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Commentary

(Special Topic)

Bombyx mori phenolamine receptors: a comparative molecular biological study

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The cDNAs coding for two biogenic amine receptors were cloned from the silkworm *Bombyx mori* and stably expressed in HEK-293 cells; these receptors were identified as an α-adrenergic-like octopamine (OA) receptor, which is linked to elevations of intracellular Ca^{2+} and cAMP levels, and a tyramine (TA) receptor, which is linked to an attenuation of intracellular cAMP production. The respective amino acid residues of OA and TA receptors involved in interactions with OA and TA were identified by functional assays of mutant receptors generated by site-directed mutagenesis. An activation mechanism of the OA receptor was proposed based on the assumption of three conformational states. © Pesticide Science Society of Japan

Keywords: octopamine, tyramine, receptor, biogenic amines, *Bombyx mori*.

Introduction

Octopamine (OA) (Fig. 1A) is a biogenic amine widely distributed in the tissue of insects and other invertebrates. This amine is regarded as an invertebrate counterpart of the vertebrate biogenic amines noradrenaline and adrenaline.[1] OA plays an important role as a neuromodulator, neurohormone, or neurotransmitter, and has been implicated in a variety of important physiological processes, such as endocrine secretion, sensory function, fight and flight, rhythmic behavior, learning and memory, social behavior, egg laying, and light production. OA receptors, membrane proteins that mediate OA-evoked signal transduction, received much attention as major sites for the action of formamidine insecticides prior to the characterization of OA receptors at the molecular level.[2] Now that it has become possible to clone receptors, it would be useful to reevaluate OA receptors as potential targets for insect pest control agents. Several years ago, we undertook studies to isolate OA receptors to understand their physiological and pharmacological importance at the molecular level. Here we review our recent progress on the characterization of receptors for two biogenic phenolamines, OA and tyramine (TA).

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Tyramine Receptor

When we set out to isolate OA receptors from the silkworm *Bombyx mori*, we noticed that the sequence of a gene named B96Bom existed in the GenBank database. This gene was annotated as coding for a *B. mori* OA receptor without functional characterization; therefore, this gene was first isolated to examine whether it indeed encodes an OA receptor.[3] We followed the usual PCR cloning protocols, using mRNA extracted from the heads of silkworm larvae. Sequencing of B96Bom showed that this gene encodes a typical seven-transmembrane G-protein-coupled receptor (GPCR) with a unique posttranslational modification site.[4]

The RT-PCR product of B96Bom was inserted into the expression vector pcDNA3 and transfected into HEK-293 cells. Cells stably expressing B96Bom were selected in the presence of the antibiotic G418. For functional analysis, we examined the levels of the intracellular second messenger cAMP and the ligand-binding ability of the selected cells. Functional assays demonstrated that OA acted on the B96Bom receptor to decrease forskolin-stimulated intracellular cAMP levels but not to increase basal cAMP levels; however, a similar effect was observed with TA (Fig. 1A), an OA synthesis precursor, at concentrations two to three orders of magnitude lower than with OA, and this attenuating effect was abolished by the addition of yohimbine, which is known as a TA receptor antagonist. Also, [^{3}H]TA specifically bound to cell membranes with high affinity, and OA and dopamine showed three orders of

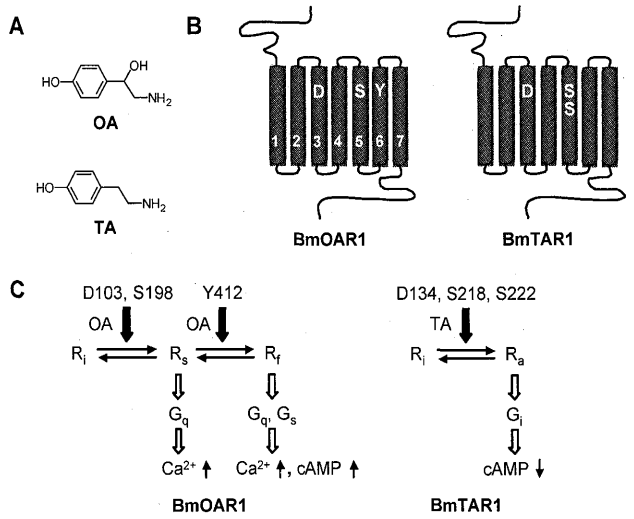

Fig. 1. Structures of OA and TA (A), agonist-interacting amino acids in TMs 3, 5, and 6 (B), and signal transduction pathways (C) of BmOAR1 and BmTAR1 receptors. R_i, receptor in an inactive state; R_s, receptor in a semi-active state; R_f, receptor in a fully active state; R_a, receptor in an active state.

