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Molecular cloning and heterologous expression of an α-adrenergic-like octopamine receptor from the silkworm *Bombyx mori*

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Abstract

A cDNA encoding an octopamine (OA) receptor (BmOAR1) was isolated from the nerve tissue of silkworm (*Bombyx mori*) larvae. Comparison of amino acid sequences showed that BmOAR1 is highly identical to OA receptors isolated from *Periplaneta americana* (Pa oa$_1$), *Apis mellifera* (AmOA1), and *Drosophila melanogaster* (OAMB or DmOA1A). BmOAR1 was stably expressed in HEK-293 cells. OA above 1 μM led to an increase in intracellular cyclic AMP concentration ([cAMP]$_i$). The synthetic OA-receptor agonist demethylchlordimeform also elevated [cAMP]$_i$ to the same maximal level (≈ 5-fold over the basal level) as that induced by OA. However, other biogenic amines, tyramine and dopamine, and chlordimeform were without effects. The [cAMP]$_i$ level raised by OA was lowered by antagonists; the rank order of antagonist activity was chlorpromazine > mianserin = yohimbine. Cyproheptadine and metoclopramide had little effect. OA above 100 nM induced a transient or sustained increase in intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$), depending on the concentration of OA. Sequence homology and functional analysis data indicate that BmOAR1 is an α-adrenergic-like OA receptor of *B. mori*.

Keywords: *Bombyx mori*, biogenic amine, octopamine, tyramine, G protein-coupled receptor.

Introduction

Biogenic amines are important chemical messengers that function as neurotransmitters, neuromodulators and neurohormones in both central and peripheral tissues of animals. D-Octopamine (OA) and tyramine (TA) are phenolic biogenic amines that are synthesized from the amino acid L-tyrosine (Starratt & Bodnaryk, 1981). They only differ from the corresponding catecholamine neurotransmitters noradrenaline and dopamine (DA) by the absence of a *meta*-hydroxyl group. Phenolic amines are found in high quantities in the nerve tissues and haemolymphs of insects (Nagao & Tanimura, 1988; Takeda *et al.*, 1991; Linn *et al.*, 1994) and other invertebrates. Phenolic amines are detected in mammals as well, but their levels in the brain are several hundred-fold lower than those of noradrenaline, DA, or serotonin; hence, they are referred to as trace amines (Berry, 2004).

OA has been implicated in numerous physiological processes in invertebrates, including feeding, flight, reproduction, learning and memory, and so on (Roeder, 1999). According to the classification proposed by Evans (1981), the two major types of OA receptors are thought to mediate the action of OA to exert various physiological effects. The activation of the type 1 OA receptor leads to the activation of phospholipase C via the G_q protein, inducing the generation of the intracellular second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol followed by the release of Ca^{2+} and the activation of protein kinase C (Blenau & Baumann, 2001). The activation of the type 2 OA receptor stimulates adenylate cyclase via the G_s protein, thereby leading to an elevation in the intracellular levels of the second messenger cyclic AMP (cAMP) in cells. Recently,

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a new classification scheme for OA receptors has been proposed on the basis of information from the newly cloned *Drosophila* OA receptors (Evans & Maqueira, 2005); in this scheme the phenolic biogenic amine receptors are classified into α-adrenergic-like and β-adrenergic-like OA receptors, and OA/TA receptors. Activation of α-adrenergic-like OA receptors leads to increases in both intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular cAMP concentration ($[cAMP]_i$), whereas activation of β-adrenergic-like OA receptors induces an increase in $[cAMP]_i$ but not $[Ca^{2+}]_i$.

The physiological role of TA is less understood than that of OA. While TA is a synthetic precursor of OA, several lines of evidence have indicated that TA is also involved in a variety of physiological processes, including carbohydrate metabolism, muscle contraction, locomotion, excretion, reproduction, oviposition, olfaction and behavioural sensitization in insects (Downer, 1979; Huddart & Oldfield, 1982; McClung & Hirsh, 1999; Kutsukake *et al*., 2000; Nagaya *et al*., 2002; Sasaki & Nagao, 2002; Blumenthal, 2003; Donini & Lange, 2004; Saraswati *et al*., 2004). Most of the cloned insect TA receptors, although categorized as OA/TA receptors in the newly proposed classification, are negatively coupled to adenylate cyclase via the G_i protein to reduce $[cAMP]_i$ (Arakawa *et al*., 1990; Saudou *et al*., 1990; Vanden Broeck *et al*., 1995; Blenau *et al*., 2000; Ohta *et al*., 2003). No information is available about cAMP production by the recently identified family of TA receptors (Cazzamali *et al*., 2005).

It has been reported that OA and TA demonstrate opposite biochemical and physiological effects (Downer, 1979; Kutsukake *et al*., 2000; Aoyama *et al*., 2001; Nagaya *et al*., 2002; Saraswati *et al*., 2004), although these two amines only differ by the presence or absence of a hydroxyl group at the β-position. It is of interest to study the mechanisms of the molecular recognition of the OA and TA receptors that lead to opposite signal transduction pathways and physiological effects. Such studies would facilitate our understanding of the physiological processes that are mediated by the two amines and amine receptors. We have recently cloned a cDNA encoding a TA receptor of the silkworm *Bombyx mori* (BmTAR1), and studied the functional and pharmacological properties of the TA receptor (Ohta *et al*., 2003, 2004, 2005). We here report the cloning of a cDNA encoding an α-adrenergic-like OA receptor from *B. mori* (BmOAR1) and the functional characteristics of BmOAR1 stably expressed in HEK-293 cells.

