Identification of Critical Structural Determinants Responsible for Octopamine Binding to the α-Adrenergic-like *Bombyx mori* Octopamine Receptor

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Received December 18, 2006; Revised Manuscript Received March 23, 2007

ABSTRACT: Octopamine (OA) is a biogenic amine with a widespread distribution in the insect nervous system. OA modulates and/or regulates various behavioral patterns of insects as a neurotransmitter, neuromodulator, and neurohormone. OA receptors (OARs) belong to one of the families of G protein-coupled receptors (GPCRs). The binding of OA to OARs is coupled to the activation of the specific G proteins, which induces the release of intracellular second messengers such as cAMP and/or calcium. We previously reported the isolation of an OAR (BmOAR1) from *Bombyx mori*. In the study presented here, five mutated BmOAR1s were constructed with a point mutation in the putative binding crevice and expressed in HEK-293 cells. The S202A mutant receptor was found to retain the cAMP response to OA as does the wild-type receptor, but such function was impaired in the other four mutants (D103A, S198A, Y412F, and S198A/S202A). Furthermore, competition binding assays using [^{3}H]OA and calcium mobilization assays gave results that were approximately consistent with those of the cAMP assays. Taken together, the results indicate that D103 and S198 are involved in the binding and activation of BmOAR1 with OA through electrostatic or hydrogen bond interactions, but S202 does not appear to participate in this process. Y412 seems to be involved in one of the active forms of BmOAR1. These findings should prove helpful in designing new pest control chemicals.

Octopamine (OA),[1] one of the most significant biogenic amines, has a wide distribution in the insect nervous system and carries out many physiological tasks, probably as a counterpart of noradrenaline and adrenaline (Figure 1), which are vertebrate biogenic amines. OA has been shown to act as a neurotransmitter, neuromodulator, and neurohormone to regulate various physiological functions in insects, such as circadian rhythms, endocrine secretion, fight and flight behaviors, and learning and memory (*1*). Numerous biochemical and pharmacological experiments suggest that OA exerts its effects by binding to specific OA receptors (OARs), which are typical rhodopsin-like G protein-coupled receptors (GPCRs) with seven α-helical transmembrane domains and share highly conserved sequence similarity. The binding of OA to the OAR is thought to activate the specifically coupled G proteins and thereby induce the release of intracellular second messengers such as cAMP and/or calcium (*2*). Since

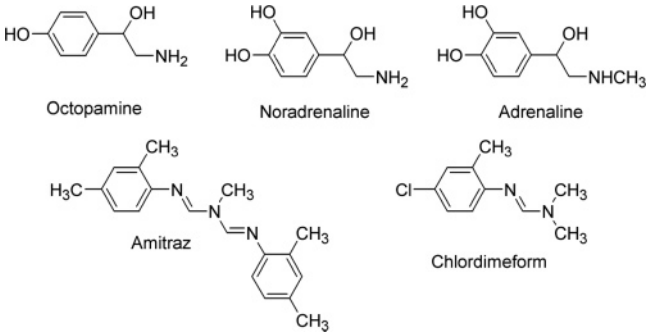

FIGURE 1: Structures of structurally related biogenic amines and OAR-targeted insecticides.

a number of OARs have been cloned and characterized, Evans and Maqueira (*3*) suggested a new classification scheme based on their similarities in structure and in signaling properties with vertebrate adrenergic receptors (ARs). In this new classification system, OARs are classified into three classes: α-adrenergic-like, *β*-adrenergic-like, and OA/tyraminergic receptors. α-Adrenergic-like OARs expressed in cell lines lead to elevation of the intracellular concentration of both calcium ($[Ca^{2+}]_i$) and cAMP ($[cAMP]_i$) in response to OA, while *β*-adrenergic-like OA receptors are selectively coupled to the production of cAMP in cells.

As OARs are restricted in their occurrence to invertebrates, they represent an attractive target for highly specific insecticides. A few insecticides such as chlordimeform and amitraz (Figure 1) are thought to exert their insecticidal activity by

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[1] Abbreviations: AR, adrenergic receptor; K_d, apparent dissociation constant; GPCR, G protein-coupled receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; $[cAMP]_i$, intracellular cAMP concentration; B_{max}, maximum binding capacity; OA, octopamine; OAR, octopamine receptor; RT-PCR, reverse transcription-polymerase chain reaction; TM, transmembrane domain.

10.1021/bi602593t CCC: $37.00
Published on Web 05/01/2007

Table 1: Mutated Amino Acids of BmOAR1 and Sequences of Oligonucleotides Used for Site-Directed Mutagenesis[a]

amino acid	substitution	TM	primer
Asp103	Ala	III	GTATGGCTAGCCGTGGCCGTGTGGATGTGCACC
Ser198	Ala	V	GGGTACGTTGTCTATGCAGCTTTGGGCTCC
Ser202	Ala	V	CAGCTTTGGGCGCCTTCTACATCCCG
Tyr412	Phe	VI	CCATTTTTCAGCGTCTTCGTGGTCCGGGCGTTC

[a] Only forward primers (from 5′ to 3′) are shown. Substituted codons are underlined.

interacting with insect OARs in metabolically activated forms (*4*). We previously reported the cloning of the first OAR (BmOAR1) from the silkworm *Bombyx mori*, which is a typical α-adrenergic-like receptor (*5*). Heterologous expression of this gene (DDBJ accession number AB255163) in HEK-293 cells resulted in the generation of receptors that exhibit a pharmacological response highly similar to that of homologous receptors (Pa oa_1, AmOA1, and OAMB) cloned from other insects (*6*−*8*). In the study presented here, we used site-directed mutagenesis to identify some of the residues involved in OA binding and activation of BmOAR1, and, on the basis of the results, proposed a potential mechanism for the activation at the molecular level. It is hoped that this work will facilitate OAR-targeted agonist/insecticide development as well as basic research into the activation mechanism of GPCRs.

