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RESEARCH ARTICLE

Characterization of a β-adrenergic-like octopamine receptor from the rice stem borer (*Chilo suppressalis*)

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SUMMARY

Octopamine, the invertebrate counterpart of adrenaline and noradrenaline, plays a key role in regulation of many physiological and behavioral processes in insects. It modulates these functions through binding to specific octopamine receptors, which are typical rhodopsin-like G-protein coupled receptors. A cDNA encoding a seven-transmembrane receptor was cloned from the nerve cord of the rice stem borer, *Chilo suppressalis*, viz. *CsOA2B2*, which shares high sequence similarity to CG6989, a *Drosophila* β-adrenergic-like octopamine receptor (DmOctβ2R). We generated an HEK-293 cell line that stably expresses CsOA2B2 in order to examine the functional and pharmacological properties of this receptor. Activation of CsOA2B2 by octopamine increased the production of cAMP in a dose-dependent manner (EC_{50}=2.33 nmol l^{-1}), with a maximum response at 100 nmol l^{-1}. Tyramine also activated the receptor but with much less potency than octopamine. Dopamine and serotonin had marginal effects on cAMP production. Using a series of known agonists and antagonists for octopamine receptors, we observed a rather unique pharmacological profile for CsOA2B2 through measurements of cAMP. The rank order of potency of the agonists was naphazoline > clonidine. The activated effect of octopamine is abolished by co-incubation with phentolamine, mianserin or chlorpromazine. Using *in vivo* pharmacology, CsOA2B2 antagonists mianserin and phentolamine impaired the motor ability of individual rice stem borers. The results of the present study are important for a better functional understanding of this receptor as well as for practical applications in the development of environmentally sustainable pesticides.

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Key words: biogenic amine, cyclic AMP, antagonist, locomotion, insecticide.

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INTRODUCTION

The biogenic amine octopamine carries out many of the functional roles in insects and other invertebrates, as an invertebrate counterpart of adrenergic ligands (Roeder, 2005; Roeder, 1999; Verlinden et al., 2010a). For example, octopamine modulates behaviors such as aggression (Hoyer et al., 2008; Zhou et al., 2008; Rillich et al., 2011), sleep (Crocker and Sehgal, 2008; Crocker et al., 2010), egg-laying (Monastirioti et al., 1996), food-seeking (Suo et al., 2006), as well as locomotion (Certel et al., 2010; Fussnecker et al., 2006; Saraswati et al., 2004).

Octopamine exerts its effects by binding to specific receptor proteins that belong to the superfamily of G-protein coupled receptors, many of which have been characterized not only from *Drosophila* but also from several other invertebrate species (Evans and Maqueira, 2005; Verlinden et al., 2010a). Based on similarities of these receptors to vertebrate adrenergic receptors in terms of amino acid sequence and signaling pathways, various octopamine receptors have been classified into three classes: (1) α-adrenergic-like octopamine receptors (OctαRs), (2) β-adrenergic-like octopamine receptors (OctβRs) and (3) octopamine/tyramine (or tyraminergic) receptors (TyrRs) (Evans and Maqueira, 2005). The first insect octopamine receptor was isolated from *Drosophila* (Han et al., 1998), which was assigned to the OctαR class. Activation of these receptors primarily leads to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and has a minor effect on intracellular cAMP concentration ($[cAMP]_i$) elevation (Grohmann et al., 2003; Han et al., 1998; Huang et al., 2010). The OctβR class is subdivided into three subclasses and they each specifically increase $[cAMP]_i$ levels in response to octopamine. The TyrR class, which shows structural and pharmacological similarities with vertebrate α_2-adrenergic receptors, was preferentially activated by tyramine to induce a decrease in $[cAMP]_i$ (Blenau et al., 2000; Ohta et al., 2003; Rotte et al., 2009; Saudou et al., 1990). For this reason, researchers thought that this class of receptors represents a class of tyramine receptors (Lange, 2009). Besides, Cazzamali et al. and Huang et al. (Cazzamali et al., 2005; Huang et al., 2009) identified a different type of G-protein coupled receptor in fruit flies and silkworms that is specific for tyramine and selectively coupled to intracellular calcium mobilization. It is now considered to be as a fourth class of receptor, namely, TyrRII, and the TyrR class was subsequently named TyrRI (Verlinden et al., 2010a).