Results

Cloning of a cDNA encoding a silkworm octopamine receptor

To obtain a full-length cDNA encoding a *B. mori* OA receptor (termed BmOAR1), a 120 bp cDNA fragment was first amplified by PCR using single-stranded cDNA synthesized from poly$(A)^+$ RNA extracted from the head of *B. mori* larvae and oligonucleotide primers based on the conserved sequence in the transmembrane domains (TMs) VI and VII of *Drosophila melanogaster* (OAMB) and *Lymnaea stagnalis* (Lym oa_1) OA receptors. 5′- and 3′ RACE (rapid amplification of cDNA ends) reactions based on the obtained sequences were carried out to determine the full-length sequence of BmOAR1, using cDNAs prepared from the head or central nervous tissue of *B. mori*. Direct sequencing of the full-length PCR product revealed that the product contained a 1521 bp open-reading frame encoding 507 amino acids (Fig. 1). The sequence data have been deposited into the DDBJ under accession no. AB255163. A BLAST search of the genomic database for *B. mori* (KAIKObase, http://sgp.dna.affrc.go.jp/KAIKO/) using this sequence revealed that the sequence is found in nine contigs (Fig. 2).

Overall sequence identities of BmOAR1 with the OA receptors of other insect species are: 59% for *Periplaneta americana* Pa oa_1 (accession no. AY333178), 56% for *Apis mellifera* AmOA1 (accession no. AJ547798), 50% for *D. melanogaster* OAMB (DmOA1A) (accession no. AF065443), 42% for *L. stagnalis* Lym oa_1 (accession no. U62771), 33% for *Aplysia californica* Ap oa_1 (accession no. AF222978), and 31% for *D. melanogaster* DmOA2 (accession no. AJ617526). Meanwhile, overall sequence identities with other biogenic amine receptors are: 37% for the *A. mellifera* TA receptor (accession no. AJ245824), 36% for the *Locusta migratoria* TA receptor (accession no. X69520), 35% for the *B. mori* TA receptor BmTAR1 (accession no. AB162828), 35% for the *B. mori* serotonin receptor (accession no. X95604), and 32% for the *D. melanogaster* TA receptor (accession no. AB073914).

A hydropathy plot of the deduced amino acid sequence predicted seven TMs connected by intracellular and extracellular loops. BmOAR1 has a relatively large loop connecting TM IV and TM V. Amino acids that are predicted to be involved in agonist binding in adrenergic receptors are conserved in BmOAR1, e.g. Ser-80 in TM II to form a hydrogen bond with the β-hydroxyl group of OA, Asp-103 in TM III to form an ion-pair with the protonated amino group of OA, Ser-198 and Ser-202 in TM V to form a hydrogen bond with the *p*-hydroxyl group of OA, and Phe-409 in TM VI to form a π–π interaction with the phenyl group of OA (Strader *et al*., 1988, 1989a,b; Li *et al*., 1995; Hieble *et al*., 1998).

Expression of BmOAR1 in HEK-293 cells

BmOAR1 was cloned into the expression vector pcDNA3 to produce the recombinant pcDNA3-BmOAR1, which was then transfected into HEK-293 cells. The expression of BmOAR1 was confirmed by RT–PCR using total RNA extracted from cells after a 3-week selection with the antibiotic G 418. A PCR product of the expected size (≈ 1.5 bp) was detected in the case of BmOAR1-transfected HEK-293 cells but not in the case of nontransfected HEK-293 cells (Fig. 3).