MATERIALS AND METHODS

Site-Directed Mutagenesis and Construction of the Expression Vectors. The BmOAR1 cDNA was amplified by PCR using the primers BmOAF1 (5′-TTTTGGTACCATGCGCTCGCTGAAC-3′) and BmOAR1 (5′-AAAGGTACCTCACCTTTCTGCCGC-3′) and pcDNA3-BmOAR1 as a template (*5*). The product was subcloned into the *Kpn*I site of the pBluescript KS(-) vector. Site-directed mutagenesis of BmOAR1 was carried out using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and pBluescript KS(-)-BmOAR1 was used as a template of cycle reactions for single-amino acid changes. The oligonucleotides used as primers were designed according to the instruction manual of the mutagenesis kit; only the forward primers are listed in Table 1. The vector for the S198A/S202A double mutant was constructed by a cycle reaction using the S198A vector as a template and oligonucleotides for the S202A mutation as primers. Each mutation was verified by DNA sequencing. To add a hemaglutinin (HA) tag at the N-terminus of BmOAR1, a Kozak sequence (*9*) and an HA tag sequence were previously introduced into the *Hin*dIII/*Kpn*I site of the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) to give pcDNA3-HA. These consecutive Kozak and HA tag sequences were generated by PCR using two 2/5-overlapping primers, *Hin*dIII-HA-f (5′-TTTAAGCTTGCCACCATGGCCTACCCCTACGACGT-3′) and *Kpn*I-HA-r (5′-TTTGGTACCGGCGTAGTCGGGCACGTCGTAGGGGT-3′). The *Kpn*I-digested fragments from the pBluescript-BmOAR1 constructs were then ligated into the *Kpn*I site of pcDNA3-HA in the correct direction.

Cell Culture, Transfection, and Stable Cell Lines. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) at 37 °C and 5% CO_2. After transfection of the wild-type and mutant BmOAR1 cDNAs into the cells with Lipofectamine (Invitrogen), the antibiotic Geneticin (G418) (1.0 mg/mL; Sigma, St. Louis, MO) was also added to select cells stably expressing the receptors before clonal culture. After 2 weeks of G418 selection, 8−12 G418-resistant colonies were trypsinized in cloning cylinders and transferred to 24-well plastic plates for expanding. These individual cell lines were analyzed for the incorporation and localization of the receptor by means of immunofluorescence and RT-PCR (reverse transcription-polymerase chain reaction) analysis (data not shown). The most intensively expressing cell lines were chosen for this study.

Immunofluorescence Analysis. Individual clonal cells, which were grown and attached to round coverslips (12 mm in diameter), were washed twice with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS for 20 min at room temperature and then were permeated with 0.2% Triton X-100 in PBS for 10 min. After being washed with PBST (PBS containing 0.1% Tween 20), the cells on the coverslips were blocked with PBST containing 10% FBS for 30 min. The cells on the coverslips were then incubated with 1:1000 anti-HA monoclonal antibody (Cell Signaling, Beverly, MA) diluted with PBST containing 10% FBS for 1 h, washed three times with PBST, and then incubated with 1:1000 Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 h and washed three times with PBST. The coverslips were mounted with a mounting medium (H1200) (Vector Laboratories, Burlingame, CA) and observed under a Nikon Eclipse TS100 fluorescent microscope with a Nikon DS-5Mc-L1 digital camera.

Membrane Preparation for Binding Assays. Cells were harvested in ice-cold 50 mM Tris-HCl (pH 7.4) and centrifuged at 25000*g* for 20 min. The cell pellet was homogenized in the buffer with a glass−Teflon homogenizer (20 strokes) and recentrifuged as described above. The resulting pellet was gently suspended in the buffer with a glass−Teflon homogenizer. The protein concentration was determined by the method of Bradford (*10*) with bovine serum albumin as the standard.

[^{3}H]Yohimbine Binding Assays. A 50 μL aliquot of 50 mM Tris-HCl (pH 7.4) for the determination of the total amount of binding or the same volume of the buffer containing various concentrations of unlabeled yohimbine was added to test tubes. Unlabeled yohimbine (final concentration of 10 μM) was added for the determination of the level of nonspecific binding. The buffer (50 μL) containing [^{3}H]yohimbine (80.5 Ci/mmol) (Perkin-Elmer Life Sciences, Boston, MA) (final concentration of 1 nM) and the buffer (150 μL) containing cell membrane homogenates (40 μg as protein) were added to all tubes. After being vortexed, the reaction mixtures were incubated for 60 min at 25 °C to allow for a binding equilibrium. The reactions were terminated by rapid filtration under reduced pressure through Whatman GF/B filters presoaked in 0.3% polyeth-

ylenimine. The filters were rapidly washed with two 3 mL portions of cold (10 °C) 50 mM Tris-HCl (pH 7.4) and then placed in 10 mL of toluene/Methyl Cellosolve-based scintillation fluid. The bound radioactivity was determined using a liquid scintillation counter. Each experiment was performed in duplicate and repeated two or three times. The maximum binding capacity (B_{max}) of each cell line and the apparent dissociation constant (K_d) of OA were estimated by fitting data to the one-site binding model using Origin Pro (Origin Lab, Northampton, MA).