Octopamine acts as a neurotransmitter, neuromodulator and neurohormone to regulate various physiological functions in insects. However, current knowledge regarding the octopamine receptor subtype(s) in mediating the effects of octopamine is limited. To date, only OctβRs from *Drosophila melanogaster* (three subtypes) and *Bombyx mori* (two subtypes but only one have function) have been cloned and characterized (Chen et al., 2010; Maqueira et al., 2005). A partial sequence of putative OctβR in the desert locust *Schistocerca gregaria* (SgOctβR) was also cloned (Verlinden et al., 2010b). In the

present study, we cloned a gene coding for a type 2 OctβR (CsOA2B2) from the rice stem borer, *Chilo suppressalis*. We expressed it in HEK-293 cells in order to pharmacologically characterize this receptor. Using *in vivo* pharmacology and a behavioral assay, we provide evidence that octopamine is involved in the regulation of rice stem borer locomotion and CsOA2B2 is a likely mediator of this effect.

MATERIALS AND METHODS

Insects and reagents

Chilo suppressalis (Walker 1863) larvae were collected from the field in Fuyang, China (119.6°E, 30.5°N), and were reared in laboratory for several generations with rice seedlings according to the method reported by Shang et al. (Shang et al., 1979). The rearing conditions were 28±1°C, >80% relative humidity and a 16 h:8 h light dark photoperiod. (±)-Octopamine hydrochloride, tyramine hydrochloride, dopamine hydrochloride, serotonin hydrochloride, naphazoline hydrochloride, clonidine hydrochloride, yohimbine hydrochloride, forskolin, G418 disulfate salt, 3-isobutyl-1-methylxanthine (IBMX), chlorpromazine hydrochloride, phentolamine hydrochloride, mianserin hydrochloride, epinastine hydrochloride and adenosine 5′-triphosphate salt (ATP) were all obtained from Sigma-Aldrich (St Louis, MO, USA).

Cloning of *CsOA2B2*

Total RNA was isolated from *C. suppressalis* nerve cord with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Single-strand cDNA, synthesized from the RNA using a ReverTra Ace-α- kit (Toyobo, Osaka, Japan), was used as a template for PCRs. We performed transcriptome sequencing of nerve cord of *C. suppressalis*. Through transcriptome sequencing, one putative OctβR (*CsOA2B2*) was annotated using BlastX [National Center for Biotechnology Information (NCBI), Bethesda, MD, USA]. Open reading frames (ORFs) were predicted with EditSeq (version 5.02, DNAstar, Madison, WI, USA).

The forward primer CSOA2B2-compF (5′-AAGCGAAG-CGTGCGAGAT-3′), located upstream of the putative start codon initiator, and the reverse primer CSOA2B2-compR (5′-CCTCAAACGGCTGGCTAT-3′), located downstream of the putative stop codon, were used to amplify the full-length gene. PCR was carried out for 3 min at 94°C (one cycle), followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 90 s at 72°C, and a final extension of 10 min at 72°C using TransTaq HiFi DNA Polymerase (TransGen Biotech, Beijing, China). PCR product was separated by electrophoresis on a 1.0% agarose gel. The purified PCR product was cloned into pGEM®-T easy vector (Promega, Madison, WI, USA) and then sequenced using the 3730 XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA).

Bioinformatic analysis

Sequence similarity/annotations and searching for orthologous genes were performed using BLAST programs from the NCBI. Multiple sequence alignments of the complete amino acid sequences were performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed with the neighbor-joining method, using 1000-fold bootstrap re-sampling, and the resulting tree was displayed graphically using MEGA 4.0 (Tamura et al., 2007). The *Homo sapiens* rhodopsin was used as an out-group.

Gene expression profile analysis

Total RNAs were extracted from different developmental stages and tissues of *C. suppressalis* with Trizol reagent (Invitrogen) according to manufacturer's instructions. Residual genomic DNA was removed by RQ1 RNase-Free DNase (Promega). Total RNA (1 μg) was reverse transcribed to cDNA with the ReverTra Ace-α- kit. RT-PCR and qPCR were performed to investigate expression of *CsOA2B2* in different developmental stages and relative expression in different tissues. A pair of gene-specific primers CsOA2B2RTF (5′-GATTTTCAAGGAGGCCAACA-3′), CsOA2B2RTR (5′-CTGCTTTGTGCTCCCTCTTC-3′) was designed and used for RT-PCR and qPCR. Elongation factor-1 (Hui et al., 2011) cDNA fragment was amplified with CsEF-1F (5′-TGAACCCCCATA-CAGCGAATCC-3′) and CsEF-1R (5′-TCTCCGTGCCAACC-AGAAATAGG-3′) primers as an internal control.

Construction of the pcDNA3-CsOA2B2 expression vector

To construct an expression plasmid, an expression-ready construct of CsOA2B2 cDNA was generated by PCR, which was performed with specific primers (CsOA2B2-HindIIIF: 5′-GGG*AAGCTT*C-ATATGGATCCCATAA-3′; CsOA2B2-XhoIR: 5′-GG*CTCGAG*T-CAAAGCGACGCCAAAT-3′). The PCR product was digested with *Hin*dIII and *Xho*I. The digested DNA fragments were then sub-cloned into pcDNA3 vector (Invitrogen) yielding pcDNA3-CsOA2B2. The correct insertion was confirmed by DNA sequencing.