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                                                                    5′ GGTGCTGGTGCAA
AATGTGTCTGGTGCGGTGACTCGTAGCGGGCGATGCGACGCGCGGCGAGACGCGCGCTGCGGCGGGCGAGGCGCGTAAT
ATG CGC TCG CTG AAC GAG TCG GCC TGT GAG GCG TTG CTC GAG GAC GTG CGC TGG GAC GAG   60
 M  R  S  L  N  E  S  A  C  E  A  L  L  E  D  V  R  W  D  E
CCC ACC AGC CTC GTG AGC CTC GCC GTG CTC GCG CTC ATC GAC GTA CTC GTG ATT GCC GGT  120
 P  T  S  L  V  S  L  A  V  L  A  L  I  D  V  L  V  I  A  G
AAC TGT CTC GTT ATC GCC GCT GTG CTC TGC TCG TCG AAG CTA CGC AGC GTC ACG AAT CTG  180
 N  C  L  V  I  A  A  V  L  C  S  S  K  L  R  S  V  T  N  L
TTT ATC GTG TCG CTA GCC GTC GCG GAC TTA CTG GTG GGA GTC GCT GTA CTG CCG TTC TCT  240
 F  I  V  S  L  A  V  A  D  L  L  V  G  V  A  V  L  P  F  (S)
GCG ACG CGG GAA GTA TTT GAG GTC TGG ATC TTC GGC GAC GTG TGG TGC TCA GTA TGG CTA  300
 A  T  R  E  V  F  E  V  W  I  F  G  D  V  W  C  S  V  W  L
GCC GTG GAC GTG TGG ATG TGC ACC GCC TCT ATT CTC AAT CTC TGC GCA ATA TCC CTG GAC  360
 A  V  (D) V  W  M  C  T  A  S  I  L  N  L  C  A  I  S  L  D
CGA TAC GTG GCT GTC ACC CGT CCT GTC AGC TAC CCG AGT ACC ATG AGC AGG AAG CGG GCT  420
 R  Y  V  A  V  T  R  P  V  S  Y  P  S  T  M  S  R  K  R  A
AAA GCG TTA ATA GCC GGT CTA TGG GTG CTC TCT TTC GTA ATT TGT TTC CCG CCA CTG GTA  480
 K  A  L  I  A  G  L  W  V  L  S  F  V  I  C  F  P  P  L  V
GGA TGG AAG GAT AAA AGG GAT GAT GAC GGA ACC CAT AAA GAA GGC TGG GCT CCC AAT CCT  540
 G  W  K  D  K  R  D  D  D  G  T  H  K  E  G  W  A  P  N  P
CCG TGC CAG TGG ACC TGC GAG TTG ACC AAT GAT GCT GGG TAC GTT GTC TAT TCA GCT TTG  600
 P  C  Q  W  T  C  E  L  T  N  D  A  G  Y  V  V  Y  (S) A  L
GGC TCC TTC TAC ATC CCG ATG TTC GTG ATG CTA TTC TTC TAT TGG AGG ATC TAC AAA GCT  660
 G  (S) F  Y  I  P  M  F  V  M  L  F  F  Y  W  R  I  Y  K  A
GCC GTT AGA ACC ACC AAG GCA ATT AAT CAA GGC TTT AGA ACT ACT AAA GGT AGA GGG CTG  720
 A  V  R  T  T  K  A  I  N  Q  G  F  R  T  T  K  G  R  G  L
GGC AGT CGT TTC GAT GAC AAC CGT CTC ACG TTG CGA ATA CAT CGC GGC AGA GGA TCC AAT  780
 G  S  R  F  D  D  N  R  L  T  L  R  I  H  R  G  R  G  S  N
CGC CCT CAC GGC TCA CCT CTG TCA ACT GCC TCT AAT CAC TCC ACA AGC ACG TCG CTG AGC  840
 R  P  H  G  S  P  L  S  T  A  S  N  H  S  T  S  T  S  L  S
GCC TCG CCA GAA CGA CTT AGA AGG CAC TCA AGC GCT CGC CGG GCC CAC GAG AAA GTC AAA  900
 A  S  P  E  R  L  R  R  H  S  S  A  R  R  A  H  E  K  V  K
ATA TCC GTC TCT TAT CCG TCA AGG GAA CAA ATC TGT CCG GCC CAC GAG AAT TCT CGG TCG  960
 I  S  V  S  Y  P  S  T  E  Q  I  C  P  A  H  E  N  S  R  S
CCC AGC CGA TCT CCA AGT CCT TCT CTG TAC GCC GTT CAT TAC GAG AGA GAC GGA AGG GAA 1020
 P  S  R  S  P  S  P  S  L  Y  A  V  H  Y  E  R  D  G  R  E
CTG ACT GAG AGC AGA CTG AGG GTC AGA CCT TCG CAT CAT TTA CAT CCC GGA CCG CTG TAC 1080
 L  T  E  S  R  L  R  V  R  P  S  H  H  L  H  P  G  P  L  Y
GAC GAT TAT GAC GAT AAG CCA CGA ACT CGA CGC ATG GGG AAG AGG AAT ATC AAA GCA CAG 1140
 D  D  Y  D  D  K  P  R  T  R  R  M  G  K  R  N  I  K  A  Q
GTG AAG CGC TTT AAG ATG GAG ACC AAG GCA GCA AAA ACC CTG GGC ATC ATA GTC GGA GGC 1200
 V  K  R  F  K  M  E  T  K  A  A  K  T  L  G  I  I  V  G  G
TTC GTG TTC TGC TGG CTG CCA TTT TTC AGC GTC TAC GTG GTC CGG GCG TTC TGT GGC GAA 1260
 F  V  F  C  W  L  P  F  (F) S  V  Y  V  V  R  A  F  C  G  E
TGC GTT ACT CCC ATA GTC TTC TCC GTA CTA TTC TGG CTG GGA TAC TGC AAC TCT GCT ATA 1320
 C  V  T  P  I  V  F  S  V  L  F  W  L  G  Y  C  N  S  A  I
AAT CCA CTC ATT TAT GCT CTA TTC TCC AAA GAT TTT CGA TTC GCG TTC AAA CGT ATT ATC 1380
 N  P  L  I  Y  A  L  F  S  K  D  F  R  F  A  F  K  R  I  I
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 C  K  C  F  C  G  G  G  G  A  R  R  E  S  D  E  G  S  A  R
CGT CCC AAC CAC AGA CCA GTG CAC TCG CAT TCT TTG GAG GAG CAG GAA CCT AGT CAG ACT 1500
 R  P  N  H  R  P  V  H  S  H  S  L  E  E  Q  E  P  S  Q  T
ACT CTA ACT GCG GCA GAA AGG TGA CGTCAAACCATGCGACGGCAACAC 3′
 T  L  T  A  A  E  R  *
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Figure 1. Nucleotide and deduced amino acid sequence of BmOAR1 cloned for functional expression. Direct sequencing of the obtained cDNA revealed nucleotide differences that are likely due to genetic polymorphism: C/T at 273; C/T at 799; C/T at 1075; and A/C at 1320. Nucleotides that differed from those in the genome database and the two consequently changed amino acids are underlined in the figure. Those are: T/C at 114; A/G at 210; C/T at 273; C/T (Thr/Ile) at 401; G/A at 426; A/G at 474; C/T at 723; C/T at 783; A/G (Thr/Ala) at 805; T/G at 813; G/A at 831; A/T at 975; C/T at 993; G/C at 1038; C/T at 1075; G/A at 1167; A/C at 1320; A/G at 1341; G/A at 1365; G/C at 1401; C/T at 1422; C/T at 1425; C/T at 1431; and G/A at 1467. The proposed TMs are indicated by dotted underlines. The amino acids that are predicted to be involved in agonist binding are circled.

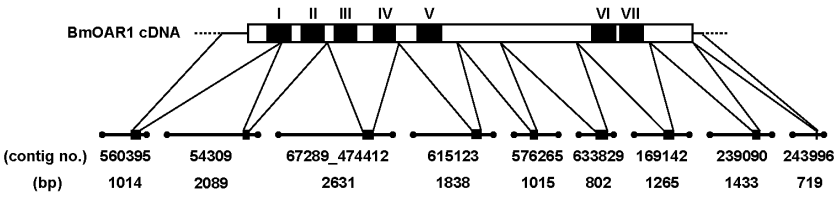

Figure 2. Correspondence of the sequence of BmOAR1 cDNA and the contig sequence of the *B. mori* genome. The TMs are indicated as black boxes I–VII.