[^{3}H]OA Binding Assays. Unlabeled OA at various concentrations was incubated together with 3 nM [^{3}H]OA (50 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO) and 100 μg of membrane protein in a total volume of 250 μL of 50 mM Tris-HCl (pH 7.4) for 120 min at 0 °C. Nonspecific binding was assessed in the presence of 1 mM unlabeled OA. The reactions were terminated by rapid filtration under reduced pressure through Whatman GF/C filters. The filters were rapidly washed with two 3 mL portions of 50 mM Tris-HCl (pH 7.4), and the bound radioactivity was determined using a liquid scintillation counter. Each experiment, done in duplicate, was repeated three to five times. IC_{50} values were estimated using the logistic function.

cAMP Assays. The cells (2×10^5) suspended in 10% FBS/D-MEM were plated on 35-mm-diameter dishes 17−21 h before the cAMP assays. Attached cells were washed once with 1 mL of Dulbecco's phosphate-buffered saline (D-PBS) (Gibco BRL) and preincubated in 1.8 mL of D-PBS containing 5 mM theophylline for 20 min at 37 °C. After the preincubation, a 200 μL aliquot of D-PBS containing various concentrations of OA was added to the dish, and then the culture was incubated for 20 min at 37 °C. The reactions were stopped by aspiration of the medium and addition of ice-cold acidic ethanol (1 M HCl/ethanol = 1/100; 300 μL). The cells in the ethanol solution were collected and homogenized in 1.5 mL microtubes. After the sample was allowed to stand for 5 min at room temperature, the debris was removed by centrifugation at 14000*g* for 5 min. The supernatant was evaporated to dryness by vacuum centrifugation for 1 h at 45 °C. The residue was suspended in 120 μL of 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM EDTA and centrifuged at 14000*g* for 5 min. The supernatant was used to determine $[cAMP]_i$. The cAMP levels were determined on the basis of the competition between unlabeled cAMP and a fixed quantity of [^{3}H]cAMP (17 Ci/mmol) (American Radiolabeled Chemicals) for binding to protein kinase A (Sigma), which has high specificity and affinity for cAMP (*11*). The radioactivity was measured by a liquid scintillation counter. Each experiment was repeated three times or more.

Calcium Mobilization Assays. The cells cultured on coverslips were loaded with Fura 2-AM (Dojindo Laboratories, Kumamoto, Japan), a calcium indicator, at a final concentration of 3−5 μM by incubating them together with 0.2% Cremophor EL (Sigma) in D-MEM for 45 min at 37 °C. The cells were subsequently washed twice with a bathing solution containing 152 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The coverslips were transferred to the microscopic chamber. The emission of Fura 2 at 510 nm was measured with Fura 2-loaded individual cells using dual-wavelength microfluorometry (OSP-3 system, Olympus). As the excitation wavelengths of Fura 2 with and without chelating Ca^{2+} peaked at 340 and 380 nm, respectively, the ratio of Fura 2 fluorescence at 510 nm upon excitation at 340 and 380 nm in a spot in a single cell (F_{340}/F_{380} ratio) correlates with intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*12*). F_{340}/F_{380} ratios were calculated in real time at 0.2 s intervals using MiCa (*13*) and used as a relative indicator of $[Ca^{2+}]_i$. Various concentrations of OA in the bathing solution were applied continuously to the cells at room temperature.

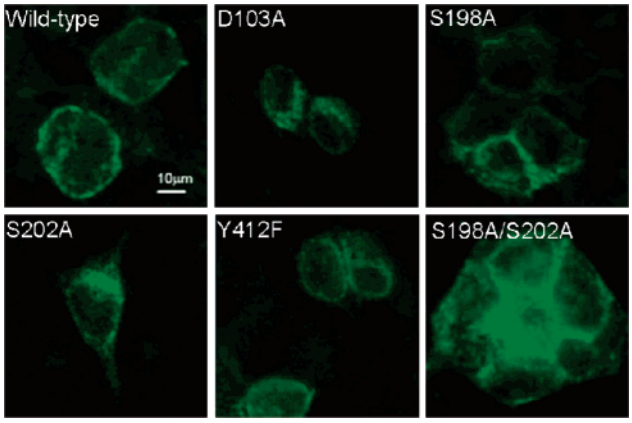

FIGURE 2: Immunofluorescence analysis of the wild-type and mutant BmOAR1s in HEK-293 cells.

Homology Modeling. The sequences of BmOAR1 and bovine rhodopsin were aligned using Clustal-W, and a nine-residue motif in TM VI was shifted forward one residue to avoid a gap. Twenty intermediate models were generated by MOE (Chemical Computing Group, Montreal, QC) on the basis of the 2.8 Å crystal structure of bovine rhodopsin (PDB entry 1HZX), and the best one was chosen as the crude template for further refining. Then this crude model was subjected to energy minimization until the root-mean-square gradient became 0.01. The OA molecule, which was optimized with the PM3 method in MOPAC in advance, was manually docked into the potential binding site in an appropriate orientation and position according to the previous experimental data.

RESULTS AND DISCUSSION

Functional Expression of the Wild-Type and Mutant BmOAR1s. Due to the lack of crystal structure data of GPCRs other than bovine rhodopsin (*14*), scientists have employed traditional and reliable mutagenesis strategies and homology modeling to identify critical residues and motifs that participate in the binding and activation of ARs. In our study, these abundant reports about α- and β-ARs were used as guidelines for choosing potential amino acids in the homologous BmOAR1. Thus, five plasmid vectors inserted with point-mutated genes of BmOAR1 as well as the wild type were constructed and transfected into HEK-293 cells for stable expression. The immunofluorescence studies clearly demonstrated that BmOAR1 was mainly expressed on the cell membrane (Figure 2). It is very important to examine the receptor expression levels on the cell surface because both the pharmacological response and the levels of ligand binding are related to the amount of receptor on the membrane to some extent. Here we chose [^{3}H]yohimbine,

Table 2: [^{3}H]Yohimbine Binding Parameters of the Wild-Type and Mutant BmOAR1s Used To Compare Their Expression Levels in HEK-293 Cells[a]

OAR	B_{max} (pmol/mg)	K_d (nM)
wild-type	110.1 ± 5.2	23.3 ± 4.5
D103A	69.7 ± 1.4	23.5 ± 2.9
S198A	106.3 ± 5.9	69.5 ± 4.4
S202A	44.7 ± 1.4	31.1 ± 4.1
Y412F	102.7 ± 3.0	19.0 ± 4.8
S198A/S202A	149.9 ± 5.9	42.7 ± 4.7

[a] Data represent the means ± standard error of two to three independent experiments, each conducted in duplicate.