magnitude lower affinity than TA.[3,5] Demethylchlordimeform (DMCDM), an active metabolite of the insecticide chlordimeform (CDM), which is also known as an OA receptor agonist, did not exert a significant effect on [^{3}H]TA binding and cAMP production in the B96Bom receptor.[6] These findings indicated that the B96Bom receptor is not an OA receptor but a TA receptor coupled to a G_i protein, which negatively modulates adenylate cyclase. Thus, the gene was named BmTAR1 (*Bombyx mori* tyramine receptor 1) (Accession No. AB162828). TA is now viewed as a biologically important biogenic amine.[7]

Octopamine Receptor

As B96Bom proved not to be an OA receptor gene, we further sought to clone an OA receptor gene from the nerve tissue of silkworm larvae.[8] A cDNA fragment was amplified by RT-PCR using primers designed based on the known sequences of *Drosophila* and *Limnaea* OA receptors, and 3′ and 5′ end sequences were obtained by RACE. The obtained full-length cDNA was found to encode a protein that contains seven transmembrane domains (TMs) and conserved amino acids characteristic of biogenic amine receptors. The cDNA was cloned into pcDNA3, and transfected into HEK-293 cells to produce cell lines stably expressing the encoded protein.

In transfected cells, OA stimulated intracellular cAMP accumulation at concentrations above 1 μM, whereas TA, OA, and CDM elicited little effect. DMCDM induced cAMP production at a level similar to that induced by OA. [^{3}H]OA specifically bound to the membranes of transfected cells. When OA was applied to cells loaded with the Ca^{2+} indicator Fura 2-AM, OA also induced an increase in the ratio of Fura 2 fluorescence at 510 nm upon excitation at 340 and 380 nm, which was proportional to the increase in intracytoplasmic Ca^{2+} levels. These findings indicated that the receptor expressed in HEK-293 cells is an OA receptor that triggers both G_s and G_q protein activations, which stimulate adenylate cyclase and phospholipase C, respectively. Thus, the gene was named BmOAR1 (*Bombyx mori* octopamine receptor 1) (Accession No. AB255163).

Agonist Recognition and Receptor Activation

To investigate how OA and TA receptors recognize a minor difference (the presence or absence of a β-hydroxyl group) in the structure between OA and TA (Fig. 1A), we performed experiments to identify critical amino acid residues involved in OA recognition of the BmOAR1 receptor.[9] We constructed five mutants (D103A, S198A, S202A, Y412F, and S198A/S202A) with one or two mutations in conserved amino acid residues in TM 3, 5, or 6 (Table 1, Fig 1B) of the BmOAR1 receptor. The receptor variants were designed to carry an N-terminal HA tag for antibody recognition. HEK-293 cells stably expressing wild-type and mutant receptors were cloned in the presence of G418. Immunostaining of cells utilizing the HA tag showed that all the receptors were localized in the plasma membrane.

We first examined [^{3}H]OA binding to the membranes of cells expressing wild-type and mutant receptors. The wild type showed ≈75% specific-to-total binding. D103A, S198A, and S198A/S202A mutants lacked the binding ability, and the Y412F mutant resulted in reduced, but statistically significant levels of binding. However, the S202A mutant retained the binding ability. Second, we determined levels of OA-induced cAMP accumulation within cells. The wild type demonstrated a cAMP production of ≈13 pmol per 2×10^5 cells. D103A, S198A, Y412F, and S198A/S202A mutants evoked no or little cAMP accumulation, but the S202A mutant produced a significant level of increase in cAMP production. Third, we examined the effect of OA on intracellular Ca^{2+} levels. The wild type evoked a quick Ca^{2+} response. D103A, S198A, and S198A/S202A mutants lacked the ability to increase Ca^{2+} levels, whereas S202A and Y412F mutants retained this ability in the same manner as did the wild type. The notable finding in Ca^{2+} assays is that the Y412F mutant maintained the

Table 1. Alignment of amino acid sequences in TM 3, 5, and 6 regions of BmOAR1 and BmTAR1 receptors

Receptor	TM		Amino acid sequence[a]	
BmOAR1	3	96	C S V W L A V **D** V W M C T A S I L N L C A I S L	119
BmTAR1	3	127	C K M W L T C **D** I M C C T S S I L N L C A I A L	150
BmOAR1	5	191	D A G Y V V Y **S** A L G S F Y I P M F V M L F F Y W R	216
BmTAR1	5	211	Q P G F V I F **S** S S G **S** F Y I P L V I M T V V Y F E	236
BmOAR1	6	394	L G I I V G G F V F C W L P F F S V **Y** V V R A F C	418
BmTAR1	6	408	L G I I M G V F V V C W L P F F V I Y L V I P F C	432

[a] Agonist-interacting amino acids are marked in bold.

