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Pheromone-binding Proteins Contribute to the Activation of Olfactory Receptor Neurons in the Silkmoths *Antheraea polyphemus* and *Bombyx mori*

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Abstract

The sensilla trichodea of the silkmoth *Antheraea polyphemus* are innervated by three types of receptor neurons each responding specifically to one of three pheromone components. The sensillum lymph of these sensilla surrounding the sensory dendrites contains three different types of pheromone-binding proteins (PBPs) in high concentrations. The sensilla trichodea of the silkmoth *Bombyx mori* are supplied by two receptor neurons each tuned specifically to one of the two pheromone components bombykol and bombykal, but only one type of PBP has been found so far in these sensilla. Recombinant PBPs of both silkmoth species in various combinations with pheromone components were applied to the receptor neurons via tip-opened sensilla during electrophysiological recordings. Over a fairly broad range of pheromone concentrations the responses of the receptor neurons depended on both, the pheromone component and the type of the PBP. Therefore, the PBPs appear to contribute to the excitation of the receptor neurons. Furthermore, bombykal in combination with the expressed PBP of *B. mori* failed to activate the corresponding receptor neuron of *B. mori,* but did so if combined with one of the PBPs of *A. polyphemus*. Therefore, a still unknown binding protein involved in bombykal transport might be present in *B. mori*.

Key words: electrophysiology, olfaction, pheromone-binding protein, silkmoth, single-sensillum recording

Introduction

Pheromone-binding proteins (PBPs) occur in concentrations up to 10 mM in the extracellular sensillum lymph surrounding the sensitive processes of olfactory receptor neurons (ORNs) in insects (Vogt and Riddiford, 1981; Klein, 1987). They solubilize and carry the odorants to the ORNs (van den Berg and Ziegelberger, 1991). Possibly they also protect the odorants from enzymatic degradation on their way to the receptor molecule, mediate the interaction between odorant and receptor molecule and, finally, deactivate the odorant (Ziegelberger, 1995; Kaissling, 1998, 2001).

In the male silkmoth *Antheraea polyphemus* the olfactory sensilla trichodea contain two or sometimes three ORNs, each responding specifically to one of the pheromone components (*E,Z*)-6,11-hexadecadienyl acetate (AC1), (*E,Z*)-6,11-hexadecadienal (AL) and (*E,Z*)-4,9-tetradecadienyl acetate (Meng *et al.*, 1989). Biochemical studies have shown, that in a competitive situation each of the three PBPs co-expressed in these sensilla (ApolPBP1, 2 and 3) preferentially binds one of the pheromone components (Maida *et al.*, 2000, 2003). Both native and recombinant ApolPBP1 bind AC1 preferentially (Maida *et al.*, 2000, 2003), while the recombinant ApolPBP1 has been shown to bind all three pheromone components (Campanacci *et al.*, 2001; Bette *et al.*, 2002). In a recent electrophysiological study (Pophof, 2002) it could be shown, that in *A. polyphemus* the PBPs contribute to the excitation of the ORNs; possibly it is the complex pheromone-PBP that activates the receptor molecule.

The olfactory hairs of the male silkmoth *Bombyx mori* contain two ORNs responding to the female pheromone components (*E,Z*)-10,12-hexadecadienol (BOL, bombykol; Kaissling and Priesner, 1970) and (*E,Z*)-10,12-hexadecadienal (BAL, bombykal; Kaissling *et al.*, 1978). In this species, only one PBP has been found so far (Maida *et al.*, 1993; Krieger *et al.*, 1996), but there are indications that more PBPs may be present although in minute amounts (Maida and Ziegelberger, 1994; Maida *et al.*, 1997). The recent analysis of the structure of the BmoriPBP suggests that the pheromone binds to an inner cavity of the protein molecule and can be removed from the cavity by a conformational

change of the PBP at low pH or upon interaction with negatively charged lipid bilayer membranes (Wojtasek and Leal, 1999; Damberger *et al.*, 2000; Sandler *et al.*, 2000; Horst *et al.*, 2001). Thus, the question arises as to whether in *B. mori*, other than in *A. polyphemus*, a single PBP could bind both pheromone components, which then would be released at the dendritic membrane before activating the receptor molecules.

Combining the recombinant PBPs of *A. polyphemus* and *B. mori* with pheromone components of both species and bringing these into direct contact with the receptor cell dendrites *in situ* opens the possibility to experimentally investigate two important questions: (i) are the results reported for *A. polyphemus* (Pophof, 2002) reproducible over a wider range of pheromone concentrations and (ii) are the two silkmoth species different as far as the direct contribution of PBPs to receptor activation is concerned?

Materials and methods

Pupae of the silkmoth *A. polyphemus* (Lepidoptera: Attacidae) were obtained from commercial breeders (M. Soroka, D. Oehlke, USA; B. Oehlke, Canada), the pupae of the silkmoth *B. mori* L. (Bombycidae) from INRA—Unité nationale séricicole (LaMulatiére, France) and Worldwide Butterflies (Dorset, UK). The animals were sexed at the pupal stage and allowed to emerge at room temperature. The male moths were then stored in a refrigerator at 12°C and used for the experiments 1–4 days after emergence.