which has been successfully used to examine the expression levels of mutants of *Drosophila* OA/tyramine receptors in CHO-K1 cells (*15*), to quantify BmOAR1 expression levels in HEK-293 cells, since the level of specific binding of [^{3}H]-yohimbine to the mock HEK-293 cells was so low (<2% of that of the wild-type BmOAR1) that it can be neglected. Yohimbine is best known as an α-AR antagonist in vertebrates (*16*) and a tyramine receptor antagonist in invertebrates (*2*). In a previous study (*5*), we found that yohimbine also binds to wild-type BmOAR1. The specific-to-total binding percentages were found to be almost the same in the wild type and all the mutants of BmOAR1 (>95%) in this study. It is reasonable to speculate that yohimbine binds to the agonist site of BmOAR1, because yohimbine has a basic nitrogen atom as does OA. However, as yohimbine is much bigger than OA, this compound probably interacts with amino acid residues other than the residues with which OA interacts (i.e., the so-called accessory binding site). This might be the reason why yohimbine binds to both the wild type and mutants of BmOAR1 without a significant effect of mutations. As shown in Table 2, the results of the [^{3}H]-yohimbine binding assays showed that the expression levels of the wild type and most mutants were in the B_{max} range of 102.7−149.9 pmol/mg, although those of two mutants, D103A (B_{max} = 69.7 pmol/mg) and S202A (B_{max} = 44.7 pmol/mg), were relatively low. The K_d values ranged from 19.0 to 42.7 nM, except in the case of S198A, for which the K_d value was as high as 69.5 nM.

As our aim in this study is to identify the amino acids involved in binding of OA, we used OA in [^{3}H]OA binding assays to compare the difference in OA binding ability among the wild type and mutants. In the meantime, wild-type BmOAR1 was well characterized by pharmacological and functional assays in determining the most appropriate OA concentration for mutant receptors. Wild-type BmOAR1 exhibited a good dose-dependent response in the [^{3}H]OA binding assays, cAMP assays, and calcium mobilization assays. The IC_{50} of OA in the [^{3}H]OA binding assays was 4.38 ± 0.40 μM, and the EC_{50}s of OA in the cAMP and calcium mobilization assays were 35.2 ± 9.8 and 1.63 ± 0.37 μM, respectively. Thus, OA was found to increase both $[cAMP]_i$ and $[Ca]_i$ in HEK-293 cells expressing BmOAR1. As there are several papers reporting that such a calcium increase would also induce an accumulation of cAMP and/or vice versa in several kinds of cells (*17*−*19*), this raised the question of whether these two responses were independent. To examine this question, OA-mediated changes in $[cAMP]_i$ levels were first compared in the absence and presence of the intracellular calcium chelator BAPTA-AM. As a result, the $[cAMP]_i$ levels following treatment with 100 μM OA were not found to be significantly different in the absence or presence of 50 μM BAPTA-AM; it was confirmed in preliminary experiments that a 20 min preincubation of the cells with 50 μM BAPTA-AM was enough to block the Ca^{2+} transient induced by 100 μM OA (data not shown). Therefore, this increase in $[cAMP]_i$ by OA likely results from direct coupling of BmOAR1 to G_s protein, which leads to activation of adenylate cyclase. On the other hand, we tested the effects of forskolin and theophylline on OA-induced elevation in $[Ca]_i$. Forskolin can directly activate adenylate cyclase, thereby increasing $[cAMP]_i$, and theophylline can also lead to the same result through inhibition of phosphodiesterase, which degrades cAMP to AMP. Neither forskolin nor theophylline led to an obvious $[Ca]_i$ increase even at a high concentration (100 μM) in wild-type BmOAR1 (data not shown), demonstrating that these second messenger productions were independent of each other in our system.

An Amino Acid Residue Interacting with the Positively Charged Amine Group of OA. The carboxyl group of Asp113 in transmembrane domain (TM) III of the β_2-AR is involved in forming an ion pair with the protonated amine moiety of agonists and antagonists (*19*). Site-directed mutagenesis of the α_{2A}-AR, the dopamine D_2 receptor, and the *B. mori* tyramine receptor (BmTAR1) also indicated that their corresponding aspartic acid residues play the same role (*21*−*23*). The corresponding amino acid residue, Asp103, is also conserved in BmOAR1 (Table 3). To examine whether Asp103 is indeed involved in interaction with OA, we generated a D103A mutant receptor, in which aspartic acid was replaced with alanine. In [^{3}H]OA binding assays, the specific-to-total binding percentage of the D103A mutant was 5.8%, whereas that of the wild type was as high as 76.1%, indicating almost complete loss of the affinity of the mutant for OA (Figure 3). When 100 μM OA was applied in examining the functional coupling to G_s protein, this mutant did not show any significant change in $[cAMP]_i$ (Figure 4). By contrast, the wild-type receptor increased $[cAMP]_i$ from 0.91 (basal) to 12.07 pmol/dish. The loss of function was also found in calcium mobilization experiments, in which the increase in $[Ca]_i$ by 10 μM OA in the D103A mutant was only 15.0% of that in the wild type (Figure 5). Taken together, our findings indicate that Asp103 is an important residue for both OA binding and receptor activation. The carboxyl group of Asp103 probably forms an ion pair with the protonated amino group of OA.

