

A Pheromone-Binding Protein Mediates the Bombykol-Induced Activation of a Pheromone Receptor *In Vitro*

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Abstract

The enormous capacity of the male silkmoth *Bombyx mori* in recognizing and discriminating bombykol and bombykal is based on distinct sensory neurons in the antennal sensilla hairs. The hydrophobic pheromonal compounds are supposed to be ferried by soluble pheromone-binding proteins (PBPs) through the sensillum lymph toward the receptors in the dendritic membrane. We have generated stable cell lines expressing the candidate pheromone receptors of *B. mori*, BmOR-1 or BmOR-3, and assessed their responses to hydrophobic pheromone compounds dissolved by means of dimethyl sulfoxide. BmOR-1-expressing cells were activated by bombykol but also responded to bombykal, whereas cells expressing BmOR-3 responded to bombykal only. In experiments employing the *B. mori* PBP, no organic solvent was necessary to mediate an activation of BmOR-1 by bombykol, indicating that the PBP solubilizes the hydrophobic compound. Furthermore, the employed PBP selectively mediated a response to bombykol but not to bombykal, supporting a ligand specificity of PBPs. This study provides evidence that both distinct pheromone receptors and PBPs play an important role in insect pheromone recognition.

Key words: *Bombyx mori*, expression, olfaction, pheromone detection, receptor

Introduction

The recognition and discrimination of volatile chemical signals in insects supposedly involve G protein-coupled olfactory receptors (ORs) residing in the dendritic membrane of olfactory neurons housed in sensillar hair structures on the antenna. In flies (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999, 2000) and mosquitos (Fox *et al.*, 2001, 2002; Hill *et al.*, 2002), multigene families have been discovered which encode 62–79 mostly broadly tuned ORs. Their diversity and nonspecific response spectra are considered as the basis for a combinatorial coding of odorants (Störtkuhl and Kettler, 2001; Keller and Vosshall, 2003; Hallem *et al.*, 2004; Couto *et al.*, 2005; Fishilevich and Vosshall, 2005). Candidate ORs for odorants and pheromones have also been identified for the moths *Heliothis virescens* (Krieger *et al.*, 2002, 2004) and *Bombyx mori* (Sakurai *et al.*, 2004; Krieger *et al.*, 2005; Nakagawa *et al.*, 2005). Two of the *B. mori* receptor types (BmOR-1 and BmOR-3) were shown to be expressed in neighboring neurons within the pheromone-sensitive hairs (long sensilla trichodea) of the male antenna (Krieger *et al.*, 2005; Nakagawa *et al.*, 2005), and injection of RNA for BmOR-1 and BmOR-3 rendered frog oocytes

responsive to components of the female sex pheromone blend (Nakagawa *et al.*, 2005).

The receptor proteins are supposed to reside in the dendrites of the sensory neurons which are bathing in the sensillum lymph. Pheromones and odorants have to traverse this aqueous fluid before reaching the chemosensory dendritic membrane. This transfer is supposed to be mediated by specific globular proteins, the pheromone-binding proteins (PBPs) or odorant-binding proteins (OBPs) (Steinbrecht, 1998; Leal, 2003; Vogt, 2003; Tegoni *et al.*, 2004). However, experimental evidence supporting this concept is still very sparse. In fact, analyses of heterologously expressed receptors were all performed in the absence of OBPs or PBPs. In these studies, hydrophobic pheromonal or odorous compounds were dissolved in the aqueous buffer solutions by means of organic solvents, such as dimethyl sulfoxide (DMSO) (Krautwurst *et al.*, 1998; Kajiya *et al.*, 2001; Sakurai *et al.*, 2004; Nakagawa *et al.*, 2005). No attempts have been made to assess a possible role of binding proteins in delivering the hydrophobic ligand. For the silkmoth *B. mori*, we have previously cloned and characterized a PBP (Krieger *et al.*, 1996) and recently identified several

putative pheromone receptor types (Krieger *et al.*, 2005). In this study, we set out to establish stable cell lines expressing BmOR-1 and BmOR-3, which allowed monitoring the responsiveness by means of calcium-imaging approaches. Heterologously expressed and purified PBP of *B. mori* was employed to assess its possible role in transferring hydrophobic pheromonal compounds to the receptors in the cell membrane.

Materials and methods

Odorants and pheromone components

Bombykol, (E10, Z12)-hexadeca-10,12-dien-1-ol, was prepared using acetylene chemistry according to published procedures (Hoskovec *et al.*, 2000; Kalinova *et al.*, 2001). The bombykol was oxidized to bombykal by pyridiniumdichromate and purified on a silica gel column. The isomeric purity of both compounds was 97% (for EZ isomer) and the chemical purity 98% (determined using gas chromatography, DB-5 phase). Using gas chromatography/mass spectroscopy, it was found that the bombykal did not contain any bombykol. Z-11-hexadecenal (Z11-C16:Al), Z-11-hexadecenol (Z11-C16:OH), and \pm linalool were purchased from Fluka (Buchs, Switzerland), benzoic acid and Z-9-hexadecenol (Z9-C16:OH) came from Sigma.

