Amino acid residues involved in the interaction with the intrinsic agonist (R)-octopamine in the β-adrenergic-like octopamine receptor from the silkworm Bombyx mori

Xi CHEN,† Hiroto OHTA,†† Kensuke SASAKI,† Fumiyo OZOE† and Yoshihisa OZOE†,*

† Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690–8504, Japan
†† Graduate School of Science and Technology, Kumamoto University, Kumamoto 860–8555, Japan

(Received June 19, 2011; Accepted July 22, 2011)

Octopamine (OA) is a biogenic amine that controls a variety of important physiological processes and behaviors of invertebrates. To identify the amino acid residues interacting with (R)-OA in a β-adrenergic-like OA receptor from the silkworm Bombyx mori (BmOAR2), the wild-type receptor and seven mutant receptors with an amino acid substitution at a potential orthosteric site were expressed in HEK-293 cells and examined for their ability to elevate intracellular cAMP levels ([cAMP]i) in response to (R)-OA. The S206A mutant receptor retained the ability to increase [cAMP], after (R)-OA treatment. In contrast, the other six mutant receptors (D115A, S202A, Y300F, Y300N, Y300L, and Y300A) lacked the ability to elevate [cAMP]. These results indicate that Asp115, Ser202, and Tyr300 participate in (R)-OA binding and the activation of BmOAR2. Homology modeling studies suggest that Ser202 and Tyr300 interact with the phenolic OH group of (R)-OA, whereas Asp115 interacts with the β-OH group and the NH2 group of (R)-OA. © Pesticide Science Society of Japan

Keywords: cAMP, G protein-coupled receptor, octopamine, orthosteric site, site-directed mutagenesis, site of action of insecticides.
insect OARs were exploited as a rational target for insecticides such as chlordimeform (Fig. 1), which is converted to a potent OAR agonist after N-demethylation.7)

Recently, we cloned a cDNA (DDBJ accession No. AB470228) encoding a $\beta$-adrenergic-like OAR (BmOAR2) from the nerve tissues of silkworm (Bombyx mori) larvae.3,9 We generated a human embryonic kidney (HEK)-293 cell line that stably expressed BmOAR2. Upon activation with OA, BmOAR2 elicited a concentration-dependent increase in intracellular cAMP levels ([cAMP]i), but not Ca2+ levels. Chlorpromazine (Fig. 1) significantly attenuated the OA-induced increase in [cAMP]. By extrapolating the results of previous studies concerning vertebrate biogenic amine GPCRs, we postulated that the binding site for the agonist and the competitive antagonists, i.e., the orthosteric site, in insect GPCRs was located in a crevice between transmembrane segments (TM). In the present study, we identified several important amino acid residues that were involved in the binding of the intrinsic agonist (R)-OA and the subsequent activation of BmOAR2. In addition, we provided an OA-binding site model that was derived from the recently elucidated active-state crystal structure of the human $\beta_2$-adrenoceptor ($\beta_2$AR).

**Materials and Methods**

1. **Site-directed mutagenesis of BmOAR2**

The BmOAR2 open reading frame (ORF) was amplified using the polymerase chain reaction (PCR) with KOD-Plus-polymerase (Toyobo), a forward primer with a KpnI site, BmOAR2-F2 (5'-TTGGTACCACTGATGGACGAGTTG-3'), and a reverse primer with a XhoI site, BmOAR2-R5 (5'-AAGCTTCTAGAAGGCGGAC-3'). The double-stranded PCR product was attached with A at the 3'-ends and then subcloned into the TA cloning vector pTA2 (Toyobo) to generate the pTA-BmAOR2 plasmid. Point mutations were introduced into the BmOAR2 gene by PCR using a QuikChange site-directed mutagenesis kit (Stratagene) and pTA-BmAOR2 as a template. Oligonucleotide primers were designed according to the instructions of the mutagenesis kit. The forward primers are listed in Table 1. Each mutation was verified by DNA sequencing using an ABI PRISM® 3100 genetic analyzer (Applied Biosystems). The KpnI/XhoI-digested fragment from the pTA-mutant_BmAOR2 plasmid was subcloned into the corresponding site of the pcDNA3-FLAG vector to produce pcDNA3-FLAG/mutant_BmAOR2. The pcDNA3-FLAG vector was constructed to add a FLAG tag (DYKDDDDK) to the N-terminus of BmOAR2 by introducing a Kozak sequence and a FLAG tag sequence into the HindIII/KpnI site of the mammalian expression vector pcDNA3 (Invitrogen). Each pcDNA3-FLAG/mutant_BmAOR2 construct was verified by DNA sequencing.