An Amino Acid Residue Interacting with the p-Phenolic Hydroxyl Group of OA. Three serine residues, Ser203, Ser204, and Ser207, in TM V of the β_2-AR were shown to contribute to the interaction with the *m*- and *p*-hydroxyl groups in the catechol moiety of agonists (*24*−*26*). The corresponding residues are Ser198, Ala199, and Ser202 in BmOAR1 (Table 3). Thus, we here made three more mutants, S198A, S202A, and a double mutant (S198A/S202A), to explore the roles of two serine residues independently or together. The results of the S198A and S198A/S202A mutants were almost the same as that of the D103A mutant in the pharmacological and functional assays (Figures 3−5). However, the S202A mutant only partially reduced the specific binding percentage of [^{3}H]OA (to 45.4%). As the expression level of the S202A mutant was much lower than

Table 3: Alignment of Amino Acid Sequences in TM III, V, and VI of BmOAR1, BmTAR1, α_{2A}-AR, and β_2-AR[a]

Receptor	TM III	TM V	TM VI
BmOAR1	CSVWLAVDVWMCTASILNLCAISL	DAGYVVYSALGSFYIPMFVMLFFYWR	LGIIVGGFVFCWLPFFSVYVVRAFC
BmTAR1	CKMWLTCDIMCCTSSILNLCAIAL	QPGFVIFSSSGSFYIPLVIMTVVYFE	LGIIMGVFVVCWLPFFVIYLVIPFC
α_{2A}-AR	CEIYLALDVLFCTSSIVHLCAISL	QKWYVISSCIGSFFAPCLIMILVYVR	LAVVIGVFVVCWFPFFFTYTLTAVG
β_2-AR	CEFWTSIDVLCVTASIETLCVIAV	NQAYAIASSIVSFYVPLVIMVFVYSR	LGIIMGTFTLCWLPFFIVNIVHVIQ
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[a] Residues conserved in all four receptors are denoted with asterisks, and the corresponding mutated sites are underlined.

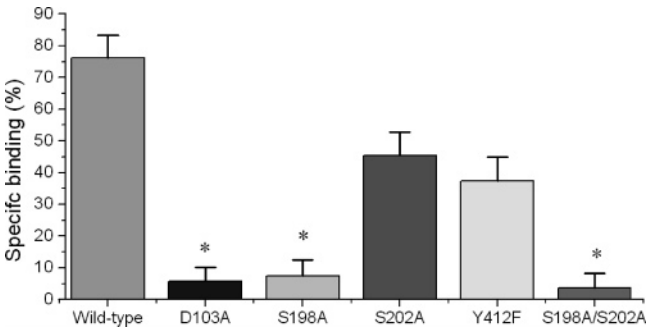

FIGURE 3: Specific binding of 3 nM [^{3}H]OA to the wild-type and mutant OARs. Specific-to-total binding percentages are shown. Asterisks indicate values significantly different from the wild-type value using one-way ANOVA followed by the Bonferroni post hoc test ($p < 0.001$). Data represent the means ± standard error of three to five independent experiments, each conducted in duplicate.

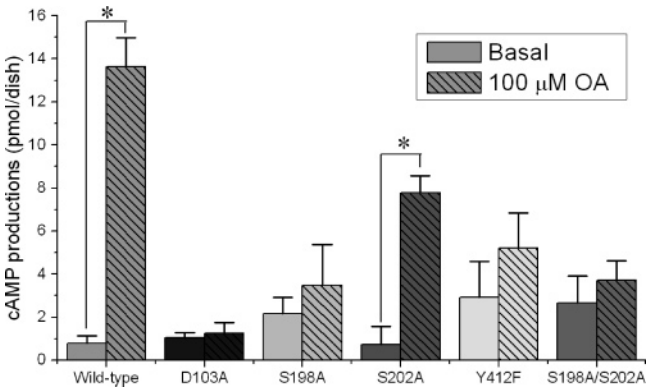

FIGURE 4: Changes in $[cAMP]_i$ in HEK-293 cells expressing the wild-type and mutant OARs by 100 μM OA. Asterisks indicate values significantly different from the basal levels using a Student's *t* test ($p < 0.05$). Data represent the means ± standard error of three or four independent experiments, each conducted in duplicate.

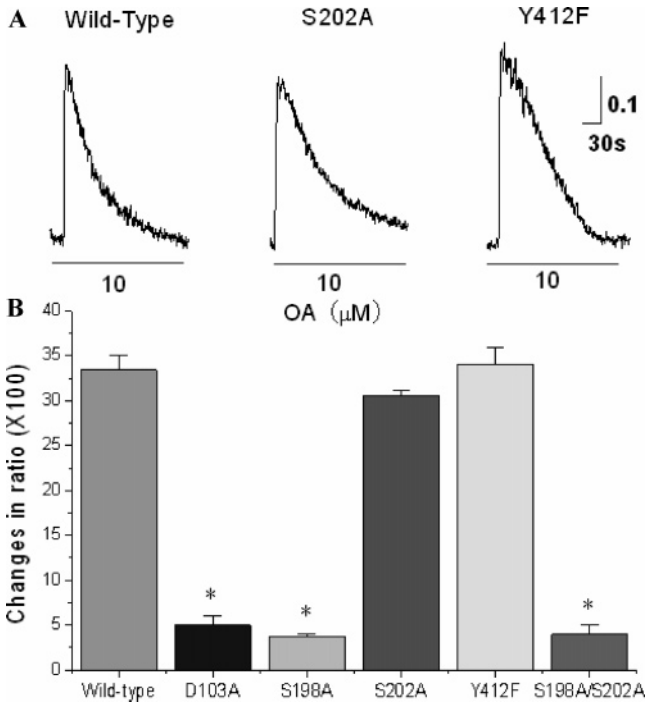

FIGURE 5: OA (10 μM)-induced $[Ca^{2+}]_i$ changes in HEK-293 cells expressing the wild-type and mutant OARs. $[Ca]_i$ is proportional to the ratio of fluorescence recorded at 340 and 380 nm excitation wavelengths. (A) Typical traces of calcium responses in the wild type and S202A and Y412F mutants during the application (underlined) of OA. The cells repeatedly responded to OA after washout of previously applied OA with a bathing solution. (B) The ratio changes in the absence and presence of 10 μM OA for the wild-type and mutant OARs. Asterisks indicate values significantly different from the wild-type value using one-way ANOVA followed by the Bonferroni post hoc test ($p < 0.001$). Data represent the means ± standard error of three independent experiments.

that of the wild type (Table 2), their actual binding abilities seem not to be significantly different after normalization. The results from the cAMP assays and calcium mobilization assays were in agreement with the binding data. The S202A mutant increased $[cAMP]_i$ and $[Ca]_i$ on application of OA. Therefore, Ser198 plays a role as significant as that of Asp103 in the OA-stimulated activation, while Ser202 adjacent to Ser198 in TM V does not appear to be involved in such a process. This finding is different from that observed with BmTAR1, in which both of the corresponding residues, Ser218 and Ser222, interacted with the *p*-phenolic hydroxyl group of tyramine (*23*).