Expression of BmORs in Flp-In T-REx293 cells

Vector constructs for the generation of stable cell lines

For stable genome integration and tetracycline-regulated heterologous expression of BmORs, the components of the Flp-In-System (Invitrogen, Paisley, UK) and a modified HEK 293 cell line carrying the $G_{\alpha 15}$ gene (Flp-In T-REx293/ $G_{\alpha 15}$) stably integrated in the genome were used. $G_{\alpha 15}$ has been shown of coupling a variety of heterologously expressed olfactory and other G protein-coupled receptors to the inositol trisphosphate cascade leading to intracellular Ca^{2+} increases (Offermanns and Simon, 1995; Krautwurst *et al.*, 1998; Kajiyi *et al.*, 2001). These can be monitored by calcium imaging with the calcium-sensitive dye fura-2. The Flp-In T-REx293/ $G_{\alpha 15}$ cell line was kindly provided by E. Tareilus and R. Gouka, Unilever R&D, Vlardingem. To monitor the expression of the BmOR receptors immunocytochemically, a FLAG-tag was added to the N-terminus.

First, the coding region of BmORs was polymerase chain reaction (PCR) amplified and integrated in frame into the pFLAG-CMV-2 vector (Kodak, New Haven, CT) using specific primers with appropriate restrictions sites (underlined). BmOR-1-*NotI*: 5'-ATT TGC GGC CGC TTA TGG TTA CTA TCC TTC AAA GA-3'; BmOR-1-*XbaI*: 5'-TGC TCT AGA GCA TTA TGT TGC CAC TGT TCG GAG-3'; BmOR-3-*HindIII*: 5'-ACA AGC TTA TGA TAT TCG TCG ACG ATG CT-3'; and BmOR-3-*EcoRI*: 5'-TTG AAT TCT TCA TTC GGA CAC GGT ACG AAG-3'. PCR conditions were 1 min 40 s at 94°C, then 21 cycles with

94°C for 1 min, 50°C or 55°C for 40 s, and 72°C for 1 min, with a decrease of the annealing temperature by 0.5°C per cycle. Subsequently, 19 further cycles at the condition of the last cycling step were performed followed by incubation for 7 min at 72°C. The PCR product was gel purified, digested, and ligated into the corresponding sites of the pFLAG-CMV-2 vector. The resulting constructs pFLAG-CMV-2/BmOR were sequenced to verify correct amplification and integration of the receptor sequence. Sequencing was performed on an ABI310 sequencing system using vector and receptor sequence-specific primers and the BIG dye cycle sequencing kit (Applied Biosystems, Foster City, Calif.). The FLAG-BmOR-encoding sequences were PCR amplified from the pFLAG-CMV-2/BmOR constructs using a combination of FLAG-*KpnI*: 5'-GGG GTA CCC CAC CAT GGA CTA CAA AGA CGA TGA CG-3' with either BmOR-1-*XhoI*: 5'-CCG CTC GAG CGG ATT ATG TTG CCA CTG TTC GGA G-3' or BmOR-3-*XhoI*: 5'-CCG CTC GAG CGG TTC ATT CGG ACA CGG TAC GAA-3'. The PCR products were gel purified, digested with *KpnI/XhoI*, and ligated into the corresponding sites of the pcDNA5-FRT/TO vector (Invitrogen). To verify correct amplification and integration into the vector, the resulting FLAG-BmOR/FRT/TO construct was sequenced.

Generation of stable cell lines

Flp-In T-REx293/ $G_{\alpha 15}$ cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, high glucose, with L-glutamine, without Na-pyruvate) supplemented with 10% fetal calf serum, 100 mg/l zeocin, 10 mg/l blasticidin, and 200 mg/l geneticin. Cells were transfected using PerFectin (Gene Therapy Systems, San Diego, CA) according to the supplier's protocol. For transfection, 3×10^5 cells were seeded into 35-mm culture dishes. After 24 h, cells were transfected with pOG44 (Invitrogen) and the BmOR/FRT/TO construct in a 10:1 ratio. Forty-eight hour posttransfection cells were maintained and selected for receptor integration into the genome using media supplemented with 100 mg/l hygromycin instead of zeocin. After 6 weeks, the presence of the BmORs sequences in the genome was tested by PCR using receptor-specific primers, genomic DNA prepared from the hygromycin resistant cells and conditions as described above. Flp-In T-Rex293/ $G_{\alpha 15}$ cells positive for BmORs were maintained and used for calcium imaging.