Stable expression of CsOA2B2 in HEK-293 cells

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) at 37°C and 5% CO_2. After transfection of pcDNA3-CsOA2B2 plasmid into the cells using Lipofectamine 2000 (Invitrogen), the antibiotic G418 (0.5 mg ml^{-1}) was added to the medium to select for cells that stably expressed the receptors. After 2 weeks of G418 selection, G418-resistant colonies were trypsinized in cloning cylinders and transferred to 24-well plastic plates for expansion. These individual cell lines were analyzed for integration of the receptor DNA by RT-PCR (data not shown). The clonal cell line that most efficiently expressing CsOA2B2 was chosen for this study.

Cyclic AMP determination

PcDNA3-CsOA2B2/HEK-293 cells were plated into 24-well tissue culture plates (Nunc, Roskilde, Dammark) at a density of 5×10^5 cells $well^{-1}$ and incubated at 37°C 10% CO_2 in a humidified incubator. The cells were washed in 1× Dulbecco's phosphate buffered saline (D-PBS) (Gibco BRL) and equilibrated for 20 min at 37°C in 100 μmol l^{-1} of the phosphodiesterase inhibitor IBMX. After the preincubation, a 200 μl aliquot of D-PBS containing various concentrations of agonists was added, and then the culture was incubated for 20 min at 37°C. The reactions were stopped by removal of agonist solutions and the immediate lysis of the cells was stopped by addition of ice-cold cell lysis buffer. The cell lysate was scraped into 1.5 ml Eppendorf tubes for collections and stored at –70°C until use. For antagonist studies, the stimulations were carried out as above except that the respective 10 μmol l^{-1} antagonists were mixed with 10 nmol l^{-1} octopamine. The amount of cAMP produced was determined using a cAMP ELISA kit (R&D Systems, Minneapolis, MN, USA).

Calcium mobilization assay

$[Ca^{2+}]_i$ was examined and analyzed with a calcium imaging system. PcDNA3-CsOA2B2/HEK-293 cells were seeded at 2×10^5 cells $well^{-1}$ in 6-well plates (Nunc) containing cover slips and allowed to grow for 48 h. Cells were then incubated for 25–35 min at 37°C in the presence of 5 μmol l^{-1} Fura-2AM (Dojindo Laboratories, Kumamoto, Japan) and 0.02% pluronic F127