An Amino Acid Residue Interacting with the β-Hydroxyl Group of OA. Studies using a homology model of the α_2-AR (*27*) and a computational model of the β_2-AR (*28*) suggested that the conserved aspartic acid residue in TM III may also be capable of forming hydrogen bonds with the β-hydroxyl group on the catecholamine side chain. Unfortunately, this proposition cannot be tested experimentally, as this amino acid is also critical for the activation via formation of the ion pair with the protonated amine moiety of agonists, as described above. A substantial loss of stereoselective binding for the β-adrenergic agonist isoproterenol was observed in the β_2-AR after substitution of Asn293 in TM VI by leucine due to the disruption of the interaction between the β-hydroxyl group of isoproterenol and the side chain of Asn293 (*29*, *30*). Replacement of the equivalent residue in the α_{1B}-AR, leucine, with the corresponding residue in the α_{1A}-AR, methionine, produced a mutant receptor with a changed agonist binding profile (*31*,

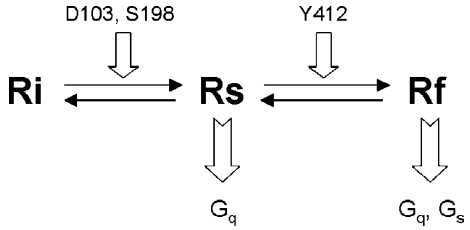

FIGURE 6: Proposed roles of the critical amino acids in the OA-dependent activation of BmOAR1. Ri represents the receptor in the inactive state, Rs the receptor in the semi-active state, and Rf the receptor in the fully active state.

32). Chatwin et al. (*15*) generated various alanine mutants for a range of receptor amino acids that could potentially form hydrogen bonds with the β-hydroxyl group of OA in the *Drosophila* OA/tyramine receptor. They found that the corresponding Y549A mutant receptor was expressed at a very low level, and thus, no conclusions could be made. Therefore, the equivalent Tyr412 residue in BmOAR1 was mutated to phenylalanine (Y412F) to test whether the function of these homologous amino acids is conserved. As a result, it was found that the specific-to-total binding percentage of [^{3}H]OA binding to the Y412F mutant receptor was 37.4%. This is almost half the binding ability of the wild-type receptor (Figure 3). In the cAMP assays, there was no significant change in $[cAMP]_i$ between basal and OA-activated levels, indicating a loss of function as seen in the D103A and S198A mutants (Figure 4). However, the Y412F mutant receptor gave a level of calcium response similar to that of the wild-type receptor (Figure 5), indicating that the mutant receptor itself can still be coupled to G proteins such as G_q protein and can induce the release of intracellular calcium.

Signal Transduction Mechanisms. The role of Tyr412 in TM VI appears to be slightly intricate, since the Y412F mutant exhibited promiscuous pharmacological and functional responses to OA. Here we propose a model that can satisfactorily explain these results. It is generally accepted that the receptor exists in two different conformations, i.e., the active and inactive states, which differ in their ability to activate G proteins (*33*). In such a two-state model, agonists have a higher affinity for the active state and stabilize this conformation. Furthermore, the demonstration that many receptors may activate distinct G proteins and that different molecular determinants of the receptor may contribute to these couplings led to the proposal that three or even more active states of the same receptor exist (*34*). In this model (a three-state or multistate model), distinct conformations of the receptor are involved in the coupling with distinct G proteins or even with intracellular proteins other than G proteins (*35*−*37*). Therefore, we propose a similar but more detailed scheme for the activation of BmOAR1 by OA (Figure 6). We hypothesize that there are two active states for BmOAR1 through the movement of critical transmembrane domains: a semi-active state that induces the activation of only G_q protein and a fully active state that leads to the activation of both G_s and G_q proteins. The binding of OA to BmOAR1 might be initiated by the formation of an ion pair between the negatively charged carboxylate of Asp103 in TM III and the positively charged aliphatic amine group of the ligand. At the same time, a hydrogen bond between the phenolic hydroxyl group of OA and the hydroxyl group of

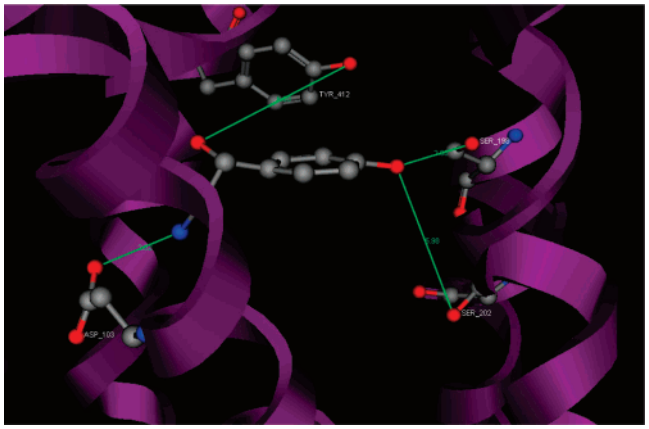

FIGURE 7: Docking OA into the binding site of BmOAR1. The distances (angstroms) of potentially interactive residues are marked.

Ser198 in TM V is formed. These two interactions are essential for achievement of the semi-active or fully active state of BmOAR1, although other interactions such as a hydrophobic interaction or $\pi-\pi$ interaction may also be involved in this binding process. For this reason, either mutation of the two residues results in the changes in the physicochemical properties of the binding pocket in BmOAR1 so that OA fails to dock into the proper binding site and to lead to any active state. However, Tyr412 in TM VI might be involved indirectly in the formation of the fully active state but not that of the semi-active state. This is why the Y412F mutant still retains the ability to stabilize the semi-active state to activate the G_q protein.