Calcium imaging of cells

For calcium imaging, 2×10^5 BmOR/Flp-In T-Rex293/ $G_{\alpha 15}$ cells were seeded onto 15-mm diameter cover slips coated with poly-L-lysine. Cells were incubated for 36 h in DMEM with 1 μ g/ml tetracycline to induce receptor expression. For imaging changes in the internal free calcium concentration of single cells, the Ca^{2+} -sensitive dye fura-2 was used. Cells were washed in Ringer solution (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3) and loaded with the dye by incubating them for 45 min at

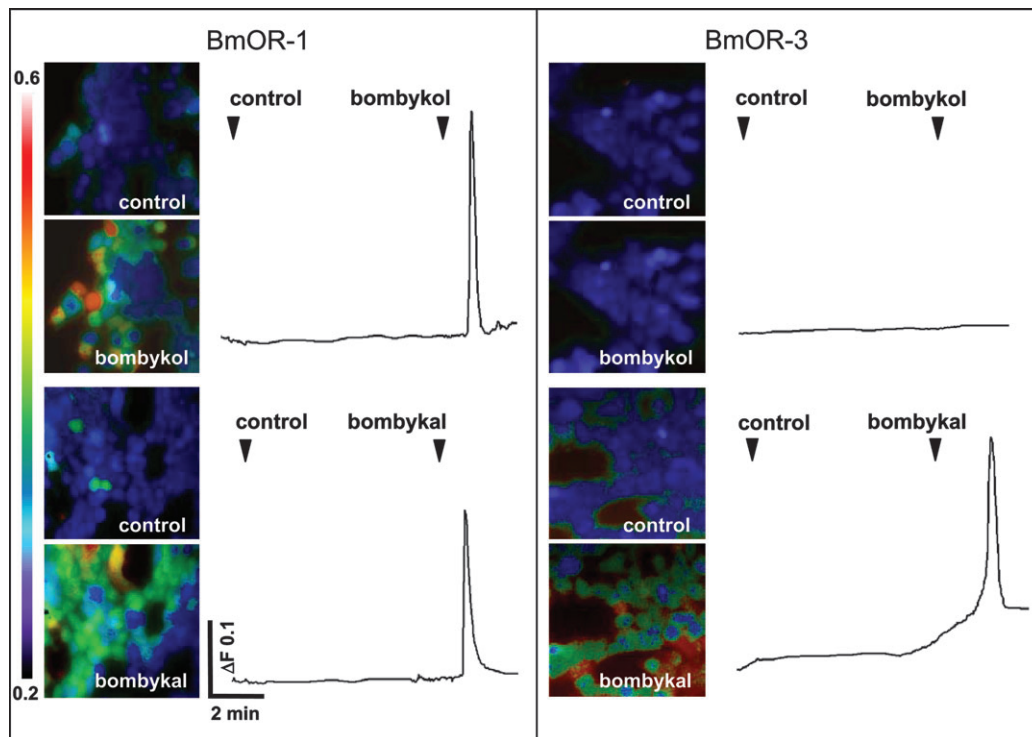


Figure 1 Pheromone-induced Ca^{2+} responses of Flp-In T-REx293/ $\text{G}\alpha_{15}$ cells expressing BmOR-1 or BmOR-3. Pseudocolor images (left panels) indicate calcium levels in receptor-expressing cells after application of DMSO/Ringer solution (control) or stimulation with bombykol or bombykal solubilized with DMSO. Response curves to the right represent changes of fura-2 fluorescence intensity ratios (340/380 nm) in individual cells from the same experiment. Pheromone components were applied (arrowheads) for 4 s at 10 nM. BmOR-1-expressing cells displayed clear responses to bombykol as well as to bombykal; however, bombykol-induced reactions showed considerably higher amplitude. Cells expressing BmOR-3 did react upon stimulation with bombykal but showed no response to bombykol.

room temperature with 4 $\mu\text{mol/l}$ of the acetoxymethyl ester of fura-2 in Ringer solution. Cover slips with fura-2-loaded cells were placed into a flow chamber on the stage of an Olympus IX70 inverted microscope equipped for epifluorescence. The chamber was rinsed with Ringer solution at a flow rate of 1 ml/10 s. Test solutions (400 μl) were applied via syringes connected to the system by three-way valves at the same flow rate.

Changes in $[\text{Ca}^{2+}]_i$ concentration in single cells upon stimulation with ligands were analyzed by monitoring the intensity of fluorescent light emission at 510 nm, using excitation at 340 and 380 nm. The ratio of fluorescence emission at 340/380 nm excitation was used as index with an increase indicating a raise in intracellular free calcium. For data analysis, fura-2 fluorescence intensity ratios of OR-expressing cells were determined before (F_0) and after stimulation (F ; peak of the response). In a single experiment, F/F_0 values of at least 30 individual cells were determined and averaged. Illumination (30 ms for each wavelength) was by an IX-FLA monochromator connected to the microscope. Cells were viewed through a UApo/340 40 \times objective, and images were detected with a MircoMax camera (Roper Scientific, Ottobrunn, Germany). To control the illuminator, the camera, and for the analysis of images, the Meta fluor imaging system

and software was used (Visitron Systems, Puchheim, Germany). The Ca^{2+} response to 10 mM adenosine triphosphate in Ringer solution was used as an internal control of cell viability. Washes with Ringer solution between stimulations were performed for at least 5 min before applying the next test solution. Dilutions of hydrophobic pheromone components were prepared from stock solutions in hexane using Ringer solution with 0.1% DMSO. Water-soluble odorants were diluted in Ringer solution with 0.1% DMSO, adding hexane to a final concentration of 0.1%. All dilutions were prepared freshly and used within 3 h. To analyze the ability of PBP to solubilize and transport bombykol or bombykal, pheromone components were added from stock solutions in hexane to recombinant protein in Ringer solution and incubated for 1 h on ice. The solution was warmed to room temperature and subsequently used for calcium imaging.