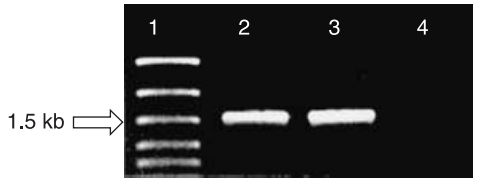

Figure 3. RT–PCR detection of transcripts from BmOAR1-HEK cells. Line 1: 100 bp DNA ladder marker; lane 2: BmOAR1-HEK cells; lane 3: pcDNA3-BmOAR1 (positive control); lane 4: Nontransfected HEK-293 cells (negative control). The primers AOF-2 and AOR-2 were used to amplify BmOAR1.

Effects of biogenic amines, agonists and antagonists on $[cAMP]_i$ in HEK-293 cells stably expressing BmOAR1

We first performed functional assays to see whether OA and related amines induce changes in $[cAMP]_i$ in HEK-293 cells stably expressing BmOAR1 (BmOAR1-HEK cells). OA above 1 μM elicited a dose-dependent increase in $[cAMP]_i$ in BmOAR1-HEK cells, whereas neither TA nor DA caused a significant increase (Fig. 4A). The $[cAMP]_i$ was ≈ 5-fold elevated over the basal level by 100 μM OA. The cAMP production reached the maximum level within 3 min after the addition of OA when examined with 100 μM OA, and remained at this elevated level for at least 40 min (Fig. 4B). Demethylchlordimeform (DMCDM), known as a potent synthetic agonist of OA receptors (Nathanson & Hunnicutt, 1981), also elevated $[cAMP]_i$ in a dose-dependent manner to the same maximal level as that demonstrated by OA, whereas chlordimeform (CDM), a weak agonist/antagonist (Nathanson & Hunnicutt, 1981), did not have such effects. No elevation in $[cAMP]_i$ in response to agonists was observed in nontransfected HEK-293 cells. These findings indicate that the interaction of OA or OA-receptor agonists with BmOAR1 leads to a significant activation of adenylate cyclase.

The effects of five antagonists of biogenic amine receptors on the OA-induced increase in $[cAMP]_i$ in BmOAR1-HEK cells were examined in order to characterize BmOAR1 pharmacologically. The $[cAMP]_i$ elevated by 100 μM OA was significantly reduced by 10 μM chlorpromazine, but not by metoclopramide and cyproheptadine (Fig. 5). Metoclopramide *per se* appeared to have a weak agonist effect rather than an antagonist effect (data not shown). Mianserin and yohimbine weakly antagonized the action of OA.

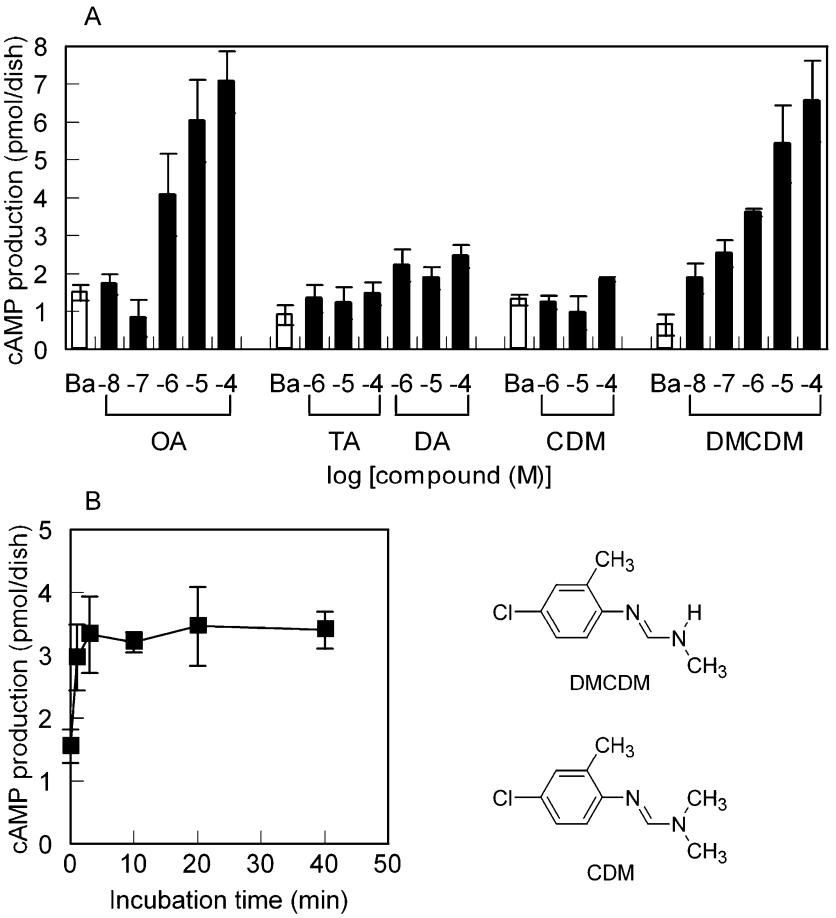

Figure 4. Effects of ligands on $[cAMP]_i$ in BmOAR1-HEK cells. (A) Effects of biogenic amines and synthetic compounds. Ba represents basal levels. (B) Effects of incubation period on OA-induced cAMP production. Basal $[cAMP]_i$ levels in HEK-293 cells were 0.74 ± 0.32 pmol/dish. Error bars indicate SEM.