Tip recordings were performed from single male sensilla trichodea of isolated antennae of *B. mori* or isolated antennal branches of *A. polyphemus* using glass capillary Ag–AgCl electrodes. The reference electrode was filled with hemolymph Ringer solution and inserted into the base of the antenna or antennal branch; the recording electrode was filled with sensillum-lymph Ringer solution (SLR) and slipped over the cut sensillum tip (Kaissling, 1995). Such a preparation can be used for recordings for at least 30 min. The preparation was held in a permanent airstream (1 m/s) filtered through charcoal and humidified by percolation through distilled water. The signals were amplified using a custom made amplifier with a low pass cutoff frequency of 2 kHz. The unfiltered data were sampled online using a Macintosh G3 computer and the data acquisition program SuperScope II 2.31 (GW Instruments).

Three PBPs of *A. polyphemus* (ApolPBP1, ApolPBP2, ApolPBP3; Maida *et al.*, 2000), and one PBP of *B. mori* (BmoriPBP; Krieger *et al.*, 1996) were heterologously expressed by J. Krieger (Stuttgart-Hohenheim) in *Escherichia coli* as described by Campanacci *et al.* (2001). The pheromone components AC1 [(*E,Z*)-6,11-hexadecadienyl acetate] and AL [(*E,Z*)-6,11-hexadecadienal] of *A. polyphemus* and BOL [(*E,Z*)-10,12-hexadecadienol] and BAL [(*E,Z*)-10,12-hexadecadienal] of *B. mori* were supplied by H.-J. Bestmann (Erlangen). In *A. polyphemus*, only the longest sensilla trichodea were used for recordings; these contain two ORNs, one tuned to AC1and the other to AL.

The pheromone components were solubilized in SLR as described previously (Kaissling *et al.*, 1991; van den Berg and Ziegelberger, 1991; Ziegelberger, 1995). Pheromone components (amounts between 5 pg and 5 µg) dissolved in 5 µl hexane were filled into standard glass test tubes and after the evaporation of the solvent 20 µl of pure SLR or SLR containing either 16 µM of one of the expressed soluble PBPs or 0.005% of the standard solubilizer dimethyl sulfoxide (DMSO) was added. The solution was incubated for 40 min at room temperature and then transferred into a plastic Eppendorf vial and stored at 8°C for between 1 and 5 days until filled into glass electrode capillaries and used for experiments. According to experiments with radioactively labeled AC1 and AL the amount of pheromone bound to PBP remains stable under these conditions (G. Ziegelberger, Seewiesen, personal communication). In the case that all of the pheromone was solubilized, the final concentrations were between 0.9 nM and 0.9 mM AC1 or AL and either 9 nM or 90 nM BOL or BAL. In *A. polyphemus*, the chosen concentrations covered the whole physiological range from almost no response to strong adaptation. In *B. mori*, the two concentrations used corresponded to those concentrations at which the influence of the PBPs could be best observed in *A. polyphemus*.

Recordings were performed on 294 single sensilla from 72 antennal branches of male *A. polyphemus* and on 284 sensilla from 43 antennae of male *B. mori*. Various control and test solutions were allowed to diffuse into the olfactory hair from the recording electrode via the cut sensillum tip. The nerve impulse frequency of the single pheromone-sensitive ORNs was measured for 1 min after the contact between electrode capillary and sensillum was established. One hundred and thirty-three sensilla of *A. polyphemus* and 178 sensilla of *B. mori* were first tested with pure SLR within the electrode to measure the spontaneous nerve impulse activity. Control experiments were performed on 44 sensilla of *A. polpyhemus* and 96 sensilla of *B. mori* either with one of the recombinant PBPs (16 µM) or with DMSO (0.005%) solubilized in SLR without pheromone added.

In *A. polyphemus*, 270 different sensilla were tested with single solutions containing between 0.9 nM and 0.9 mM of the pheromone components AC1 or AL. The pheromone components were dissolved in SLR either without solubilizer or with either DMSO or one of the recombinant PBPs of *A. polyphemus*. In *B. mori*, 199 sensilla were tested with solutions containing 9 or 90 nM BOL or BAL, dissolved in SLR without solubilizer, or with DMSO or one of the expressed PBPs from both *B. mori* and *A. polyphemus*. Unpaired *t*-tests were used to confirm whether the nerve impulse frequency increased over the spontaneous level after application of single test or control solutions and whether the responses were significantly different from each other.

Regarding the high variability of the data, the alpha-level for significance was set to $P < 0.01$.