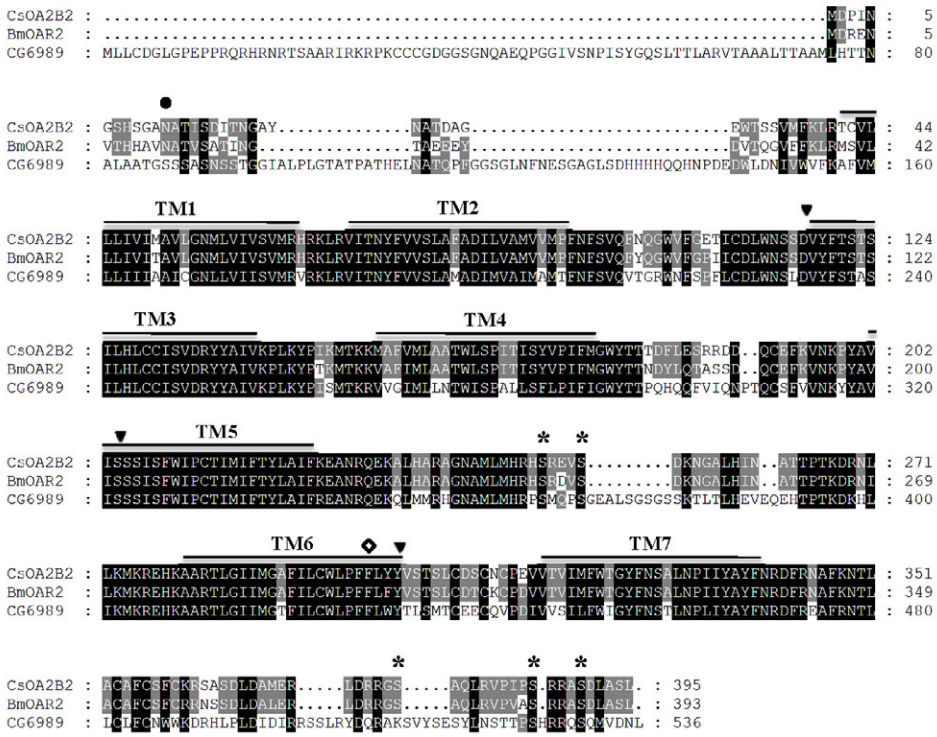

Fig. 1. Amino acid sequence alignment of CsOA2B2 and orthologous receptors from *Bombyx mori* (BmOAR2; GenBank accession number BAJ06526) and *Drosophila melanogaster* (CG6989, DmOctβ2R; AAF54835). The amino acid position is indicated on the right. The predicted seven transmembrane regions are indicated by TM1–7. The alignment was performed by ClustalX and the shaded sequences highlight the identity level of amino acids between the receptors. Potential *N*-glycosylation sites and potential phosphorylation sites for protein kinase C are labeled by filled circles and asterisks, respectively. Amino acids that are predicted to be involved in agonist binding are indicated by filled triangles. The second phenylalanine after the FxxxWxP motif in TM6, which is a unique feature of aminergic receptors, is indicated by a diamond.

(Invitrogen). The cells were subsequently washed twice with a bathing solution (152 mmol l^{-1}NaCl, 5.4 mmol l^{-1}KCl, 5.5 mmol l^{-1} glucose, 1.8 mmol l^{-1} CaCl2, 0.8 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} HEPES, pH 7.4) (Huang et al., 2009). The cover slips were transferred to the microscopic chamber constantly perfused with the bathing solution at ~2 ml min^{-1}. The fluorescence at 510 nm by excitation at 340 or 380 nm with a xenon lamp was measured with individual cells using an Easy Ratio Pro calcium imaging system (Photon Technology International, Birmingham, NJ, USA).

Behavioral assay and data collection

The effects of drug treatments on crawling larvae were assessed and quantified in locomotion assays. We used the three compounds that have been proven to have significant antagonistic effects on CsOA2B2, chlorpromazine, mianserin and phentolamine. To dilute these compounds, we used acetone and distilled water with 1:1 (v/v) mixture as solvent. For drug treatments, fifth-instar larvae of rice stem borers (9–11 mg) were topically treated with 1 µl of each drug solution (10 µmol l^{-1}) and control solution (solvent) onto the middle-abdomen notum of the larvae with a hand microapplicator (Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China).

The locomotion assay method was modified from Min and Condron (Min and Condron, 2005). Briefly, insects were placed in a 15 cm Petri dish. A small amount of diet was spread along the other sides of the dish prior to running the assay. After washing with a small amount of distilled water, the larvae were gathered with a brush and transferred onto a plate. At the start of the assay, the larvae were placed and spread out 1.5 cm from the edge of the plate. The assay was run for 5 min. As the larvae crawled towards the diet, the time it took larvae to reach the edge of the diet was recorded. Larvae that crawled out of the diet were scored only once and those larvae that did not make it to the diet but were still mobile within the 5 min were marked with a time of infinity. The time at which half the total number of larvae in the assay reached the food was then determined ($t_{1/2}$). All data were normally distributed and were analyzed using one-way ANOVA. To determine which data sets had significantly different means, the Tukey–Kramer multiple comparisons test was performed as a *post hoc* test.

RESULTS

Cloning of *CsOA2B2* and phylogenetic analysis

The ORF of *CsOA2B2* was identified from transcriptome analysis of *C. suppressalis* nerve cord, and then confirmed by PCR and DNA sequencing (supplementary material Fig. S1). The cDNA (1188 bp) encodes a protein of 395 amino acids with a calculated molecular weight of 44.8 kDa (GenBank accession number JN620367). The deduced amino acid sequence of CsOA2B2 shows the hallmarks of G-protein coupled receptors that have typical seven transmembrane domains. Amino acid sequence comparisons between CsOA2B2 and other insect biogenic amine receptors show high amino acid similarity with BmOAR2, 87.5% (Chen et al., 2010), and DmOctβ2R, 59.5% (Maqueira et al., 2005). Sequence motifs, which are essential for the phosphorylation by protein kinase C, potential *N*-glycosylation, ligand binding and signal transduction of the receptor, are well conserved in CsOA2B2 (Fig. 1).