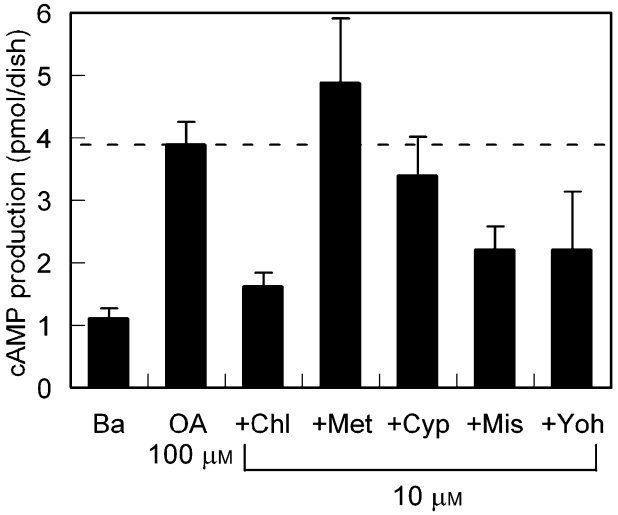

Figure 5. Effects of antagonists on OA-induced cAMP production. Ba indicates basal levels. Chl, Chlorpromazine; Met, metoclopramide; Cyp, cyproheptadine; Mis, mianserin; Yoh, yohimbine. Error bars indicate SEM.

Effects of OA on $[Ca^{2+}]_i$ in BmOAR1-HEK cells

We examined whether OA induces a change in $[Ca^{2+}]_i$ in BmOAR1-HEK cells, using Oregon Green 488 BAPTA-5N, a fluorescent $[Ca^{2+}]_i$ indicator. OA at 1 μM induced a sharp and sustained increase in $[Ca^{2+}]_i$, while OA at 300 nM produced a sharply transient increase in $[Ca^{2+}]_i$ followed by a lower but more sustained increase (Fig. 6). OA at 100 nM induced a delayed, transient increase in $[Ca^{2+}]_i$. No increase in $[Ca^{2+}]_i$ in response to 1 μM OA was observed in nontransfected HEK-293 cells.

Effects of biogenic amines, agonists, and antagonists on [^{3}H]yohimbine binding to BmOAR1

To examine whether BmOAR1 is a receptor specific for OA, we performed binding assays using 3 nM [^{3}H]OA and the membrane homogenates of BmOAR1-HEK cells. However, [^{3}H]OA showed only low levels of specific binding (≈ 5%). Therefore, we employed [^{3}H]yohimbine as an alternative radio-ligand. [^{3}H]Yohimbine displayed a high level of binding to the membranes of BmOAR1-HEK cells with a high specific-to-total binding ratio (0.87 ± 0.04 under standard assay conditions), while specific [^{3}H]yohimbine binding to the membranes of nontransfected HEK-293 cells was negligible (< 1% of that to the membranes of BmOAR1-HEK cells). We determined the abilities of biogenic amines, OA-receptor agonists, and antagonists to inhibit specific [^{3}H]yohimbine binding to BmOAR1. IC_{50} values for the inhibition of specific [^{3}H]yohimbine binding are listed in Table 1. Yohimbine was found to be the most potent inhibitor of [^{3}H]yohimbine binding with an IC_{50} of 25 nM. The rank order of potency of antagonists did not agree with that in cAMP assays. BmOAR1 did not show specificity for the agonist OA in the case of [^{3}H]yohimbine binding assays.

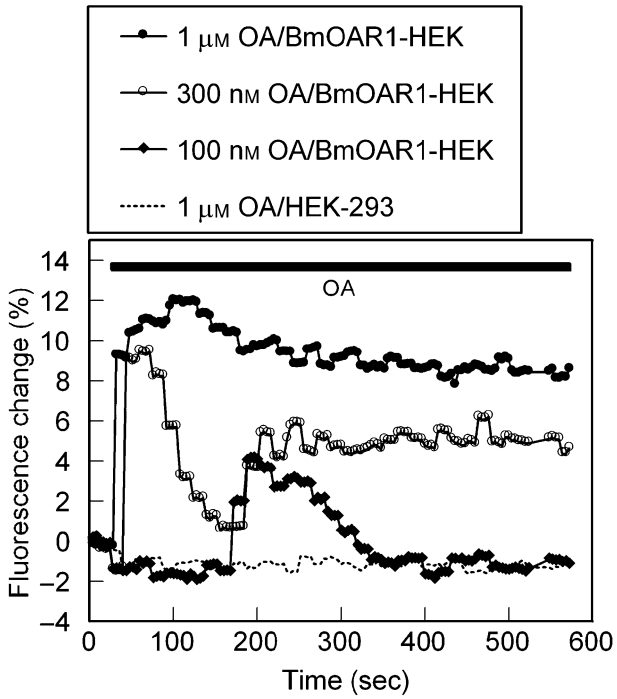

Figure 6. Effects of OA on $[Ca^{2+}]_i$ levels in BmOAR1-HEK cells. Changes in $[Ca^{2+}]_i$ are shown as the fractional change $\Delta F/F_b$, i.e. the change in fluorescent intensity ($\Delta F = F_b - F_t$) divided by the background fluorescent intensity (F_b), where F_t is the fluorescent intensity at t s. Data were collected every 4 s. The horizontal bar above the plots represents the duration of the OA application.

Table 1. Inhibition of [^{3}H]yohimbine binding to membranes of BmOAR1-HEK cells by agonists and antagonists

Agonist/antagonist	IC_{50} values (μM)	95% Confidence interval (μM)
OA	42.2	29.7–58.4
TA	58.3	41.9–79.9
DA	60.0	41.5–85.2
CDM	23.9	17.7–32.8
DMCDM	3.72	2.68–5.19
Chlorpromazine	1.07	0.788–1.46
Metoclopramide	24.6	17.4–34.5
Cyproheptadine	1.12	0.834–1.52
Mianserin	0.157	0.108–0.228
Yohimbine	0.0250	0.0178–0.0338

TA and DA had IC_{50} values comparable with that of OA (42.2 μM). DMCDM was found to be a more potent inhibitor (IC_{50} = 3.72 μM) than OA, and CDM also had inhibitory activity, although it was inactive in cAMP assays.