2. Stable transfection of wild-type and mutant BmAOR2 cDNAs into HEK-293 cells

HEK-293 cells were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (D-MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Cells (2 × 10^5 cells) were plated on 35 mm-diameter dishes one day before transfection. The plated cells were transfected with pcDNA3-FLAG/wild-type_BmAOR2 or mutant_BmAOR2 (2 µg each) with Lipofectamine® (4 µL; 2 mg/mL; Invitrogen) in Opti-MEM® I reduced serum medium (1 mL; Invitrogen). After incubation for 5 hr at 37°C, the medium was replaced with medium containing FBS. After one day, the cells, which were reseeded at low density, were cultured in the presence of the antibiotic G418 (Sigma-Aldrich) at 1.0 mg/mL for 2–3 weeks. G418-resistant colonies were trypsinized in cloning cylinders and transferred to 24-well plastic plates for cell expansion. These individual clonal cell lines were analyzed for stable expression and localization of the receptor using RT-PCR, immunofluorescence analysis, and radioligand binding assays.

3. Immunofluorescence imaging of clonal HEK-293 cells transfected with wild-type and mutant BmAOR2 cDNAs

Individual clonal cells, which were grown on round coverslips (Warner Instruments; 12 mm in diameter), were washed twice

**Table 1.** Substituted amino acids of BmOAR2 and sequences of oligonucleotides used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Amino acid of WT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Substituted amino acid</th>
<th>TM&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Forward primer sequence (&lt;sup&gt;5’&lt;/sup&gt; to &lt;sup&gt;3’&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp115</td>
<td>Ala</td>
<td>3</td>
<td>CGATTTTTGGAATCATACGCGGCTACTTTTAC</td>
</tr>
<tr>
<td>Ser202</td>
<td>Ala</td>
<td>5</td>
<td>CCATACGCGATCGACGTTGCTATCTCATC TTAC</td>
</tr>
<tr>
<td>Ser206</td>
<td>Ala</td>
<td>5</td>
<td>GTTCCTATAGCATCTGGATACCCCTGACG</td>
</tr>
<tr>
<td>Tyr300</td>
<td>Phe</td>
<td>6</td>
<td>CATTCTTTCTGTTTCTGGCTGACTCTTTTG</td>
</tr>
<tr>
<td>Tyr300</td>
<td>Asn</td>
<td>6</td>
<td>CATTCTTTCTGTTTCTAGTCTGACTCTTTTG</td>
</tr>
<tr>
<td>Tyr300</td>
<td>Leu</td>
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<td>CATTCTTTCTGTTTCTAGTCTGACTCTTTTG</td>
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<tr>
<td>Tyr300</td>
<td>Ala</td>
<td>6</td>
<td>CATTCTTTCTGTTTCTAGTCTGACTCTTTTG</td>
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</tbody>
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<sup>a</sup> WT, wild type. <sup>b</sup> TM, transmembrane segment. <sup>c</sup> Codons for substituted amino acids are underlined.
with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 20 min at room temperature. After being washed with PBS containing 0.1% Tween 20 (PBST), the cells were blocked with PBST containing 10% FBS for 30 min. The cells on the coverslips were then incubated with 1:1000 anti-FLAG monoclonal antibody (Sigma-Aldrich), diluted with PBST containing 10% FBS for 1 hr, washed three times with PBST, incubated with 1:1000 Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) for 1 hr, and washed three times with PBST. The coverslips were mounted with mounting medium (H1200) (Vector Laboratories), and the cells were observed under an Olympus BX51 microscope equipped with a DP70 digital camera.