Results

Antheraea polyphemus

In *A. polyphemus* the spontaneous nerve impulse frequency measured with pure SLR in the recording electrode was 0.22 ± 0.37 imp./s ($n = 133$) in the ORN with the larger nerve impulse amplitude, which is tuned to AC1 (AC1-cell). In the ORN with the smaller nerve impulse amplitude, specialized to AL (AL-cell), the spontaneous nerve impulse frequency was 0.18 ± 0.36 imp./s ($n = 133$). Neither 0.005% DMSO nor 16 µM of any of the three recombinant PBPs of *A. polyphemus* solubilized in SLR had any effect on the nerve impulse frequency of these two pheromone-sensitive ORNs when presented without pheromone (Table 1).

The pheromone components AC1 and AL were tested at a broad range of concentrations from 0.9 nM to 0.9 mM (Figure 1). The nerve impulse frequencies of the responses elicited in the AC1-cell by AC increased with increasing pheromone concentration from 0.9 to 90 nM; at a concentration of 9 µM and more, the nerve impulse frequencies were lower again, probably due to adaptation (see Discussion). The nerve impulse frequencies elicited in the AL-cell by AL varied only little with increasing pheromone concentration.

AC1 (0.9 nM) diluted in SLR without any solubilizer failed to activate the AC1-cell. At the same pheromone concentration but in the presence of a solubilizer, AC1 activated the AC1-cell significantly and independently of the solubilizer used (Figure 1A), i.e. the responses to AC1 with DMSO or any of the recombinant PBPs of *A. polpyhemus* were similar. Surprisingly, the combination AC1/ApolPBP2 significantly excited the AL-cell. AL activated the AL-cell at the concentration of 0.9 nM only if combined with DMSO or with ApolPBP2 (Figure 1B). Without a solubilizer or combined with ApolPBP1 or ApolPBP3 it had no effect on the nerve impulse response of the AL-cell. 0.9 nM AL did not activate the AC1-cell.

At the pheromone concentration of 9 nM, AC1 activated the AC1-cell without a solubilizer, or when combined with DMSO, ApolPBP1 and ApolPBP3 (Figure 1C). However, in combination with ApolPBP2, 9 nM AC1 did not activate the AC1-cell. The AL-cell was not activated by 9 nM AC1, regardless in which combination it was offered. AL at the concentration of 9 nM activated the AL-cell without a solubilizer or when combined with DMSO, ApolPBP2 and ApolPBP3 (Figure 1D). It should be noted that the combination AL/ApolPBP1 had no effect on the nerve impulse frequency of the AL-cell, but activated significantly the AC1-cell.

At a pheromone concentration of 90 nM and more (Figure 1E–J), the specificity of both pheromone-sensitive ORNs of *A. polyphemus* was considerably lowered and both pheromone-sensitive ORNs responded to both AC1 and AL. AC1 at 90 nM activated the AC1-cell without a solubilizer or combined with any of the recombinant PBPs of *A. polyphemus* (Figure 1E). The combinations AC1/ApolPBP1 and AC1/ApolPBP3 elicited stronger responses than pure AC1 or AC1/ApolPBP2. Furthermore, AC1 excited the AL-cell without a solubilizer, or combined with ApolPBP1 or ApolPBP2. AL at 90 nM activated the AL-cell without a solubilizer or combined with any of the PBPs (Figure 1F). However, AL did not activate the AC1-cell without a PBP; it did so in combination with any of them, but in combination with ApolPBP1 it elicited a significantly larger response than in combination with ApolPBP2.

AC1 at 9 µM did not activate any of the pheromone-sensitive ORNs of *A. polyphemus* without a solubilizer (Figure 1G); it excited the AC1-cell in combination with ApolPBP1 or ApolPBP2, but the AL-cell was excited only in combination with ApolPBP2. AL at 9 µM did not elicit any significant responses in both, the AC1-cell and AL-cell (Figure 1H); possibly the number of sensilla tested with this concentration of AL was too low.

At the pheromone concentration of 0.9 mM, the specificity of the two pheromone-sensitive ORNs of *A. polyphemus* was completely lost, both of them responded to both pheromone components solubilized by either ApolPBP1 or ApolPBP2 (Figure 1I,J).

Table 1 Average spontaneous nerve impulse frequencies (imp./s) of the two pheromone-sensitive receptor neurons of *A. polyphemus* measured over 1 min with sensillum-lymph Ringer solution (SLR) in the recording electrode and with 0.005% DMSO or 16 µM of one of the three recombinant pheromone-binding proteins (PBPs) of *A. polyphemus* diluted in SLR

	AC1-cell	AL-cell	*n*
SLR	0.2220 ± 0.3710	0.1790 ± 0.3570	133
DMSO	0.1770 ± 0.3540	0.0380 ± 0.0570	12
ApolPBP1	0.2150 ± 0.5570	0.1270 ± 0.1280	11
ApolPBP2	0.1300 ± 0.1890	0.0660 ± 0.0800	10
ApolPBP3	0.2420 ± 0.7490	0.1060 ± 0.1560	11

Neither DMSO nor any of the PBPs alone activated significantly any of the receptor neurons (unpaired *t*-test, $P < 0.05$).
Means ± SD; n = number of tested sensilla.