Discussion

In the current study, we cloned a cDNA encoding a *B. mori* OA receptor, BmOAR1, and stably expressed it in HEK-293 cells. We found that OA and DMCDM cause a several-fold

elevation in $[cAMP]_i$ in BmOAR1-HEK cells, whereas TA and DA had no significant effect. OA also induced an elevation of $[Ca^{2+}]_i$ in BmOAR1-HEK cells. The cloning of a cDNA encoding a *B. mori* OA receptor (B96Bom) was reported previously (von Nickisch-Rosenegk *et al*., 1996), but we have demonstrated that the B96Bom receptor is not an OA receptor, but a TA receptor (Ohta *et al*., 2003). Therefore, BmOAR1 is the first OA receptor cloned from *B. mori*.

To date, cDNAs encoding OA receptors have been isolated from the pond snail *L. stagnalis* (Gerhardt *et al*., 1997a,b), the fruit fly *D. melanogaster* (Han *et al*., 1998; Balfanz *et al*., 2005), the marine molluscs *A. californica* and *A. kurodai* (Chang *et al*. 2000), the honeybee *A. mellifera* (Grohmann *et al*., 2003) and the American cockroach *P. americana* (Bischof & Enan, 2004). Recently, three novel OA receptors (DmOct1β1R (or DmOA2), DmOct1β2R, and DmOct1β3R) have been identified from *D. melanogaster* (Balfanz *et al*., 2005; Maqueira *et al*., 2005). All these OA receptors were positively coupled to adenylate cyclase to raise $[cAMP]_i$ when heterologously expressed in cell lines, although the OA receptor of the tobacco budworm *Heliothis virescens* expressed in LLC-PK_1 cells was reported to reduce $[cAMP]_i$ in response to OA (von Nickisch-Rosenegk *et al*., 1996). An *L. stagnalis* OA receptor that couples to chloride channels in HEK-293 cells has also been reported (Gerhardt *et al*., 1997b).

As a variety of OA receptors have been identified, a new classification system has been proposed for insect OA receptors (Evans & Maqueira, 2005). In the new classification, insect OA receptors are classified into three classes on the basis of sequence similarities and in terms of similarities to adrenergic receptors in their signalling pathway: α-adrenergic-like, β-adrenergic-like and OA/TA receptors. α-Adrenergic-like OA receptors expressed in cell lines lead to elevations of both $[Ca^{2+}]_i$ and $[cAMP]_i$ in response to OA, while β-adrenergic-like OA receptors are selectively coupled to the production of cAMP in cells. A third class of a *Drosophila* receptor has been shown to lead to both attenuation of $[cAMP]_i$ and generation of a Ca^{2+} signal (Enan, 2005), probably in an agonist-specific manner in some cases (Robb *et al*., 1994; Reale *et al*., 1997). The *L. migratoria* TA receptor also mediated the same agonist-induced changes in second messenger levels (Poels *et al*., 2001), whereas this class of receptors from other insects and nematodes has been found to be coupled to the reduction of forskolin-stimulated $[cAMP]_i$ in response to TA (Blenau *et al*., 2000; Rex & Komuniecki, 2002; Ohta *et al*., 2003; Rex *et al*., 2004). According to this classification scheme, BmOAR1 cloned in the present study is categorized in the class of α-adrenergic-like receptors in terms of its sequence similarity to Pa oa_1, AmOA1, and OAMB, which are classified into this class, and its capability of elevating both $[Ca^{2+}]_i$ and $[cAMP]_i$. All these α-adrenergic-like receptors, when expressed in HEK-293 cells, induced an increase in $[cAMP]_i$ in response to OA above 1 μM. However, there is a difference in the levels of cAMP production. BmOAR1 led to a ≈ 5-fold elevation in $[cAMP]_i$ over basal levels in response to 100 μM OA, while Pa oa_1, AmOA1 and OAMB (DmOA1) led to ≈ 900-, ≈ 7- and ≈ 10 (≈ 2)-fold increases, respectively, at maximum OA concentrations tested. Clearly, Pa oa_1 causes an extraordinary increase compared with other receptors. We have also observed that DMCDM, which was shown to be a potent partial agonist of firefly OA receptors (Nathanson & Hunnicutt, 1981), acts as a full agonist of BmOAR1. CDM, a weak agonist/antagonist (Nathanson & Hunnicutt, 1981), showed no activity against BmOAR1. It would be interesting to examine the effects of these compounds on $[cAMP]_i$ in β-adrenergic-like OA receptors. BmOAR1 caused an elevation of $[Ca^{2+}]_i$ in response to OA above 100 nM. The elevation of $[Ca^{2+}]_i$ levels was observed at lower OA concentrations (10–50 nM) in the case of other α-adrenergic-like OA receptors expressed in HEK-293 cells (Grohmann *et al*., 2003; Bischof & Enan, 2004; Balfanz *et al*., 2005), although one group reported an increase in $[Ca]_i$ at 10 μM OA (Han *et al*., 1998). Particularly notable is the observation of Ca^{2+} oscillations in AmOA1 and DmOA1. More detailed experiments will be needed to elucidate whether or not the delayed $[Ca^{2+}]_i$ elevation observed in BmOAR1 is due to Ca^{2+} oscillation. It also remains to be determined whether Ca^{2+} comes from intracellular stores or extracellular media. In the case of DmOA1, the elevation of $[Ca^{2+}]_i$ was attributed to release from intracellular stores (Balfanz *et al*., 2005). Detailed analysis is also important to determine whether elevations of $[Ca^{2+}]_i$ and $[cAMP]_i$ occur independently, although our preliminary experiments using BAPTA-AM, an intracellular calcium chelator, suggested that the increase in $[cAMP]_i$ was independent of the increase in $[Ca^{2+}]_i$ (data not shown). A similar finding was previously reported in the case of Pa oa_1 expressed in HEK-293 cells (Bischof & Enan, 2004). All these issues will be addressed in a subsequent study.