Expression and purification of PBPs

Recombinant *B. mori* PBP was expressed in *E. coli* and purified from a periplasmic preparation of the bacteria as described previously (Wojtasek and Leal, 1999; Campanacci *et al.*, 2001; Oldham *et al.*, 2001). To remove hydrophobic ligands which have been found to copurify with *B. mori*

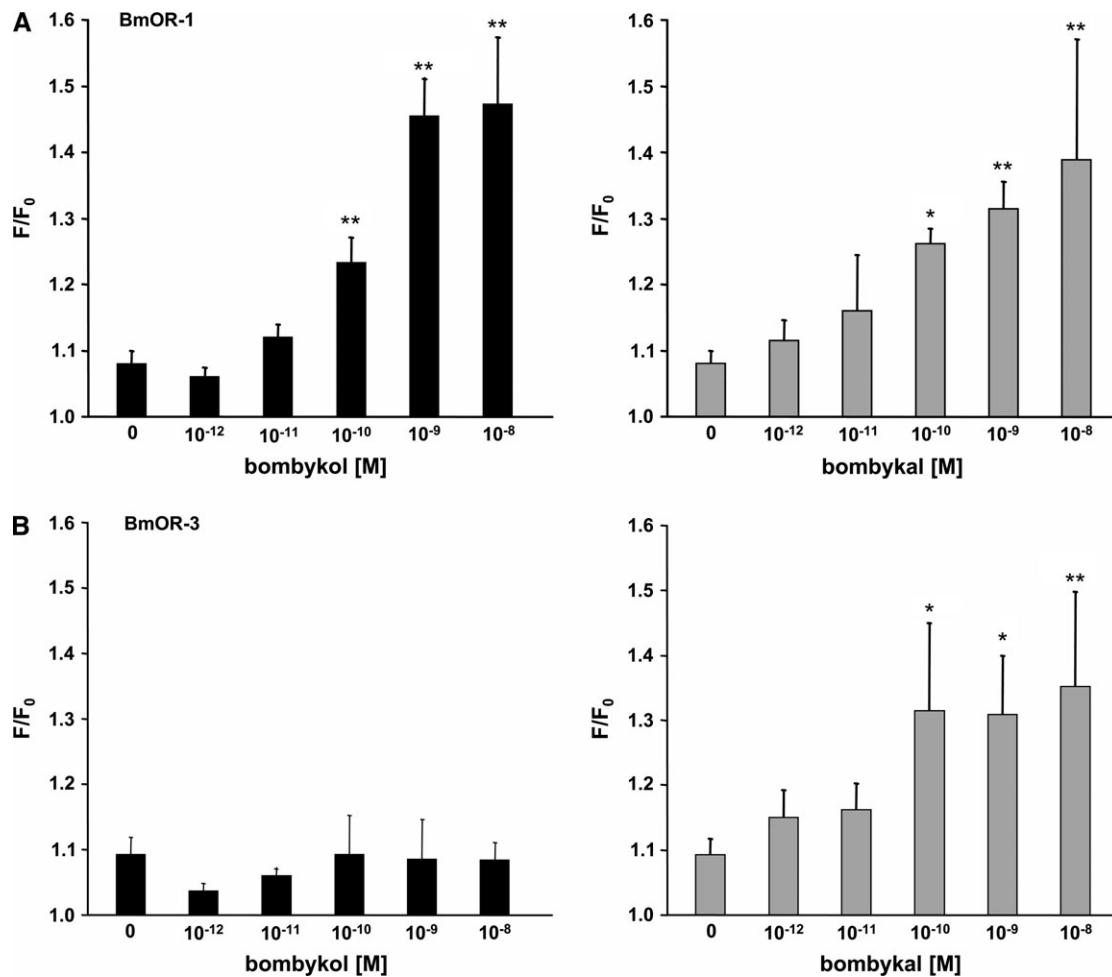


Figure 2 Bombykol and bombykal dose-response profiles of BmOR-expressing cells. Fura-2 fluorescence intensity ratios of BmOR-1– (A) or BmOR-3 (B)–expressing cells were determined before (F_0) and after stimulation (F ; peak of the response) approaching different concentrations of bombykol (black bars) or bombykal (gray bars). Bars represent the mean responses of cells to the pheromone concentration indicated expressed as F/F_0 . Each bar is based on the mean $F/F_0 \pm$ SD from three to eight independent experiments. BmOR-1–expressing cells (A) respond to bombykol and bombykal in a dose-dependent manner. In contrast, cells expressing BmOR-3 (B) respond only to bombykal; no response was elicited by bombykol even at high concentration. Significant increases in comparison to no pheromone (0) are indicated by asterisks; * $P < 0.05$, ** $P < 0.01$ in a one-way ANOVA followed by Dunnett's posttest.

PBP recombinant-binding protein was dilipidated following the protocol described earlier (Oldham *et al.*, 2001) and finally dissolved in Ringer solution. The protein concentration was determined spectrometrically at 280 nm using absorption coefficients determined for the protein by the ProtParam program (ExpASY molecular biology server; Swiss Institute of Bioinformatics).