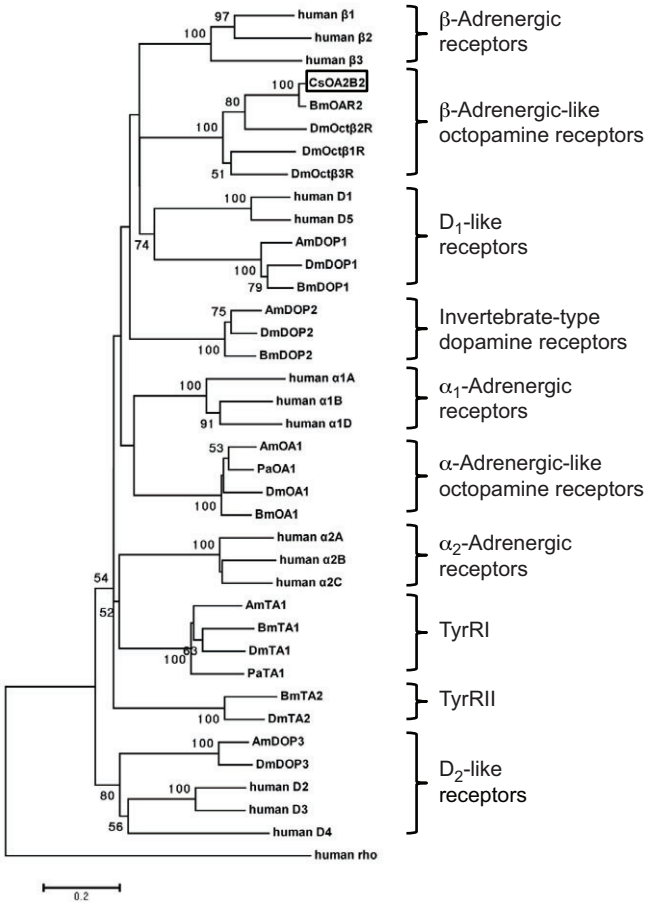

Fig. 2. Phylogenetic tree of CsOA2B2 and various biogenic amine receptors. Neighbor-joining trees were constructed in ClustalW2 using 1000-fold bootstrap re-sampling, and the resulting tree was displayed graphically using MEGA 4. The numbers at the nodes of the branches represent the level of bootstrap support for each branch. The receptor sequences followed by their GeneBank accession numbers are listed in the order illustrated: *Homo sapiens* β_1-adrenergic (NP_000675); *H. sapiens* β_2-adrenergic (NP_000015); *H. sapiens* β_3-adrenergic (NP_000016); *Chilo suppressalis* CsOA2B2 (JN620367; present study); *Bombyx mori* BmOAR2 (BAJ06526); *Drosophila melanogaster* DmOctβ2R (AJ880689); *D. melanogaster* DmOctβ1R, splice variant A (AJ880687); *D. melanogaster* DmOctβ3R, splice variant A (AJ884591); *H. sapiens* dopamine D1 (NP_000785); *H. sapiens* dopamine D5 (NP_000789); *Apis mellifera* AmDOP1 (NP_001011595); *D. melanogaster* DmDOP1 (CAA54451); *B. mori* BmDOP1 (NP_001108459); *A. mellifera* AmDOP2 (NP_001011567); *D. melanogaster* DmDOP2 (NP_733299); *B. mori* BmDOP2 (NP_001108338); *H. sapiens* α_{1A}-adrenergic isoform 1 (NP_000671); *H. sapiens* α_{1B}-adrenergic (NP_000670); *H. sapiens* α_{1D}-adrenergic (NP_000669); *A. mellifera* AmOA1 (NP_001011565); *Periplaneta americana* PaOA1 (AAP93817); *D. melanogaster* DmOA1/oamb (NP_732541); *B. mori* BmOA1 (NP_001091748); *H. sapiens* α_{2A}-adrenergic (NP_000672); *H. sapiens* α_{2B}-adrenergic (NP_000673); *H. sapiens* α_{2C}-adrenergic (NP_000674); *A. mellifera* AmTA1 (NP_001011594); *B. mori* BmTA1 (BAD11157); *D. melanogaster* DmTA1 (BAB71788); *P. americana* PaTA1 (AM990461); *B. mori* BmTA2 (BAI52937); *D. melanogaster* DmTA2 (NP_650652); *A. mellifera* AmDOP3 (NP_001014983); *D. melanogaster* DmDOP3 (AAN15955); *H. sapiens* dopamine D2 isoform short (NP_057658); *H. sapiens* dopamine D3 isoform a (NP_000787); *H. sapiens* dopamine D4 (NP_000788). Human rhodopsin receptor (NP_000530) was used as an outgroup.

Together with human catecholamine receptors, insect catecholamine receptors were used to generate a phylogenetic tree. CsOA2B2 clustered with BmOAR2, DmOctβ2R, DmOctβ1R and DmOctβ3R in a distinct clade. A closely related clade contained the human β-adrenergic receptors. Phylogenetic analysis also showed that arthropod OctβRs and human β-adrenergic receptors are closely related to the D_1-like dopamine receptors (Fig. 2). All of above-reported receptors are coupled with G_s protein and induce increasing of $[cAMP]_i$.