We examined the effects of antagonists on an OA-induced elevation of $[cAMP]_i$ in BmOAR1-HEK cells. The rank order of activity reducing OA-induced $[cAMP]_i$ at 10 μM was: chlorpromazine > mianserin = yohimbine. Although at present not much is known about the pharmacology of cloned OA receptors, the high activity of chlorpromazine suggests that BmOAR1 belongs to the classical type 1 OA receptor family proposed on the basis of physiological and pharmacological studies at the tissue level. The classical type 3 OA receptor and *Drosophila* β-adrenergic-like OA receptor (DmOctβRs) families are characterized by a high potency of mianserin (Evans, 1981; Maqueira *et al*., 2005), and metoclopramide was reported to be the most potent antagonist in the type 2 OA receptor family (Evans, 1981). As pharmacological characterization of Pa oa_1 was attempted using [^{3}H]yohimbine binding assay, we also performed similar assays using the same radio-ligand. In binding assays, biogenic amines

including OA exhibited lower potencies than antagonists, and not only OA but other amines bound to BmOAR1. Although antagonists except metoclopramide showed high potencies, the rank order of potency was not in agreement with the rank order of antagonist effects on OA-induced cAMP production. Similar findings were reported in the case of Pa oa_1. More suitable radio-ligands than [^{3}H]yohimbine will be needed to study the structure–activity relationships of ligands acting on OA receptors in the future.

In conclusion, we have cloned an α-adrenergic-like OA receptor from *B. mori* and successfully expressed it in HEK-293 cells. This receptor, together with BmTAR1, should prove to be a useful tool for studies on positive and negative regulation mediated by biogenic amine receptors.

Experimental procedures

Reagents

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), OPTI-MEM® I reduced-serum medium, fetal bovine serum (FBS), Lipofectamine, and pcDNA3 were purchased from Invitrogen Corp. (Carlsbad, CA, USA). KOD-Plus DNA polymerase was from Toyobo, Co., Ltd (Osaka, Japan). A first-strand cDNA synthesis kit, an Oligotex-dT30 <Super> mRNA purification kit, a-3′-full RACE Core Set, and a 5′-full RACE Core Set were obtained from Takara Bio Inc. (Shiga, Japan). Isogen was from Nippon Gene, Co., Ltd (Tokyo, Japan). The Thermo Sequenase II dye terminator cycle sequencing premix kit and BigDye terminator V3.1/1.1 cycle sequencing kit were purchased from Amersham Bioscience K.K. (Tokyo, Japan). CDM and DMCDM were synthesized in our laboratory. DL-OA and DA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Oligo(dt) cellulose and theophylline were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). G 418, TA, yohimbine, and Cremophor EL were from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Metoclopramide, mianserin, and chlorpromazine were obtained from RBI Research Biochemicals, Inc. (Natick, MA, USA). Oregon Green 488 BAPTA-5N AM was from Molecular Probes, Inc. (Eugene, OR, USA). [^{3}H]cAMP (17 Ci/mmol) and [^{3}H]yohimbine (80.5 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA) and PerkinElmer Life Sciences, Inc. (Boston, MA, USA), respectively. Other general reagents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Insects

Eggs of *B. mori* (Kinshu-Showa), purchased from Ueda Silkworm-Eggs Coop (Ueda, Japan), were hatched and reared on an artificial diet at 25 °C.

Cloning of cDNA encoding BmOAR1

Total RNA was extracted with Isogen from 30 mg of the central nervous tissue (a mixture of brains, suboesophageal ganglia, and prothoracic ganglia) of 145 fourth-instar *B. mori* larvae, and mRNA was isolated with Oligotex-dT30 <Super> mRNA purification kit. Alternatively, mRNA was extracted from the head homogenates of ≈ 50 fifth-instar *B. mori* larvae, using oligo(dt) cellulose, as described previously (Badley *et al.*, 1988).

Single-stranded cDNA was synthesized from head mRNA using the first-strand cDNA synthesis kit and was used as the template of the first PCR in nested PCR. Each primer was designed from the nucleotide sequences of *Lymnaea* Lym oa_1 (Gerhardt *et al.*, 1997a) and *Drosophila* OAMB (Han *et al.*, 1998), encoding their TM VI to TM VII. The first PCR was performed using 5′-AGGCT-GCAAARACCCTAGCC-3′ as a forward primer and 5′-TTGCAG-TAGCCCAGCCAGAA-3′ as a reverse primer. The second PCR was performed using 5′-ACCCTAGCCATCATAGT(ACGT)GGC-3′ as a forward primer and 5′-CCAGCCAGAAGAGCA(CG)GA(AG)A-3′ as a reverse primer. As the PCR product (≈ 120 bp) was found to encode a region 80% homologous with the TM VI–VII region of *Drosophila* OAMB, 5′RACE reactions were carried out to amplify the 5′-end of the cDNA, using primers designed based on the sequence of the 120 bp product and the 5′-Full RACE Core Set. 3′RACE was carried out to amplify the 3′-end of the cDNA, using primers designed based on the sequence of the 5′RACE product and the 3′-Full RACE Core Set. Single-stranded cDNA for RACE was synthesized from head or central nervous tissue mRNA.