The OA molecule was manually docked into the potential binding site of the homology model of BmOAR1 according to the mutagenesis data (Figure 7). The size of the binding pocket is just suitable for the OA molecule, especially the distance of Asp103 in TM III and Ser198 in TM V. It is possible for OA to interact with both residues. In hydrogen bonds (D−H···A, where D−H is an acidic donor group and A is an acceptor atom), the D···A distance is normally in the range of 2.7−3.1 Å (*38*). Ser202 seems too distant (5.98 Å) to interact with the phenolic hydroxyl group of OA, unlike Ser198. Tyr412 seems too far (6.60 Å) from the β-hydroxyl group in the inactive state to form a hydrogen bond between them, when the location of OA is confined to a specific area in which OA interacts with the above-mentioned Asp103 and Ser198. Specific movements of the transmembrane helices are essential for activation of GPCRs, especially TMs III, V, and VI in the rhodopsin-like families (*39*). Therefore, Tyr412 probably gets close to the binding site after the formation of the semi-active state and comes to interact with OA through the movement of TM VI, ultimately resulting in full activation of BmOAR1.

Another potential mechanism that accounts for the selectivity of G protein coupling revealed by the Y412F mutant could be the dimerization of BmOAR1. It is widely accepted that many GPCRs assemble as dimers, a process that affects intracellular trafficking, ligand recognition, signaling, and regulation (*40*). The use of receptor blocking peptides suggests that receptor-specific dimerization motifs are present in TM VI and TM VII of the D_2 dopamine receptor (*41*) and TM VI of the β_2-AR (*42*). There are a few reports that examine the influence of receptor dimerization on the functional coupling to multiple G proteins (*43*). Therefore,

we cannot exclude the possibility that native BmOAR1 forms dimers and that the mutation of Tyr412 in TM VI affects or disrupts the dimerization, resulting in the inactivation of G_s protein. Further experiments aimed at examining whether this mechanism also holds true of BmOAR1 are needed.

Conclusions. All pharmacological and functional assays in this study clearly confirmed the involvement of Asp103 and Ser198 in the binding of OA leading to the activation of BmOAR1. The data from cAMP assays, binding assays, and calcium mobilization assays clearly demonstrated that the D103A, S198A, and S198A/S202A mutants lost nearly all their binding affinity and pharmacological responses, which means that these mutant receptors cannot form an effective binding crevice for OA to trigger the activation of the receptor and the coupled G proteins and thus do not activate transduction pathways via second messengers such as cAMP and calcium. This deduction is also consistent with the homology modeling in which Asp103 and Ser198 lie in the proper position and orientation of the OA binding pocket. In contrast, Ser202 adjacent to Ser198 in TM V does not appear to participate in the OA-stimulated activation, which accounts for the fact that the OA response of the S202A mutant was as intact as in the wild-type receptor, whereas Tyr412 might play a significant role in one of the active states of BmOAR1.

ACKNOWLEDGMENT

Part of this work was conducted at the Department of Molecular and Functional Genomics, Center for Integrated Research in Science, Shimane University.

