC assays reflect the most stable ligand binding at the completely enclosed internal binding site, whereas the increase in the NPN fluorescence intensity in PBP.NPN complexes (relative to free NPN) reflects both internally and externally bound NPN (Gong et al. 2010). Externally bound NPN is probably easier to be displaced by another ligand than internally bound NPN, given the multistep mechanism of ligand binding and release (Gong et al. 2009). Therefore, ligands with lower K_i values probably displaced more accessible, externally bound NPN readily. Ligands with high K_i values most likely bind to the PBP internally, which requires a multistep entry mechanism. Because this is a more complex (less likely) process, a higher concentration of ligand is necessary to displace the NPN reporter ligand.

Previous studies with minilibraries of aromatic compounds have shown blend effects (Paduraru et al. 2008), presumably because multiple ligands are competing in the multistep binding mechanism of PBPs. Therefore, the fluorophore displacement constants, K_i, reflect only the ability of a competing compound to displace either externally or both externally and internally bound NPN. Therefore, NPN displacement data should be used with caution when inferring the ligand-binding selectivity of insect PBPs.

In conclusion, we have attempted to link in vitro detectable PBP–ligand interactions to the electrophysiological responses induced by a ligand. The binding affinities to PBPs of a series of derivatives of general odorant compounds did not show a straightforward relationship with the EAG responses to mixed stimuli of pheromone and the test compounds. Therefore, it is impossible to predict an antagonist or an agonist from the PBP-ligand affinities. A breakthrough came from a series of compounds that, when given with the pheromone, cause a delayed activation of the antenna, concurrent with an antagonism of the antennal depolarization. For these compounds, a particular conformation of the PBP-pheromone complex appeared to be stabilized. We conclude that the PBP conformations induced by the ligand interaction appear to influence the ability of the system to first activate and later deactivate the signaling cascade that gives rise to the EAG signal.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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