Bombyx mori

In *B. mori* the spontaneous nerve impulse frequency measured with pure SLR in the recording electrode was 0.27 ± 0.62 imp./s ($n = 178$) in the ORN with the larger nerve impulse amplitude, which is tuned to BOL (BOL-cell, Figure 2C). In the ORN with the smaller nerve impulse amplitude, responding to BAL (BAL-cell, Figure 2C), the spontaneous nerve impulse frequency was 0.20 ± 0.68 imp./s ($n = 178$). Addition of 16 µM of recombinant ApolPBP1 increased the nerve impulse frequency of the BOL-cell significantly by

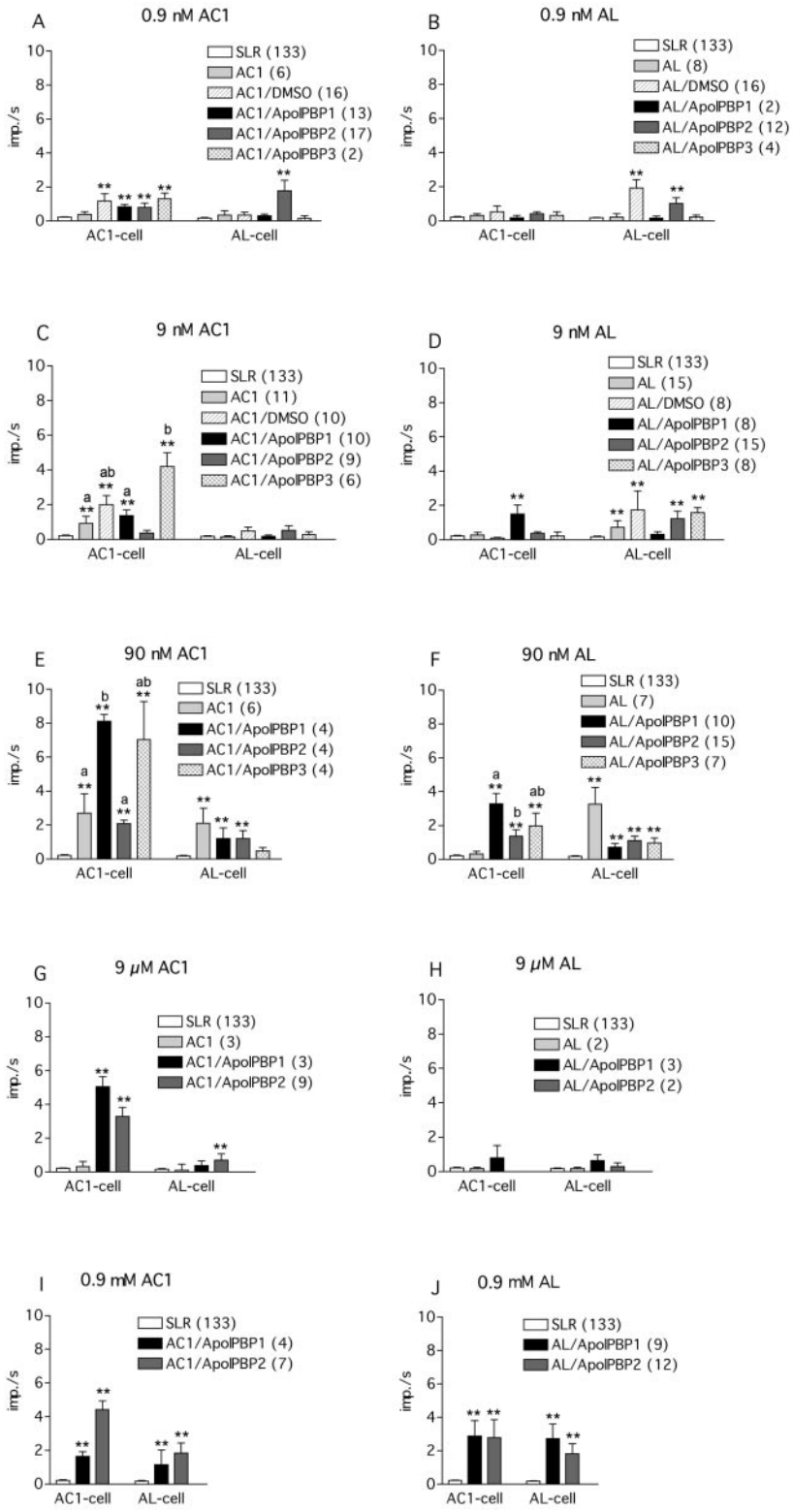

Figure 1 Average nerve impulse frequencies (imp./s) of the two pheromone-sensitive receptor neurons of *A. polyphemus* elicited by 1 min application of the pheromone components (*E,Z*)-6,11-hexadecadienyl acetate (AC1) and (*E,Z*)-6,11-hexadecadienal (AL) diluted in the concentration range 0.9 nM–0.9 mM in sensillum-lymph Ringer solution (SLR) either alone or in combination with DMSO (0.005%) or one of the three recombinant pheromone-binding proteins of *A. polyphemus* (ApolPBP1–3, 16 μM). Columns indicate means ± SE. Numbers in parentheses give numbers of tested sensilla. Nerve impulse frequencies significantly higher than under SLR are marked by asterisks: $*P < 0.01$, $**P < 0.001$ (unpaired *t*-test). These responses, if significantly different from each other, are marked by different letters (unpaired *t*-test, $P < 0.01$).