REFERENCES

1. Roeder, T. (1999) Octopamine in invertebrates, *Prog. Neurobiol. 59*, 533−561.
2. Blenau, W., and Baumann, A. (2001) Molecular and pharmacological properties of insect biogenic amine receptors: Lessons from *Drosophila melanogaster* and *Apis mellifera*, *Arch. Insect Biochem. Physiol. 48*, 13−38.
3. Evans, P. D., and Maqueira, B. (2005) Insect octopamine receptors: A new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors, *Invertebr. Neurosci. 5*, 111−118.
4. Roeder, T. (2005) Tyramine and octopamine: Ruling behavior and metabolism, *Annu. Rev. Entomol. 50*, 447−477.
5. Ohtani, A., Arai, Y., Ozoe, F., Ohta, H., Narusuye, K., Huang, J., Enomoto, K., Kataoka, H., Hirota, A., and Ozoe, Y. (2006) Molecular cloning and heterologous expression of an α-adrenergic-like octopamine receptor from the silkworm *Bombyx mori*, *Insect Mol. Biol. 15*, 673−772.
6. Han, K.-A., Millar, N. S., and Davis, R. L. (1998) A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies, *J. Neurosci. 18*, 3650−3658.
7. Grohmann, L., Blenau, W., Erber, J., Ebert, P. R., Strünker, T., and Baumann, A. (2003) Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain, *J. Neurochem. 86*, 725−735.
8. Bischof, L. J., and Enan, E. E. (2004) Cloning, expression and functional analysis of an octopamine receptor from *Periplaneta americana*, *Insect Biochem. Mol. Biol. 34*, 511−521.
9. Kozak, M. (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells, *J. Mol. Biol. 196*, 947−950.
10. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
11. Munirathinam, G., and Yoburn, B. C. (1994) A simple procedure for assaying cAMP, *Pharmacol., Biochem. Behav. 48*, 813−816.
12. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J. Biol. Chem. 260*, 3440−3450.
13. Enomoto, K., Furuya, K., Yamagishi, S., and Maeno, T. (1992) Mechanically induced electrical and intracellular calcium responses in normal and cancerous mammary cells, *Cell Calcium 13*, 501−511.
14. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor, *Science 289*, 739−745.
15. Chatwin, H. M., Rudling, J. E., Patel, D., Reale, V., and Evans, P. D. (2003) Site-directed mutagenesis studies on the *Drosophila* octopamine/tyramine receptor, *Insect Biochem. Mol. Biol. 33*, 173−184.
16. Alexander, S. P. H., Mathie, A., and Peters, J. A. (2006) Guide to receptors and channels, 2nd edition, *Br. J. Pharmacol. 147* (Suppl. 3), S12.
17. Gorbunova, Y. V., and Spitzer, N. C. (2002) Dynamic interactions of cyclic AMP transients and spontaneous Ca^{2+} spikes, *Nature 418*, 93−96.
18. Dyachok, O., Isakov, Y., Sågetorp, J., and Tengholm, A. (2006) Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells, *Nature 439*, 349−352.
19. Liu, G., Badeau, R. M., Tanimura, A., and Talamo, B. R. (2006) Odorant receptors directly activate phospholipase C/inositol-1,4,5-trisphosphate coupled to calcium influx in Odora cells, *J. Neurochem. 96*, 1591−1605.
20. Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S., and Dixon, R. A. F. (1988) Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function, *J. Biol. Chem. 263*, 10267−10271.
21. Wang, C.-D., Buck, M. A., and Fraser, C. M. (1991) Site-directed mutagenesis of α_{2A}-adrenergic receptors: Identification of amino acids involved in ligand binding and receptor activation by agonists, *Mol. Pharmacol. 40*, 168−179.
22. Mansour, A., Meng, F., Meador-Woodruff, J. H., Taylor, L. P., Civelli, O., and Akil, H. (1992) Site-directed mutagenesis of the human dopamine D_2 receptor, *Eur. J. Pharmacol. 227*, 205−214.
23. Ohta, H., Utsumi, T., and Ozoe, Y. (2004) Amino acid residues involved in interaction with tyramine in the *Bombyx mori* tyramine receptor, *Insect Mol. Biol. 13*, 531−538.
24. Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S., and Dixon, R. A. F. (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor, *J. Biol. Chem. 264*, 13572−13578.
25. Sato, T., Kobayashi, H., Nagao, T., and Kurose, H. (1999) Ser^{203} as well as Ser^{204} and Ser^{207} in fifth transmembrane domain of the human β_2-adrenoceptor contributes to agonist binding and receptor activation, *Br. J. Pharmacol. 128*, 272−274.
26. Liapakis, G., Ballesteros, J. A., Papachristou, S., Chan, W. C., Chen, X., and Javitch, J. A. (2000) The forgotten serine. A critical role for Ser-$203^{5.42}$ in ligand binding to and activation of the β_2-adrenergic receptor, *J. Biol. Chem. 275*, 37779−37788.
27. Nyrönen, T., Pihlavisto, M., Peltonen, J. M., Hoffrén, A.-M., Varis, M., Salminen, T., Wurster, S., Marjamäki, A., Kanerva, L., Katainen, E., Laaksonen, L., Savola, J.-M., Scheinin, M., and Johnson, M. S. (2001) Molecular mechanism for agonist-promoted α_{2A}-adrenoceptor activation by norepinephrine and epinephrine, *Mol. Pharmacol. 59*, 1343−1354.
28. Freddolino, P. L., Kalani, M. Y. S., Vaidehi, N., Floriano, W. B., Hall, S. E., Trabanino, R. J., Kam, V. W. T., and Goddard, W. A., III (2004) Predicted 3D structure for the human β2 adrenergic receptor and its binding site for agonists and antagonists, *Proc. Natl. Acad. Sci. U.S.A. 101*, 2736−2741.
29. Wieland, K., Zuurmond, H. M., Krasel, C., Ijzerman, A. P., and Lohse, M. J. (1996) Involvement of Asn-293 in stereospecific agonist recognition and in activation of the β_2-adrenergic receptor, *Proc. Natl. Acad. Sci. U.S.A. 93*, 9276−9281.
30. Zuurmond, H. M., Hessling, J., Blüml, K., Lohse, M., and Ijzerman, A. P. (1999) Study of interaction between agonists and Asn293 in helix VI of human β_2-adrenergic receptor, *Mol. Pharmacol. 56*, 909−916.
31. Hwa, J., Graham, R. M., and Perez, D. M. (1995) Identification of critical determinants of α_1-adrenergic receptor subtype selective agonist binding, *J. Biol. Chem. 270*, 23189−23195.

32. Hwa, J., Graham, R. M., and Perez, D. M. (1996) Chimeras of α_1-adrenergic receptor subtypes identify critical residues that modulate active state isomerization, *J. Biol. Chem. 271*, 7956−7964.
33. Krumins, A. M., and Barber, R. (1997) The stability of the agonist β_2-adrenergic receptor-G_s complex: Evidence for agonist-specific states, *Mol. Pharmacol. 52*, 144−154.
34. Leff, P., Scaramellini, C., Law, C., and McKechnie, K. (1997) A three-state receptor model of agonist action, *Trends Pharmacol. Sci. 18*, 355−362.
35. Hall, R. A., Premont, R. T., and Lefkowitz, R. J. (1999) Heptahelical receptor signaling: Beyond the G protein paradigm, *J. Cell Biol. 145*, 927−932.
36. Kenakin, T. (2001) Inverse, protean, and ligand-selective agonism: Matters of receptor conformation, *FASEB J. 15*, 598−611.
37. Christopoulos, A., and Kenakin, T. (2002) G protein-coupled receptor allosterism and complexing, *Pharmacol. Rev. 54*, 323−374.
38. Voet, D., and Voet, J. G. (1995) *Biochemistry*, 2nd ed., John Wiley and Sons, Inc., New York, p 176.
39. Gether, U., and Kobilka, B. K. (1998) G protein-coupled receptors. II. Mechanism of agonist activation, *J. Biol. Chem. 273*, 17979−17982.
40. Rios, C. D., Jordan, B. A., Gomes, I., and Devi, L. A. (2001) G-Protein-coupled receptor dimerization: Modulation of receptor function, *Pharmacol. Ther. 92*, 71−87.
41. Ng, G. Y. K., O'Dowd, B. F., Lee, S. P., Chung, H. T., Brann, M. R., Seeman, P., and George, S. R. (1996) Dopamine D2 receptor dimers and receptor-blocking peptides, *Biochem. Biophys. Res. Commun. 227*, 200−204.
42. Hebert, T. E., Moffett, S., Morello, J.-P., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation, *J. Biol. Chem. 271*, 16384−16392.
43. Hermans, E. (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors, *Pharmacol. Ther. 99*, 25−44.

BI602593T