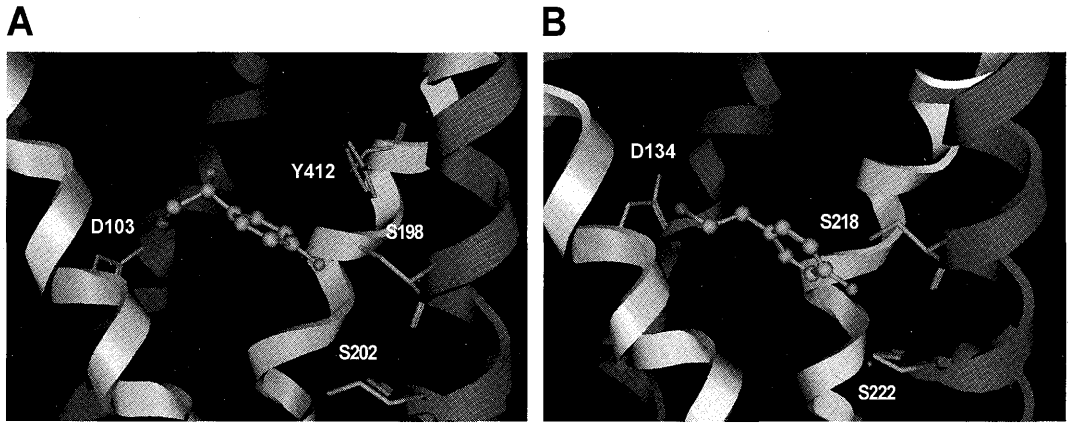

Fig. 2. Docking of OA into the BmOAR1 receptor (A) and TA into the BmTAR1 receptor (B). Homology models were constructed based on the crystal structure of bovine rhodopsin, using MOE® (Chemical Computing Group). TM 4 is omitted from both displays for clarity.

full ability to increase Ca^{2+} levels. This is in marked contrast to the case of the cAMP response of this mutant.

An OA molecule was manually docked into the putative binding site of a homology model of the BmOAR1 receptor according to the findings from site-directed mutagenesis experiments (Fig. 2A). In this docking model, Asp 103 and Ser 198 were observed in places where the amino group and the *para*-hydroxyl group of OA are capable of forming ionic interaction and hydrogen bonding with these amino acids, respectively; however, Ser 202 and Tyr 412 were likely too distant to interact with OA. Taken together with the finding that the S202A mutant retained all functional abilities, this indicates that Ser 202 is not involved in the interaction with OA. By contrast, Tyr 412 is promiscuous. Mutation of this tyrosine to phenylalanine led to loss of the ability to increase cAMP levels, but retention of the ability to bind OA and to increase Ca^{2+} levels. These results led us to reason that OA first interacts with Asp 103 and Ser 198, and that thereby conformation changes of the BmOAR1 receptor are induced in such a manner that the *para*-hydroxyl group of this tyrosine comes close to the β-hydroxyl group of OA to form a hydrogen bond.

On the basis of the above results, we hypothesize that the BmORA1 receptor adopts at least three states of conformation (Fig. 1C). The receptor in the inactive state would be converted to the semi-active state *via* the interaction of Asp 103 and Ser 198 with OA, and transition of the semi-active state to the fully active state would require additional interaction between Tyr 412 and the β-hydroxyl group of OA. The semi-active state would lead to increased Ca^{2+} levels *via* G_q protein activation, and the fully active state would lead to increases in both Ca^{2+} and cAMP levels *via* G_q and G_s protein activation. Our data support a multistep process reported in vertebrate GPCRs.[10] In a previous study,[11] several mutants of the BmTAR1 receptor were expressed in HEK-293 cells and examined for their ability to bind TA and to attenuate forskolin-stimulated cAMP production in response to TA. The results of the study indicated that Asp 134 interacts with the amino group of TA, and that both Ser 218 and Ser 222 form hydrogen bonds with the *para*-hydroxyl group of TA, as supported by a docking model (Fig. 2B). The difference of agonist-interacting amino acids between BmOAR1 and BmTAR1 receptors might be considered as one of the reasons why these receptors transduce signals by different pathways (Table 1, Fig. 1B, C); however, the mechanism by which these receptors discriminate their own agonists from other similar biogenic amines remains to be elucidated.

In *Drosophila*, four OA/TA receptor family genes have been identified to date.[12,13] We worked on *B. mori* receptors, and cloned an α-adrenergic-like OA receptor, which is linked to elevations of intracellular Ca^{2+} and cAMP levels, and a TA receptor, which is linked to an attenuation of intracellular cAMP production. We also identified the respective amino acid residues of OA and TA receptors involved in interactions with OA and TA for the first time. Detailed pharmacological analysis of BmOAR1 and BmTAR1 receptors is in progress. The physiological implications of each signaling pathway remain to be studied in the future. The assay systems presented in this study might prove useful for screening novel insect bioregulators.

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