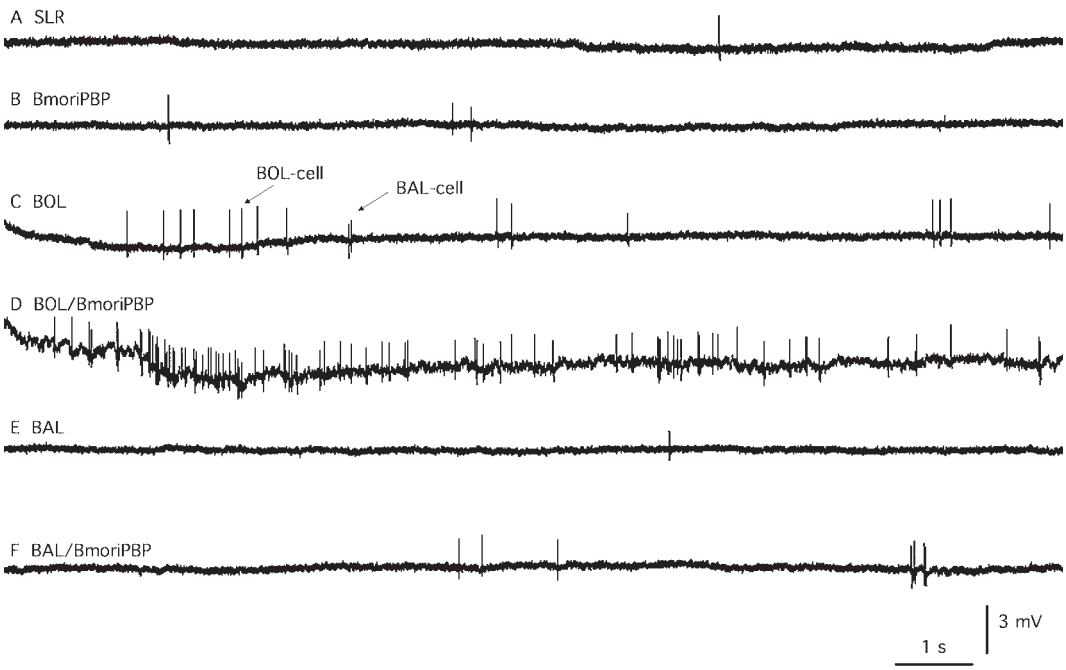

Figure 2 Responses of the two pheromone-sensitive ORNs of *B. mori* to the pheromone components (*E,Z*)-10,12-hexadecadienol (BOL) and (*E,Z*)-10,12-hexadecadienal (BAL) applied in physiological saline via the recording electrode with and without addition of BmoriPBP. **(A)** Spontaneous nerve-impulse activity under pure sensillum-lymph Ringer solution. **(B)** BmoriPBP diluted in SLR (16 μM) did not activate any of the ORNs. **(C)** BOL (9 nM) diluted in SLR without any PBP weakly activated the BOL-cell (large nerve impulse amplitude). **(D)** The same concentration of BOL combined with BmoriPBP activated the BOL-cell much more strongly. **(E)** BAL (9 nM) in SLR without PBP had no effect on any of the ORNs. **(F)** The same concentration of BAL combined with BmoriPBP elicited few nerve impulses in the BOL-cell, but had no effect on the BAL-cell. Traces A–D were recorded from one sensillum, traces E–F from another sensillum of the same antenna. The recordings started at the moment when contact between the electrode and the sensillum was established and continuous stimulation started.

about a factor of 10 (Figure 3A). Neither DMSO nor any of the other recombinant PBPs of *B. mori* or *A. polyphemus* had any effect on the nerve impulse frequency of the pheromone-sensitive ORNs of *B. mori* (Figure 3A).

The pheromone components BOL and BAL were tested in *B. mori* at the concentrations of 9 nM and 90 nM only (Figures 2 and 3B–E). In this species, not only the species-specific PBP (BmoriPBP), but also the three recombinant PBPs of *A. polyphemus* were tested for their ability to solubilize BOL and BAL. At the pheromone concentration of 9 nM, BOL increased the nerve impulse frequency of the BOL-cell significantly without a solubilizer or combined with DMSO or any of the recombinant PBPs (Figures 2C,D and 3B). The combination BOL/BmoriPBP elicited significantly stronger responses of the BOL-cell than BOL combined with the other solubilizers and weakly also excited the BAL-cell. BAL at 9 nM excited the BAL-cell very weakly when applied without a solubilizer (Figure 3C). When combined with either DMSO or any of the PBPs, 9 nM BAL did not activate the BAL-cell. However, the combination of 9 nM BAL with BmoriPBP significantly activated the BOL-cell (Figures 2F and 3C).