4. [3H]Chlorpromazine binding to the membranes of HEK-293 cells transfected with wild-type and mutant BmOAR2 cDNAs

Stably transfected clonal HEK-293 cells were harvested in ice-cold 50 mM Tris-HCl containing 120 mM NaCl (pH 7.4) and centrifuged at 25,000 g for 20 min. The cell pellets were homogenized in the buffer using a glass-Teflon homogenizer (20 strokes) and centrifuged at 25,000 g for 20 min. The resulting pellets were gently suspended in the buffer using a glass-Teflon homogenizer. The protein concentration was determined using the method described by Bradford and bovine serum albumin as the standard. A 50-μL aliquot of the buffer was added to test tubes for the determination of total binding. A 50-μL aliquot of unlabeled chlorpromazine (RBI Research Biochemicals; final conc., 10 μM) in the buffer was added to different test tubes to measure nonspecific binding. The buffer (50 μL) containing [3H]chlorpromazine (Vitrax; 574 GBq/mmol; final conc., 10 nM) and the buffer (150 μL) containing cell membranes (40 μg of protein) were added to all tubes. After vortexing, the reaction mixtures were incubated for 90 min at 25°C to allow the samples to reach binding equilibrium. Reactions were terminated by rapid filtration of the samples under reduced pressure through Whatman GF/B filters, which were presoaked in 0.3% polyethylenimine, using a Brandel M-24 cell harvester. The filters were rapidly washed with three 2-mL aliquots of cold buffer, and the bound radioactivity was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. Each experiment was repeated at least three times.

6. Homology modeling of BmOAR2 and docking studies with (R)-OA

The homology model of BmOAR2 was generated using the crystal structure of the human β2AR in the active state (PDB ID 3POG) and MOE 2010.10 software (Chemical Computing Group). The alignment of the two protein sequences was determined using CLUSTAL W to generate the multiple sequence alignment of BmOAR2, the human β2AR, and five proteins (EMBL-EBI accession No. E2BQJ9, E2AS88, E0VX90, Q9VCZ3, and E1JIT6) with high sequence similarities to BmOAR2. Geometry optimization was performed using the AMBER99 force field. The structure of (R)-OA was built using the MOE molecular builder, and the most stable conformation was obtained by a conformational search. The (R)-OA molecule was initially placed in the putative binding site using the data from site-directed mutagenesis to ensure that the NH2 and the phenolic OH groups of (R)-OA were close to the potential interacting amino acid residues D11532 and S20242, respectively (superscripts denote the Ballesteros-Weinstein index number of amino acids; i.e., TM number followed by the position number relative to a reference conserved amino acid residue14). This receptor-ligand complex was minimized using the MMFF94x force field and by fixing the receptor atoms in place. The OA-interacting atoms were then permitted to move while the other receptor atoms remained fixed. Finally, the resulting complex was minimized by allowing the receptor atoms within the distance of 4.5 Å from (R)-OA-interacting atoms to move while the other atoms remained fixed to produce the final docking model.
Results

1. Stable expression of wild-type and mutant BmOAR2 in HEK-293 cells

To define the function of BmOAR2, we generated HEK-293 cell lines stably expressing the wild-type and seven mutant receptors. The sequence information of human ARs and insect OA/TA receptors was used to predict potential (R)-OA-interacting amino acids in BmOAR2. Four conserved amino acids were mutated in the present study (Fig. 2). The pcDNA3-FLAG plasmids inserted with ORFs encoding the wild-type and mutant receptors were transfected into HEK-293 cells. Clonal cell lines obtained after culturing in the presence of the antibiotic G418 for 2-3-weeks were screened using RT-PCR (data not shown). Immunofluorescence analysis using an anti-FLAG antibody was performed to examine the localization of BmOAR2 in the selected cells. The cell lines transfected with the wild-type and seven mutant BmOAR2 plasmids showed intense fluorescence on the surface of the plasma membrane (Fig. 3a). Mock (empty vector)-transfected cells showed no fluorescence (data not shown).