Results

We have generated stable cell lines expressing BmOR-1 or BmOR-3 to scrutinize their ligand specificity. To promote coupling of activated receptors to an effective intracellular reporter system, receptor cDNAs were stably integrated into the genome of modified HEK 293 cells (Flp-In T-REx293/ $G\alpha_{15}$ cells); $G\alpha_{15}$ has been shown to facilitate the coupling of heterologously expressed receptors to the phospholipase

C pathway leading to an increase of intracellular Ca^{2+} concentration (Offermanns and Simon, 1995; Krautwurst *et al.*, 1998; Kajiya *et al.*, 2001). For monitoring changes in intracellular calcium levels, BmOR/Flp-In T-REx293/ $G\alpha_{15}$ cells were loaded with fura-2 and fluorescence ratios were recorded, with an increase of the fluorescence ratio indicating elevated levels of calcium (Grynkiewicz *et al.*, 1985). Due to their hydrophobicity, the putative ligands bombykol and bombykal were solubilized by means of DMSO. Application of low doses of bombykol (10 nM) elicited a significant increase of intracellular calcium levels in cells expressing BmOR-1 but not in BmOR-3 cells (Figure 1). In contrast, application of 10 nM bombykal induced a calcium response in both BmOR-1– and BmOR-3–expressing cells. As indicated by the false color pictures in Figure 1, superfusing patches of BmOR-1 cells with 10 nM bombykol or patches of BmOR-3 cells with 10 nM bombykal induced a strong

response in most of the cells. Monitoring the response kinetics of individual BmOR-1 cells and BmOR-3 cells to bombykol or bombykal revealed in each case a transient time course (Figure 1). Flp-In T-REx293/G α_{15} cells without a BmOR receptor or expressing the OR type BmOR-2 (Krieger *et al.*, 2003) did not show any response to the pheromone components (data not shown). Altogether, the calcium signals of BmOR-1 and BmOR-3 cells upon stimulation with bombykol and bombykal, respectively, indicate that the cells have generated functionally active receptors which render them responsive to the pheromonal compounds.

For a more detailed analysis of the bombykol- and bombykal-induced responses of BmOR-1 and BmOR-3 cells, recordings were performed at different concentrations. In a single stimulation experiment, fura-2 fluorescence intensity ratios of at least 30 OR-expressing cells were measured before (F_0) and after stimulation (F ; peak of the response) to calculate a mean F/F_0 ratio. Mean F/F_0 values from single independent experiments were averaged to determine the responsiveness of cells. Upon application of control stimuli (e.g., application of Ringer/DMSO/hexane), this value was determined to be slightly above 1.0 due to slight fluctuations intrinsic to calcium-imaging measurements. Application of increasing concentrations of bombykol to BmOR-1 cells led to an increase in the F/F_0 values in a dose-dependent manner (Figure 2A) resulting from a rise in the number of reacting cells as well as higher amplitudes of the bombykol-induced calcium signals. Half-maximal effects were observed at ~ 100 pM. Threshold concentration for calcium responses of individual cells was at about 10 pM bombykol. BmOR-1 cells also respond to bombykal; however, compared to bombykol, signals were lower at higher concentrations. BmOR-3 cells selectively respond to bombykal; bombykol elicited no response in BmOR-3 cells even at concentrations as high as 10 nM (Figure 2B). To further assess the specificity of the receptors, BmOR-expressing cells were challenged with pheromone components from other moth species as well as with general odorants which are recognized by female *B. mori* (linalool, benzoic acid) (Heinbockel and Kaissling, 1996). It was found that neither the BmOR-1- nor the BmOR-3-expressing cells responded to 10 nM Z9-C16:OH (Figure 3A,B). Also weak reactions observed after applying the same high concentration of the pheromone components Z11-C16:Al and Z11-C16:OH or the odorants linalool and benzoic acid were not significantly different from control. These results indicate a selective interaction of BmOR-1 and BmOR-3 with components of the *B. mori* pheromone blend.

In the aqueous sensillum lymph of the sensory hairs, the hydrophobic pheromone components are supposed to be solubilized by means of PBPs. To scrutinize this long-standing hypothesis, we have examined if the PBP of *B. mori* may fulfill this role *in vitro*. Recombinant *B. mori* PBP was employed to solubilize bombykol or bombykal in the buffer medium substituting the organic solvent DMSO. Activation of recep-