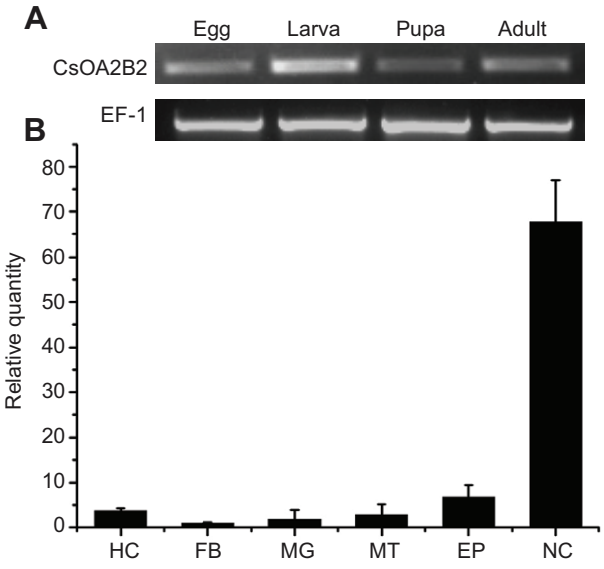

Fig. 3. Expression profile of *CsOA2B2* analyzed by RT-PCR and qPCR. (A) Expression pattern of *CsOA2B2* in different developmental stages. (B) Expression patterns of *CsOA2B2* mRNA levels in tissues of fifth-instar larvae were quantified by qPCR and normalized against levels of elongation factor-1 (EF-1) mRNA. Tissues tested are hemocytes (HC), fat body (FB), midgut (MG), Malpighian tubules (MT), epidermis (EP) and nerve cord (NC). Data represent means ± s.e.m. (*N*=3 repetitions).

Expression pattern of *CsOA2B2*

CsOA2B2 was expressed in all developmental stages including egg, larva, pupa and adult (Fig. 3A). In the larval stage, *CsOA2B2* was expressed in all tested tissues but was highly expressed in the nerve cord, followed by the epidermis, hemocytes, Malpighian tubules, midgut and fat body (Fig. 3B), suggesting that it might play an important role in the central nervous system of insects.

Functional and pharmacological characterization of CsOA2B2

A HEK-293 cell line stably expressing CsOA2B2 was generated in order to examine the signaling and pharmacological properties of the receptor. A wide range of naturally occurring biogenic amines were tested for their abilities to change $[cAMP]_i$. Octopamine and tyramine significantly induced the increase of cAMP production in CsOA2B2-expressing cells (Fig. 4A), but not in mock-transfected cells (supplementary material Fig. S2). The dose–response relationships of octopamine and tyramine on $[cAMP]_i$ were examined. It can be seen that octopamine was three orders of magnitude more potent than tyramine (EC_{50}: octopamine, 2.33×10^{-9} mol l^{-1}; tyramine, 2.76×10^{-6} mol l^{-1}) and also had a lower threshold than tyramine on this receptor (Fig. 4B). Dopamine and serotonin at 1 μmol l^{-1} had no significant effect on cAMP production. Thus, CsOA2B2 is likely to be an endogenous *C. suppressalis* octopamine receptor mediating its action *via* increases in $[cAMP]_i$.

In order to characterize the pharmacological profile of CsOA2B2, we then examined the effects of various potential octopamine