2. Determination of BmOAR2 expression levels by [3H]chlorpromazine binding experiments

We next performed [3H]chlorpromazine binding experiments to examine the expression levels of the receptors. Chlorpromazine is a BmOAR2 antagonist. The specific-to-total binding of [3H]chlorpromazine to the membranes of each mutant (p<0.05 by unpaired t-test). The ratio of the specific binding of [3H]chlorpromazine to the membranes of mock-transfected HEK-293 cells (0.16±0.03) was significantly lower than that of cell lines transfected with cDNAs for the wild-type receptor (Fig. 3b). These findings indicate that mutant receptors are expressed in cells at levels that are not significantly different from those of the wild-type receptor.

3. Effects of (R)-OA on [cAMP], in cell lines expressing wild-type or mutant BmOAR2

To identify OA-interacting amino acid residues of BmOAR2, we performed functional analysis of cell lines expressing wild-type or mutant receptors by determining the changes in [cAMP]. The intrinsic agonist (R)-OA was employed in the present study. The R-enantiomer, which accounted for approximately 95% of OA in various insect species, was over 200-fold more potent than the S-enantiomer in stimulating OA-sensitive adenylate cyclase to produce cAMP in homogenates of the brain of the American cockroach. In our previous cAMP assays, (R)-OA was found to be approximately 100-fold more potent than (S)-OA in BmOAR2. (R)-OA at concentrations above 0.1 nM elicited a concentration-dependent increase in [cAMP], with an EC50 value of approximately 1.0 nM in the wild-type cell line, and [cAMP], reached a maximum of approximately 100 pmol/dish at 10 nM (R)-OA (Fig. 4). (R)-OA-induced cAMP elevations were lowered at concentrations above 1 μM. No elevation in [cAMP], in response to (R)-OA was observed in mock-transfected HEK-293 cells (data not shown). These findings indicate that BmOAR2 activation with (R)-OA is coupled to a significant activation of adenylate cyclase, probably via Gs protein. In sharp contrast to the wild-type receptor, six mutant receptors (D115^332A, S2025^42A, Y300^6^55F, Y300^6^55N, Y300^6^55L, and Y300^6^55A) did not show any significant change in [cAMP], after the application of (R)-OA (Fig. 4). The S206^46A mutant showed lower efficacy and potency than the wild-type receptor. The maximum cAMP level was approximately 22 pmol/dish, and the EC50 value was approximately 11 nM using (R)-OA concentration ranges below 100 nM.

4. Docking of (R)-OA into the putative binding site of a BmOAR2 homology model

A BmOAR2 homology model was constructed using the recently solved crystal structure of the βAR in an active state as a template. (R)-OA was docked into the potential binding site of the homology model. Ser202^46 in TM5 and Tyr300^65 in TM6 were predicted to form a hydrogen bond with the phenolic OH group.

Fig. 2. Alignment of amino acid residues in TM3, TM5, and TM6 of biogenic amine GPCRs. The residue numbers are indicated on the left of each sequence. Identical residues among all six receptors are denoted with asterisks, and the amino acids at the mutation sites are shown in bold. BmOAR1, B. mori α-adrenergic-like octopamine receptor (DDBJ: AB255163); BmOAR2, B. mori β-adrenergic-like octopamine receptor (DDBJ: AB470228); DmOCTβ2, D. melanogaster β-adrenergic-like octopamine receptor (GenBank: A880689); α1aAR, human α1a-adrenoceptor (EMBL: P08913); β2AR, human β2-adrenoceptor (EMBL: P07550); and BmTAR1, B. mori type-1 tyramine receptor (DDBJ: AB162828).
of (R)-OA, and Asp1153.32 in TM3 might form a hydrogen bond with the NH$_2$ group of (R)-OA (Fig. 5). Asp1153.32 may interact not only with the NH$_2$ group but also with the $\beta$-OH group. By contrast, Ser2065.46 appeared to be distantly located from any of these functional groups of (R)-OA.