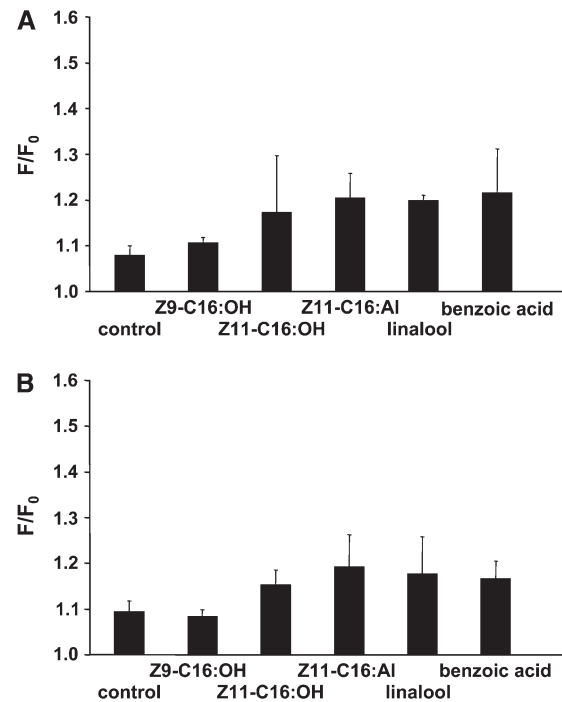


Figure 3 Stimulation of BmOR-expressing cells with pheromonal compounds and general odorants. Bars represent the responses of cells expressing BmOR-1 (A) and BmOR-3 (B) upon stimulation with 10 nM solutions of pheromone components from other moth species or general odorants relevant to *Bombyx mori*. At this high ligand concentrations, structurally related pheromone components (Z9-C16:OH, Z11-C16:OH, and Z11-C16:Al) elicited no significant response. Also no significant reaction of BmOR-1 or BmOR-3 cells was obtained with benzoic acid and linalool. All components tested were solubilized in 0.1% DMSO (control). F/F_0 ratios represent the mean $F/F_0 \pm$ SD of cells from three to five independent replicates. Statistic analysis by one-way ANOVA followed by Dunnett's posttest revealed no significant increases of F/F_0 values compared to control.

tors upon interaction with pheromone components was monitored by calcium imaging of the cells. Superfusion of BmOR-1 cells with Ringer solution containing *B. mori* PBP (1 μ M) preincubated with bombykol (100 pM) led to a clear calcium response in the cells (Figure 4A), with a transient time course on the single-cell level. The mean calcium signal intensity in BmOR-1 cells elicited by application of *B. mori* PBP/bombykol was comparable to the response obtained with the same concentration of bombykol dissolved by means of DMSO (Figure 4B). Bombykol without addition of solvent in Ringer solution did not induce any response; also PBP alone did not have any effect. Applying PBP (1 μ M) together with bombykal (100 pM) also elicited no response in BmOR-1 cells (Figure 4C,D), suggesting that *B. mori* PBP may selectively bind bombykol. This notion was further supported by experiments demonstrating that also BmOR-3-expressing cells were not activated by 1 μ M *B. mori* PBP preincubated with 100 pM bombykal (Figure 4E). The response of BmOR-1 cells to bombykol mediated by the PBP also showed a clear dose dependence. Application of a solution with the same concentration of PBP (1 μ M)