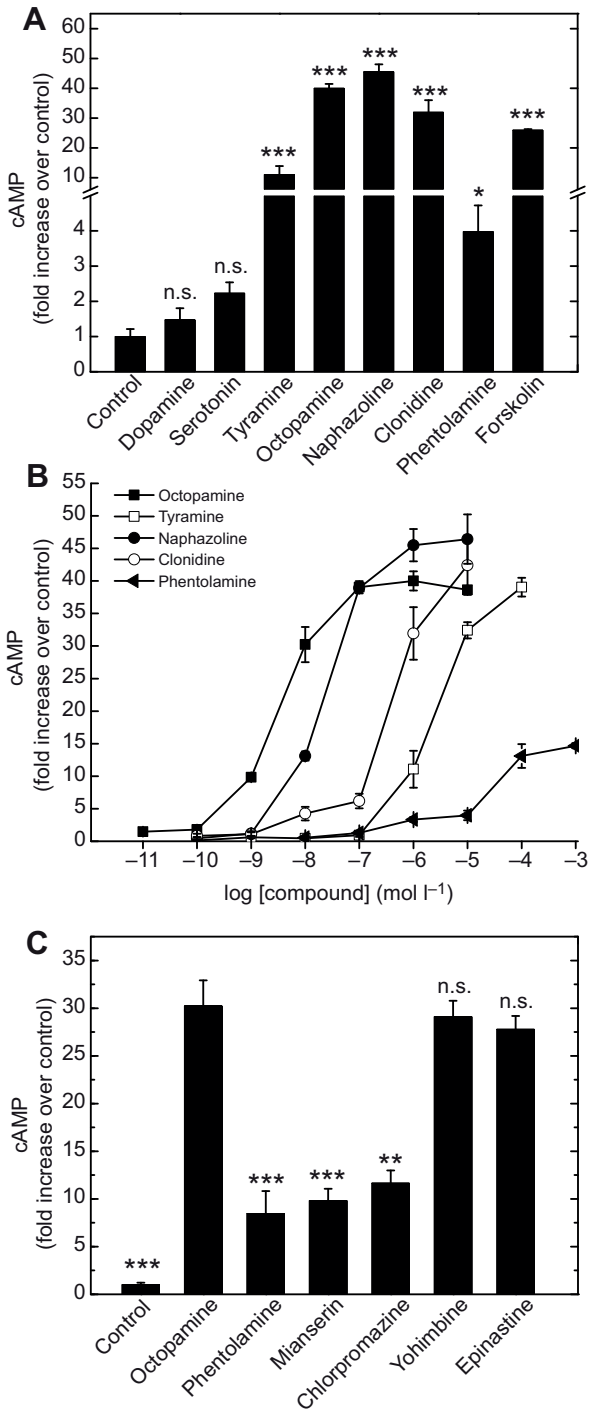

Fig. 4. cAMP levels in HEK-293 cells expressing CsOA2B2 and treated with various aminergic ligands. The cAMP production of control is 2.65±0.53 pmol well^{-1}. (A) HEK-293 cells transfected with pcDNA3-CsOA2B2 construct were treated with the indicated compounds (1 μmol l^{-1}) or with forskolin (10 μmol l^{-1}) as a positive control. Results are expressed as the fold increase of cAMP relative to untreated cells. Data represent means ± s.e.m. of four to six experiments. (B) Dose–response relationships of the effects of biogenic amines and synthetic compounds on $[cAMP]_i$ in pcDNA3-CsOA2B2/HEK-293 cells. (C) Effects of a range of synthetic antagonists (10 μmol l^{-1}) on cAMP levels in HEK-293 cells stably expressing CsOA2B2. Data represent means ± s.e.m. of four to six experiments. Asterisks indicate values significantly different from the control value using one-way ANOVA with the Tukey–Kramer multiple comparisons test (*P<0.05, **P<0.001, ***P<0.0001).

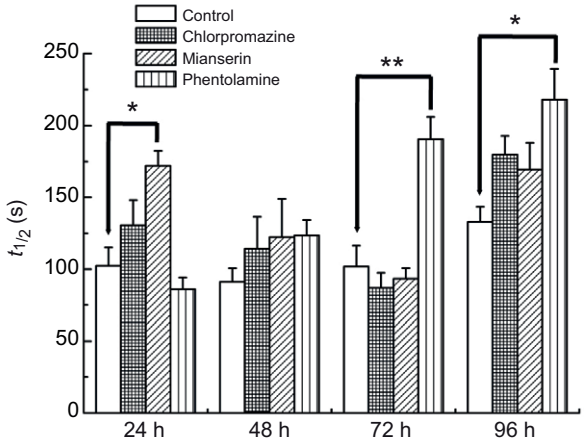

Fig. 5. Behavioral assay. Half-times ($t_{1/2}$) of control populations, chlorpromazine-treated populations, mianserin-treated populations and phentolamine-treated populations at 24, 48, 72 and 96 h after treatment. Statistical tests were performed using one-way ANOVA with Tukey–Kramer multiple comparisons test. Data represent means ± s.e.m. of the $t_{1/2}$ values (*P<0.05, **P<0.001).

receptor agonists and antagonists. It can be seen that these agonistic effects were concentration dependent (Fig. 4B). The EC_{50} values for naphazoline and clonidine were 2.08×10^{-8} and 6.06×10^{-7} mol l^{-1}, respectively. These results were very similar to those found in BmOAR2, but no biphasic dose–response relationship was found (Chen et al., 2010). Phentolamine, a classical α-adrenergic antagonist, is generally known as an antagonist for octopamine receptors (Roeder, 1999). But recently it has been shown that phentolamine is capable of showing agonistic effects on OctβRs, resulting in increases in cAMP levels in the transfected cell lines (Chen et al., 2010; Maqueira et al., 2005). In our assay, this compound showed weak agonist activity, with an EC_{50} of 5.25×10^{-5} mol l^{-1} (Fig. 4B). For assays with antagonists, the elevation of $[cAMP]_i$ caused by 10 nmol l^{-1} octopamine was significantly reduced by 10 μmol l^{-1} phentolamine, mianserin and chlorpromazine. However, the inhibitory effects of these three compounds are similar. Yohimbine and epinastine did not significantly antagonize the action of octopamine (Fig. 4C). The results were little different than those for *Drosophila* (DmOctβ2R: mianserin > phentolamine) (Maqueira et al., 2005) and *B. mori*, in which only chlorpromazine showed inhibition effects (Chen et al., 2010).