**Discussion**

We performed site-directed mutagenesis experiments to identify amino acid residues that participated in (R)-OA-binding followed by the activation of BmOAR2. Eight plasmid vectors inserted with ORFs encoding the BmOAR2 wild type and mutants were constructed and transfected into HEK-293 cells for stable expression. Immunofluorescence imaging showed the clear expression of the receptors on the plasma membrane of HEK-293 cells (Fig. 3a). We further examined receptor expression levels as the magnitudes of ligand binding and signaling responses are affected by the expression levels of receptors on the membrane. To determine the receptor expression levels, we first tested $[^3H]$yohimbine binding to cell membranes, which was successfully used for the same purpose in the studies of the *Drosophila* OA/TA receptor expressed in CHO-K1 cells and the *B. mori* $\alpha$-adrenergic-like OAR (BmOAR1) expressed in HEK-293 cells$^{16,17}$; however, no specific binding was detected in cell lines expressing BmOAR2. In our previous study,$^8$ the effects of six potential antagonists on an (R)-OA-induced increase in [cAMP]$_i$ in BmOAR2-expressing HEK-293 cells were examined to characterize the pharmacological properties of this receptor. Of the tested antagonists, only chlorpromazine significantly reduced (R)-OA-induced elevation of [cAMP]$_i$; therefore, we
examined [3H]chlorpromazine binding to the membranes of cells expressing wild-type BmOAR2, and demonstrated a significantly higher percentage of specific vs. total binding (=50%) compared with that of mock-transfected HEK-293 cells (=16%) in the current study. In contrast, there were no significant differences in the specific binding percentages between wild-type and mutant BmOAR2 (Fig. 3b), indicating that these receptors are expressed at comparable levels. We attributed the lack of mutation-mediated effects on the binding of [3H]chlorpromazine to possible additional interactions between the phenothiazine ring of chlorpromazine and a hydrophobic/aromatic pocket of BmOAR2. Similar interactions are observed in the histamine H1 receptor.18)

The dissociated carboxyl group of Asp113 3.32 located in TM3 of βAR is involved in an electrostatic interaction with the ammonio group of agonists and antagonists.19) Site-directed mutagenesis studies of the human α2a-AR, the human dopamine D2 receptor, the B. mori type-1 TA receptor (BmTAR1), and the B. mori α-adrenergic-like OAR (BmOAR1) have revealed that their corresponding Asp

Fig. 5. Docking of (R)-OA to a potential binding site of BmOAR2 using the homology model. (a) Side view of the (R)-OA/BmOAR2 complex. (R)-OA is shown in cyan. (b) Extracellular view of the binding site. (R)-OA is shown in cyan. Asp1153.32, Ser2025.42, S2065.46, and Tyr3006.55 are shown in green, yellow, yellow, and magenta, respectively. (c) Diagram of the putative interaction of (R)-OA with amino acids in BmOAR2. Dotted outline surrounding (R)-OA denotes the proximity contour, which shows the distance to the active site interior, and dotted arrows in green indicate a hydrogen bond donor and acceptor. Arene-H interaction is shown as a green dotted line with a phenyl/H symbol. Polar, greasy, and acidic amino acids are highlighted in magenta, green, and magenta with a red circle, respectively. Shadowed circles indicate receptor contact.
residues play roles similar to that of βAR.17,20–22 Because the Asp residue of 3.32 is also conserved in BmOAR2 (Fig. 2), we generated the D1153.32A mutant receptor to examine whether Asp1153.32 was involved in the interaction with (R)-OA. After the application of (R)-OA in the range from 100 pM to 10 μM, the D1153.32A mutant receptor did not show significant changes in [cAMP], (Fig. 4). This finding indicates that Asp1153.32 is an important residue for both (R)-OA binding and receptor activation.