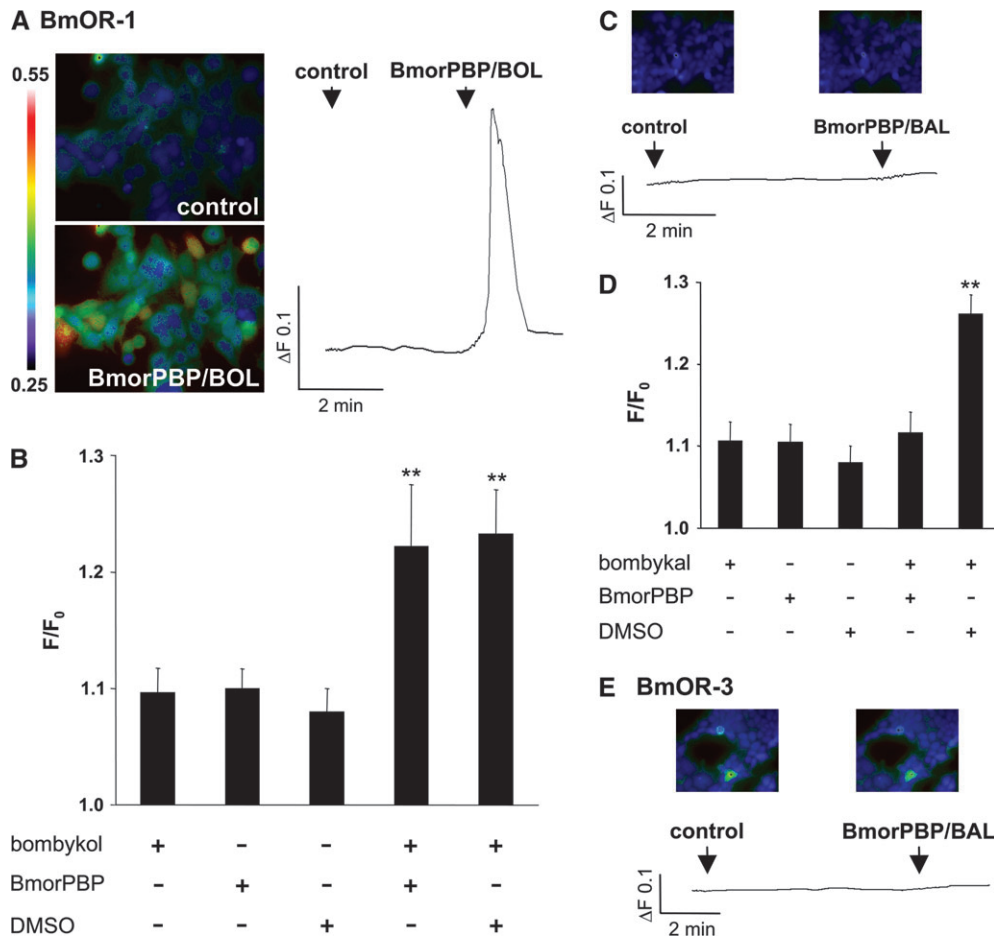


Figure 4 *Bombyx mori* pheromone-binding protein (BmorPBP) mediated responses of BmOR-1 cells to bombykol but not to bombykal. **(A)** Pseudocolor images (left panels) indicate calcium levels in BmOR-1-expressing cells after application of DMSO/Ringer solution (control) or stimulation with BmorPBP/bombykol (BOL) in Ringer. The response curve to the right represent changes of fura-2 fluorescence intensity ratios (340/380 nm) of an individual cell from the same experiment. **(B)** BmOR-1 cells were stimulated with bombykol, BmorPBP and DMSO alone, or in combinations. *Bombyx mori* PBP with bombykol elicited a significant response; the signal intensity was similar to bombykol solubilized with DMSO (data from Figure 2). Bombykol, BmorPBP, or DMSO (control) alone did not elicit any responses. **(C, D)** Same experiments as shown in A and B but approaching BmorPBP and bombykal (BAL) on BmOR-1 cells. BmOR-1 cells did not respond to a combination of BmorPBP/bombykal. Only stimulation of BmOR-1 cells with bombykal solubilized by DMSO (values from Figure 2) revealed a clear response. **(E)** BmOR-3-expressing cells did not respond to stimulation with BmorPBP/bombykal. Responses of cells in B and D are displayed as mean $F/F_0 \pm$ SD ratios determined from a minimum of three independent measurements. Significant increases in comparison to stimulation with bombykol or bombykal alone are indicated by asterisks; $**P < 0.01$ in a one-way ANOVA followed by Dunnett's posttest. Concentrations used in stimulation experiments shown in A–E were 100 pM for bombykol or bombykal, 1 μ M for *B. mori* PBP, or 0.1% for DMSO.

loaded with increasing concentrations of bombykol (1 pM to 1 nM) led to dose-dependent calcium signals (Figure 5A). Similarly, the response of BmOR-1 cells to a PBP/bombykol combination was also dependent on the concentration of PBP in the solution (Figure 5B). The same concentration of bombykol (100 pM) elicited a stronger response with increasing concentrations of PBP (10 nM to 10 μ M). Together, these results indicate that *B. mori* PBP in a ligand-specific manner increases the solubility and availability of bombykol.

Discussion

The present study demonstrates a specific interaction of the two candidate pheromone receptors BmOR-1 and BmOR-3

with pheromone components of the silkmoth *B. mori*, bombykol and bombykal *in vitro*. This result is in line with the previous observation that BmOR-1 and BmOR-3 are expressed in neighboring neurons of long trichoid hairs on male silkmoth antenna (Krieger *et al.*, 2005) as well as with earlier electrophysiological recordings from sensilla trichodea, indicating that these long olfactory hairs contain two sensory neurons, one responsive to bombykol and the second to bombykal (Kaissling *et al.*, 1978). The functional characterization of the BmOR-3 receptor, heterologously expressed in modified HEK 293 cells, has demonstrated its highly sensitive and selective responsiveness to bombykal but not to bombykol. A similar result has recently been reported after injecting a combination of RNAs for BmOR-3 and BmOR-2