At the pheromone concentration of 90 nM, BOL activated the BOL-cell if solubilzed by DMSO or BmoriPBP. In combination with BmoriPBP, BOL excited also the BAL-cell (Figure 3D). BAL (90 nM) alone or solubilized by DMSO weakly activated the BAL-cell (Figure 3E). The combination BAL/BmoriPBP had no effect on the nerve impulse frequency of the BAL-cell, but a strong activation was achieved by the combination BAL/ApolPBP1. In combination with BmoriPBP and ApolPBP1, 90 nM BAL excited also the BOL-cell; the combination BAL/ApolPBP1 elicited in the BOL-cell a nerve impulse frequency of 1.65 ± 1.41 imp./s (n = 10, Figure 3E). This was not significantly different (P = 0.3198, unpaired t-test) from the nerve impulse frequency elicited in the BOL-cell during control experiments by ApolPBP1 alone (2.28 ± 1.76 imp./s, n = 26, Figure 3A).

Discussion

van den Berg and Ziegelberger (1991) for the first time applied via opened sensillum tips pheromone in combination with the isolated native PBP of *A. polyphemus* and showed that the PBP acts as a solubilizer and carrier of the pheromone. This method was used later to test different combinations of recombinant PBPs and pheromone components of *A. polyphemus* (Pophof, 2002) with the result that the PBPs appeared to be directly involved in receptor activation. Possibly, the pheromone components activate the ORNs in complex with the PBPs.