In the case of β2AR, both Ser2035.42 and Ser2045.43 interact with the m-OH group of catecholamine agonists, and Ser2075.46 interacts with the p-OH group.23–25 Ser1985.42 but not Ser2055.46 of BmOAR1 is involved in the interaction with the p-OH group of (R)-OA.26 In BmTar1, both Ser2185.42 and Ser2225.46 residues are important for the interaction with the p-OH group of TA22); therefore, we mutated the equivalent Ser residues to investigate their interaction with BmOAR2. The S2065.46A mutant of BmOAR2 lacked the ability to increase [cAMP] in response to (R)-OA (Fig. 4). In the S2065.46A mutant, however, the ability to respond to (R)-OA was retained, although it was diminished in terms of both efficacy and potency. The efficacy and potency of (R)-OA were one-fifth and one-tenth of those of the wild-type cacy and potency. The efficacy and potency of (R)-OA were retained, although it was diminished in terms of both efficacy and potency. Eventually, all of the mutants failed to elicit (R)-OA-stimulated cAMP elevation (Fig. 4), indicating that Tyr4126.55 was irreplaceable for the activation of BmOAR2.

In recent years, significant progress has been made in X-ray crystallography to resolve the structures of membrane proteins such as GPCRs and channels; however, most of the resolved structures of GPCRs were in the inactive state. Although crystalization of active-state GPCRs has been hampered due to their indigenous flexibility in the absence of a G protein, the crystal structure of an agonist-bound, active-state β2AR has been recently obtained as a complex with an agonist and an antibody fragment that behaves as a functional G-protein surrogate.29 Using this structure as a template, we created a BmOAR2 homology model (Fig. 5a). In this model, Asp1153.32, together with Thr1193.36, interacted with the NH₂ group of (R)-OA, and Ser2055.42 interacted with the phenolic p-OH group. Asp1153.32 also participated in forming a hydrogen bond with the β-OH group of (R)-OA, indicating that this amino acid recognized the stereochemistry of the β-position of (R)-OA (Fig. 5b). Ser2065.46 did not directly interact with (R)-OA but formed a hydrogen bond with Ser1203.37 located near Asp1153.32. The S2065.46A mutation may weaken the TM3/TM5 interaction, thereby giving rise to a debased receptor. In our initial hypothesis, Tyr3006.55 was postulated to interact with the β-OH group of (R)-OA; however, the homology model showed that Tyr3006.55 was not responsible for interacting with the β-OH group of (R)-OA but, together with Ser2055.42, interacted with the phenolic p-OH group of (R)-OA. It is also notable that the amino acid residues with a hydrophobic/aromatic side chain in TM3, TM5, TM6, and TM7 surrounded the (R)-OA molecule (Fig. 5c). In general, these results are similar to those observed in the crystal structures of the agonist-bound β1 and β2AR.29,31

In conclusion, the current work provided a functional characterization of the wild type and mutants of a β-adrenergic-like OAR. Together with the functional data, the homology model of BmOAR2 revealed important amino acid residues that interacted with the intrinsic agonist (R)-OA. These findings will be helpful in designing novel pest control chemicals.

Acknowledgements

This work was supported in part by KAKENHI (a Grant-in-Aid for Scientific Research) (C). Part of this work was conducted at the Department of Molecular and Functional Genomics at the Center for Integrated Research in Science at Shimane University.

References

2) M. Monastirioti, C. E. Linn, Jr. and K. White: J. Neurosci. 16,