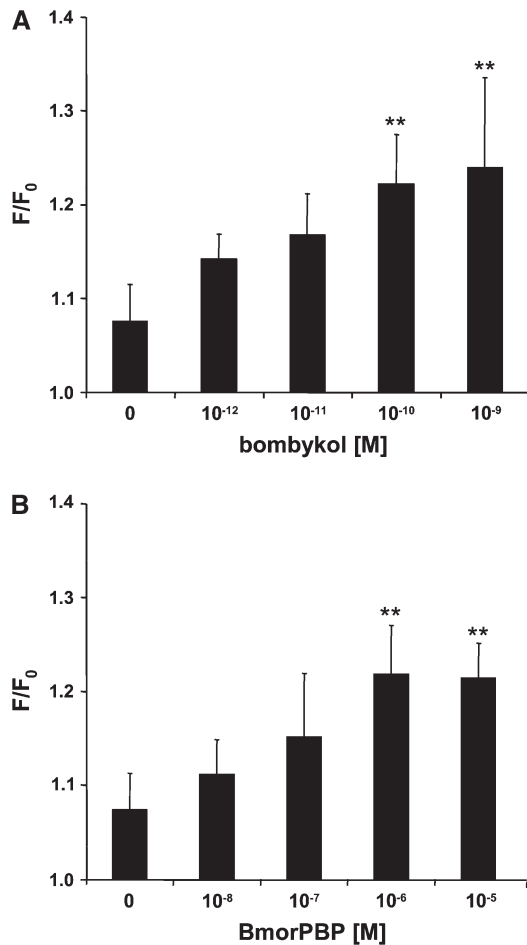


Figure 5 Responses of BmOR-1 cells to varying BmorPBP/bombykol concentrations. **(A)** Response profile of cells upon stimulation with 1 μ M BmorPBP preincubated with different bombykol concentrations (1 pM to 1 nM). At constant BmorPBP concentration, the response of BmOR-1 cells was dependent on the bombykol dose. **(B)** Stimulation of BmOR-1 cells with varying amounts of BmorPBP (10 nM to 10 μ M) loaded with 100 pM bombykol each. Applying a fixed concentration of the pheromone component but varying the amount of BmorPBP, the response of BmOR-1 cells was dependent on the PBP concentration. Data represent the mean responses of cells expressed as $F/F_0 \pm$ SD ratios determined from at least three independent experiments. Significant increases in comparison to no pheromone or no PBP are indicated by asterisks; $**P < 0.01$ in a one-way ANOVA followed by Dunnett's post test.

in frog oocytes; expression of BmOR-3 receptor made the oocytes responsive to bombykal but only very weakly to bombykol (Nakagawa *et al.*, 2005). In the same study, it was shown that oocytes expressing BmOR-1 led to a strong response to bombykol but elicited only a weak response to bombykal. BmOR-2 improved functional expression of BmOR-1 and BmOR-3 in oocytes (Nakagawa *et al.*, 2005), and its *Drosophila* homologue (Or83B) was found necessary for membrane targeting and function of other odorant receptor types (Larsson *et al.*, 2004; Neuhaus *et al.*, 2005; Benton *et al.*, 2006). Functional expression of BmOR-1 and BmOR-3 in modified HEK 293 cells was possible without BmOR-2,

probably due to the ability of HEK 293 cells to correctly process G protein-coupled receptors (Couve *et al.*, 2002; Ivic *et al.*, 2002; Thomas and Smart, 2005). We have found that BmOR-1 expressed in modified HEK 293 cells did respond to both bombykol and bombykal, although at higher doses (1–10 nM), the response to bombykol was stronger. If this difference in ligand specificity of BmOR-1 in HEK 293 cells compared to oocytes is due to the different host cells is elusive.

Analyzing the responsiveness of heterologously expressed receptors for pheromones and odorants in cultured cells is always hampered by the strong hydrophobicity of the appropriate ligands. Usually, organic solvents are employed to minimize this problem. In fact, the solvent DMSO has been used in most studies with insect receptors (Wetzel *et al.*, 2001; Sakurai *et al.*, 2004; Nakagawa *et al.*, 2005). Under these circumstances, the solubilized pheromones or odorants were capable to activate the receptor (see also Figures 1 and 2). For the insect antennae, it has been proposed that globular proteins in the sensillum lymph, PBPs and OBPs, act as solubilizer for the hydrophobic ligands (Vogt and Riddiford, 1981; Leal, 2003; Vogt, 2003). In view of the fact that experimental evidence for this concept is still very weak, the results of this study demonstrate that in the presence of PBP, bombykol elicited a response of BmOR-1-expressing cells comparable to bombykol dissolved by DMSO, indicating that the PBP can completely substitute the organic solvent (Figure 4). This result suggests that indeed one of the functions of PBPs in the sensillum lymph may be solubilization of the pheromonal compounds.

The observation that stimulation of BmOR-expressing cells by bombykal only occurs when it was solubilized by means of DMSO but not in the presence of the *B. mori* PBP (Figure 4) strongly suggests that the *B. mori* PBP specifically interacts with bombykol but not with bombykal. This finding indicates that the binding proteins are not just general solubilizer but are tuned to interact with certain compounds, thus confirming results obtained in previous binding assays. It has been shown that different PBPs of a moth display distinct binding specificity for various pheromonal compounds (Du and Prestwich, 1995; Feixas *et al.*, 1995; Plettner *et al.*, 2000; Maida *et al.*, 2003); moreover, a binding protein can interact differentially with different pheromone components (Bette *et al.*, 2002; Mohl *et al.*, 2002). In addition, electrophysiological studies have demonstrated that the *B. mori* PBP applied in combination with bombykol to receptor neurons via tip-opened sensillar hairs led to the activation of the bombykol-sensitive neuron, whereas application of bombykal with PBP failed to have any effect (Pophof, 2004). Similarly, the OBP LUSH was shown to be absolutely necessary for activation of pheromone-sensitive neurons in *Drosophila* by 11-*cis* vaccenyl acetate (Xu *et al.*, 2005).

The selective role of the PBP for bombykol rather than bombykal implies that another binding protein of *B. mori* may exist. The presence of multiple PBPs have been demonstrated

for several other moths species (Krieger *et al.*, 1991; Merritt *et al.*, 1998; Robertson *et al.*, 1999; Maida *et al.*, 2000). Forthcoming studies have to show if indeed a further PBP exists in *B. mori* which could be specialized for bombykal.

Acknowledgements

The authors are deeply grateful to Prof. H. Breer for valuable advice and generous support. We thank Erwin Tareilus and Robin Gouka, Unilever R&D, Vlardinggen for expert help with the heterologous expression system and Gesa Dreesman for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (grant KR1786/3-2).

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Accepted April 13, 2006