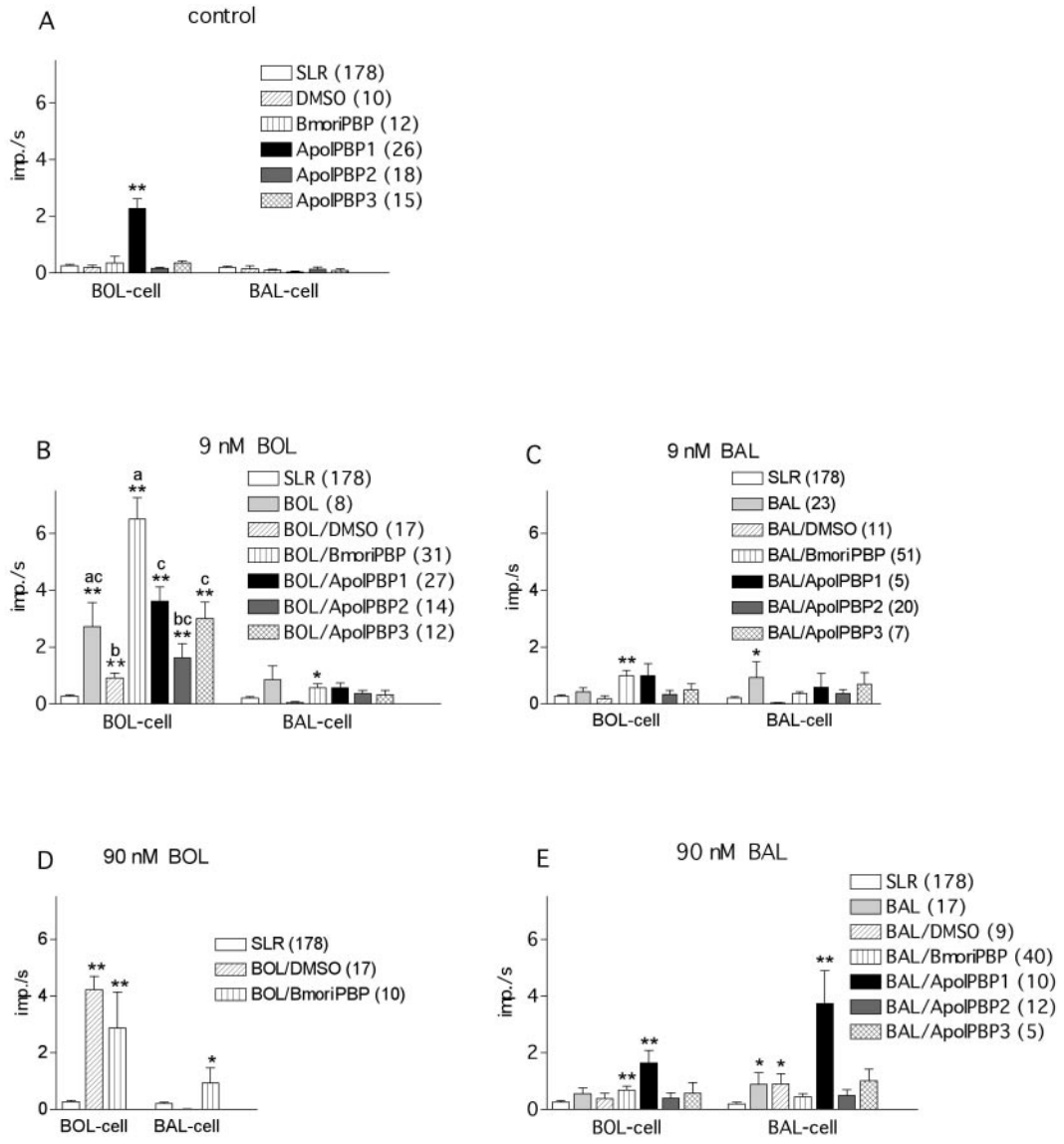

Figure 3 Average nerve impulse frequencies (imp./s) of the two pheromone-sensitive receptor neurons of *B. mori* measured for 1 min. **(A)** Control recordings measured with sensillum-lymph Ringer solution (SLR) in the recording electrode and with 0.005% DMSO or 16 μM of one of the recombinant pheromone-binding proteins (PBPs) diluted in SLR. **(B–E)** Responses elicited by the pheromone components (*E,Z*)-10,12-hexadecadienol (BOL) and (*E,Z*)-10,12-hexadecadienal (BAL) diluted at a concentration of either 9 nM (B, C) or 90 nM (D, E) in SLR either alone or in combination with DMSO or one of the recombinant PBPs of *B. mori* and *A. polyphemus*. For further explanations see Figure 1.

The results described here give a complex picture of ORN-responses resulting from the interactions between single ORNs, pheromone components and PBPs of two silkmoth species, which varies with pheromone concentration. Certain general tendencies, which were not completely stable over the whole concentration range, are discussed below.

Effects of pheromone-binding proteins without pheromone

With one exception, none of the PBPs had an effect on the response of the pheromone-sensitive ORNs when presented without pheromone. Surprisingly, ApolPBP1 excited the BOL-cell (but not the BAL-cell) in *B. mori* even without addition of a pheromone component (Figure 3A). This might point to a direct interaction of ApolPBP1 with the BOL-cell, but it might also have been caused by an unknown internal ligand present in the binding cavity of the recombinant ApolPBP1. The presence of an internal ligand (*cis*-vaccenic acid) within the recombinant BmoriPBP-molecule and a delipidation procedure for its removal have been described by Oldham *et al.* (2001). The PBPs used in the present study were not subjected to such a delipidation procedure. In the experiments with pheromone added, any

internal ligand probably was competitively removed by the respective pheromone component, but in control experiments with pure PBPs the presence of internal ligands cannot be excluded.

Effects at low pheromone concentrations

At low pheromone concentrations, the responses of the ORNs corresponded well to the binding preferences of the PBPs known from binding tests; for native as well as for recombinant PBPs of *A. polyphemus* it has been shown that both, ApolPBP1 and ApolPBP3 bind AC1, but only ApolPBP2 binds AL (Maida *et al.*, 2000, 2003). These binding affinities are in accordance with the results of the presented electrophysiological recordings, which show that, in the concentration range of 9 nM–9 μM AC1, the combinations AC1/ApolPBP1 and AC1/ApolPBP3 elicited stronger responses of the AC1-cell than the combination AC1/ApolPBP2 (Figure 1C,E,G), suggesting that ApolPBP1 and ApolPBP3 solubilized more AC1 than ApolPBP2. Similarly, 0.9 nM AL activated the AL-cell significantly only if solubilized by DMSO or ApolPBP2, but remained inactive if combined with either ApolPBP1 or ApolPBP3 (Figure 1B).

In *B. mori*, the general pattern of responses to pheromone components, when combined with different PBPs, was comparable to that obtained in *A. polyphemus* and was corresponding to the binding properties of BmoriPBB, which are described by the binding of BOL within an internal binding pocket of BmoriPBP (Wojtasek and Leal, 1999; Damberger *et al.*, 2000; Horst *et al.*, 2001; Klusák *et al.*, 2003). At 9 nM BOL, the combination BOL/BmoriPBP elicited a stronger response than BOL solubilzed by DMSO or combined with any of the PBPs of *A. polyphemus* (Figure 3B), suggesting that BmoriPBP indeed functions as the natural transporter of BOL. The combination BAL/Bmori PBP, however, failed to activate the BAL-cell at both pheromone concentrations (Figures 2F and 3C,E); therefore, BmoriPBP does not appear to solubilize BAL under natural conditions. This is in accordance with the results of chemical studies: BOL binds to BmoriPBP and the BOL–BmoriPBP complex has been identified by mass-spectrometry (Oldham *et al.*, 2000), but binding between BAL and BmoriPBP has not been described so far.