The open-reading frame of BmOAR1 cDNA, which was amplified by PCR using the DNA polymerase KOD-Plus, a single-stranded cDNA template synthesized from the central nervous tissue, a forward primer with a *Kpn*I site (5′-TTTTGGTACCATGCGCTCGCT-GAAC-3′) (AOF-2), and a reverse primer with a *Xba*I site (5′-AAAATCTAGATCACCTTTCTGCCGCAG-3′) (AOR-2), was ligated into the corresponding site of the mammalian expression vector pcDNA3 to produce pcDNA3-BmOAR1. The insertion was confirmed again by DNA sequencing.

DNA sequences were analysed with an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Norwalk, CT, USA). The sequencing reaction was performed using the Thermo Sequenase II dye terminator cycle sequencing premix kit or BigDye terminator V3.1/1.1 cycle sequencing kit. These kits were used according to vendors' protocols.

Stable expression of BmOAR1 in HEK-293 cells

HEK-293 cells were grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS. Cells (2×10^5/2 ml) were plated on 35-mm diameter dishes on day 1, the day before transfection (day 1). On day 2, the plated cells were transfected with pcDNA3-BmOAR1 (2 μg) along with 4 μl of Lipofectamine (2 mg/ml) in 1 ml of OPTI-MEM® I reduced-serum medium. After incubation for 5 h at 37 °C, the cells in the medium were re-fed by the addition of 1 ml of 20% FBS/DMEM and cultured. On day 3, the medium was replaced with fresh 10% FBS/DMEM. On days 4–5, the culture medium was replaced with 2 ml of 10% FBS/DMEM containing the antibiotic G 418 (1.0 mg/ml). The vector-integrated cells were selected in the presence of G 418 for ≈ 4 weeks, while the selection medium was renewed every 5 days.

RT–PCR using BmOAR1-HEK cells

Total RNA was extracted from nontransfected HEK-293 and BmOAR1-HEK cells according to the Isogen manual. Single-stranded cDNA was synthesized from RNA (10 μg) using the first-strand cDNA synthesis kit, and employed as a template for PCR using the primers AOF-2 and AOR-2.

Determination of $[cAMP]_i$ levels

Nontransfected HEK-293 and BmOAR1-HEK cells (2×10^5/2 ml) suspended in 10% FBS/DMEM were plated on 35-mm diameter

dishes 17–21 h before cAMP assays. Attached cells were washed once with 1 ml of DPBS and preincubated in 1.8 ml of DPBS containing 5 mM theophylline for 20 min at 37 °C. After the preincubation, a 200-μl aliquot of DPBS containing a compound (OA, TA, etc.) was added to the cells, and then the culture was incubated for 20 min at 37 °C. Yohimbine, metoclopramide, mianserin and chlorpromazine used in antagonist experiments were added along with OA. The reactions were stopped by aspiration of the medium followed by addition of 3 ml of ice-cold acidic ethanol (1 M HCl/ethanol = 1/100). 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 15 000 ***g*** for 5 min. The supernatant was evaporated to dryness by vacuum centrifuging for 1 h at 45 °C. The residue was suspended in 50 mM Tris–HCl buffer (pH 7.4) with 4 mM EDTA and centrifuged at 15 000 ***g*** for 5 min. The supernatant was used to determine $[cAMP]_i$ levels, which were quantified by the method of Munirathinam & Yoburn (1994). The radioactivity was measured in a POPOP/DPO/Methyl Cellosolve/toluene scintillation fluid by a Beckman LS 6000SE liquid-scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA) or a Packard Tri-Carb 2100TR liquid-scintillation analyser (PerkinElmer Life Sciences, Inc.). Each experiment was repeated three times or more.

Determination of $[Ca^{2+}]_i$ levels

Effects of OA on changes in $[Ca^{2+}]_i$ were examined. BmOAR1-HEK cells cultured on coverslips were loaded with a membrane-permeant acetoxymethyl ester of Oregon Green 488 BAPTA-5N, at a final concentration of 1 μM by incubating together with 0.05–2% Cremophor EL for 30–45 min at 37 °C. After the loading, the coverslips with the cells were transferred to fresh DMEM containing 1 mM HEPES and set in the microscopic chamber. Laser-scan microscopy was used to observe the fluorescence of Oregon Green 488 BAPTA-5N with emission of 515 nm under irradiation of 488 nm with Argon laser. A fluorescence image was acquired using an image board (AG-5, Scion Corporation, Frederick, MD, USA) and analysed using MACRO commands of the image processor program 'Scion Image' (Scion Corporation). This method is suitable for evaluating changes in $[Ca^{2+}]_i$ in a real-time manner by measuring fluorescence changes.

[^{3}H]Yohimbine binding assays

BmOAR1-HEK cells were cultured for 7–10 days, and the cells at 100% confluency were used for binding assays. Cells were harvested in 50 mM ice-cold Tris–HCl (pH 7.4) and centrifuged at 25 000 ***g*** for 20 min. The cell pellet was homogenized in the buffer with a glass-Teflon homogenizer and re-centrifuged as 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 (1976). To determine the abilities of ligands to inhibit specific [^{3}H]yohimbine binding in BmOAR1-HEK cells, a 50-μl aliquot of Tris–HCl (pH 7.4) for the determination of total binding or the same volume of the buffer containing various concentrations of unlabelled yohimbine or other ligands was added to 1.5-ml microtubes. Unlabelled yohimbine (final concentration 10 μM) was added for the determination of nonspecific binding. Buffer (50 μl) containing [^{3}H]yohimbine (final concentration 1 nM) and buffer (150 μl) containing cell membrane homogenates (40 μg as protein) were added to all tubes. After vortexing, 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% polyethylenimine. The filters were rapidly washed with two 3-ml portions of cold 50 mM Tris–HCl (pH 7.4) and then placed in 10 ml of a 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. IC_{50} values were estimated by the probit method.

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