Adaptive effects at high pheromone concentrations

At high pheromone concentrations, the response magnitudes elicited by the single pheromone/PBP combinations were smaller than at the lower concentrations, which might be a result of adaptation of the tonic part of the response during prolonged continuous stimulation via the recording electrode lasting for 1 min. Furthermore, the dependence of the response magnitude on the type of the PBP seemed to be reversed at high pheromone concentrations in *A. polyphemus*: at a pheromone concentration of 0.9 mM, AC1/ApolPBP2 elicited in the AC1-cell a stronger response than AC1/ApolPBP1 (Figure 1I) and AL/ApolPBP1 activated the AL-cell more than the AL/ApolPBP2 (Figure 1J). These effects possibly are also due to adaptation; apparently, AC1/ApolPBP1 as the stronger stimulus for the AC1-cell and AL/ApolPBP2 as the stronger stimulus for the AL-cell, caused stronger adaptation and, therefore, weaker tonic responses than the other combinations. In *B. mori,* already at 90 nM BOL (Figure 3D) the response to BOL/BmoriPBP was decreased in comparison to a lower concentration of BOL/BmoriPBP (Figure 3B) or to 90 nM BOL/DMSO (Figure 3D), suggesting that adaptation occurs in the very sensitive BOL-cell as a result of prolonged stimulation.

Effects on response specificity

In accordance with a previous study (Pophof, 2002), not only the magnitude, but also the specificity of the responses of the pheromone-sensitive ORNs was influenced by the PBPs. In *A. polyphemus*, at low concentrations the combination AC1/ApolPBP2 excited the AL-cell more than the AC1-cell (Figure 1A) and the combination AL/ApolPBP1 activated the AC1-cell stronger than the AL-cell (Figure 1 D,F). Under natural conditions (airborne stimulation of intact sensilla), AC1 activates the AL-cell only at concentrations ~1000-fold above threshold. Likewise, AL activates the AC1-cell only at very high concentrations. At any event, each ORN always responds to that pheromone component to which it is tuned much stronger than to any other compound. The observed responses to the artificially produced unnatural pheromone/PBP combinations as described above suggest, that not only the pheromone components themselves, but also the PBPs are involved in the activation of the ORNs. PBPs which naturally (under competitive conditions) preferentially bind a certain ligand tend to activate the ORN specialized to this particular ligand even when carrying another compound.

In competitive binding studies with native and recombinant PBPs of *A. polyphemus* (Maida *et al.*, 2000, 2003) no binding of AC1 to ApolPBP2 was found, but according to the responses observed here some binding must occur. The binding of AL to ApolPBP1 was observed earlier (Campanacci *et al.*, 2001); however, the shift in the endogenous tryptophan fluorescence of ApolPBP1 after binding of AL differs strongly from that observed after binding of AC1, suggesting different functional states of the two complexes (Bette *et al.*, 2002).

In *B. mori*, the combination BAL/Bmori PBP elicited at both pheromone concentrations tested a weak but significant excitation of the BOL-cell, suggesting at least some binding of BAL to BmoriPBP, and a dependence of the ORN-response not only on the pheromone component, but also on the type of PBP involved. This is in accordance with the results obtained in *A. polyphemus* and suggests also for *B. mori* an interaction of the pheromone/PBP complex with the receptor molecule, rather than a release of BOL in the

vicinity of the dendritic membrane, as proposed by Damberger *et al.* (2000) and Horst *et al.* (2001).

Surprisingly, BAL strongly activated the BAL-cell in combination with ApolPBP1 (Figure 3E). Possibly another binding protein solubilizing BAL is present in the long sensilla trichodea of *B. mori* males. The presence of minor amounts of several PBPs has been already suggested (Maida and Ziegelberger, 1994). Another member of the large family of the insect odorant-binding proteins with yet unknown binding properties, the ABPX (Krieger *et al.*, 1996), was found to be co-localized with BmoriPBP in the sensilla trichodea of *B. mori* males (R.A. Steinbrecht, Seewiesen, personal communication). Therefore, ABPX could be another candidate solubilizer of bombykal.

It has been proposed here, that the PBPs do participate in the activation of pheromone-sensitive ORNs in several silkmoth species. Even another molecule found in the dendritic membrane, the sensory neuron membrane protein (Rogers *et al.*, 2001a,b) which belongs to the family CD36 of protein docking proteins, might be involved in this interaction. Whether this actually is the case, remains to be shown. Also many other aspects of PBP function still await conclusive explanation and incorporation into a unifying model. Among these are the release of the pheromone components from the binding cavity (Wojtasek and Leal, 1999; Damberger *et al.*, 2000; Horst *et al.*, 2001) and the formation of dimers (Leal, 2000), both known from *B. mori*. According to recent biochemical studies performed with *Lymantria dispar*, the multimerization of PBPs may lead to enhanced ligand binding at high pheromone concentrations and could be involved in the attenuation of very strong stimuli (Honson *et al.*, 2003; Plettner *et al.*, 2003). Another mechanism, possibly involved in pheromone deactivation (Kaissling, 1998, 2001), is the redox-shift of the PBP described from *A. polyphemus* (Ziegelberger, 1995).

Conclusions

It has been shown here, in accordance with preliminary work (Pophof, 2002), that the PBPs not only solubilize the pheromone components but also contribute to the activation of ORNs. This phenomenon was more conspicuous at low pheromone concentrations, at high concentrations the specificity of the ORNs was reduced and adaptive effects predominated. These results were obtained in both silkmoth species *A. polyphemus* and *B. mori*. In *B. mori*, the presence of a PBP is suggested, which solubilizes BAL and is different from BmoriPBP.

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