We then tested whether any Ca^{2+} response could be induced upon binding of octopamine to CsOA2B2. However, we were not able to demonstrate any Ca^{2+} response in the cells expressing the cloned receptor with 10 μmol l^{-1} octopamine. However, application of 10 μmol l^{-1} ATP, as positive control, induced a rapid significant increase of Ca^{2+} over the basal level (supplementary material Fig. S3).

Locomotion assay with *in vivo* pharmacology

We designed a behavioral assay to investigate the locomotion ability of drug-treated rice stem borers. Three octopamine receptor antagonists were used to examine their effects on larval locomotion. Mianserin-treated larvae took more time (172±10.4 s, mean ± s.e.m.) to reach the edge of the diet than that in control treatments (102.4±12.7 s) at 24 h after treatment and phentolamine-treated larvae also took more time (72 h: 172±10.4 s; 96 h: 217.9±21.5 s) than larvae in control treatments (72 h: 101.8±14.4 s; 96 h: 132.9±10.5 s) at 72 h and 96 h after treatment (Fig. 5).

DISCUSSION

In the present study, we have characterized an octopamine receptor from the rice stem borer. Because this receptor could be mapped into the subgroup of the insect OctβRs, we therefore named it CsOA2B2 and functional studies supported this sequence-based classification. The phylogenetic analysis, including insect and human catecholamine receptors, strengthens the view of four distinguishable classes of octopamine and tyramine receptors [OctαRs, OctβRs, octopamine/tyramine receptors (TyrRI) and tyramine receptors (TyrRII)] (Evans and Maqueira, 2005; Verlinden et al., 2010a).

The distribution pattern of *CsOA2B2* revealed a ubiquitous expression in all developmental stages and examined tissues, indicating the possibility of a vast array of physiological functions for CsOA2B2. However, qPCR results indicated that *CsOA2B2* is expressed predominantly in the nervous cord of the fifth-instar larvae, suggesting a putative role in the modulation of neuronal activity. Similar results were also found in DmOctβ2R, which is highly expressed in heads (Maqueira et al., 2005). However, in the desert locust *S. gregaria*, the transcript levels of SgOctβR are the highest in the flight muscles, followed by the central nervous system (Verlinden et al., 2010b).

CsOA2B2 was stably expressed in HEK-293 cells, which have been used successfully in previous studies to examine the pharmacological properties of cloned insect GPCRs (Bischof and Enan, 2004; Han et al., 1998; Huang et al., 2010; Rotte et al., 2009). The potency of octopamine on CsOA2B2 is similar to that in DmOctβ2R and BmOAR2 in terms of EC_{50} values (10^{-9} to ~10^{-8} mol l^{-1}) determined in CHO and HEK-293 cells, respectively. In agonist assays, naphazoline was less potent than octopamine but more potent than clonidine, which is consistent with that of the classical type-2A octopamine receptors (Evans, 1981). Of all the antagonists tested, phentolamine, mianserin and chlorpromazine significantly inhibit cAMP productions induced by 10 nmol l^{-1} octopamine. However, phentolamine, which acts as a full agonist with antagonistic effects smaller than mianserin at DmOctβ2R (Maqueira et al., 2005) and has weak agonist activity but no antagonistic effects at BmOAR2 (Chen et al., 2010), plays dual roles at CsOA2B2. It acts as a partial agonist and has significantly antagonistic effects at CsOA2B2. Yohimibine and epinastine were ineffective on CsOA2B2 in cAMP assays. Thus, CsOA2B2 showed an agonist profile similar to that of DmOctβ2R and BmOAR2 but the antagonist profile did not match either one exactly.

There are many papers that suggest that octopamine plays a number of important roles in the regulation of various behaviors, including motor function in invertebrates (Fox et al., 2006; Fussnecker et al., 2006; Saraswati et al., 2004). Octopamine has been shown to modulate the food-seeking behavior in *Caenorhabditis elegans* through a G_q-coupled octopamine receptor (SER-3) (Suo et al., 2006). In the honey bee, disruption of an OctαR (AmOA1) by mianserin and AmOA1 dsRNA both inhibited olfactory acquisition and recall (Farooqui et al., 2004; Fussnecker et al., 2006). It is also demonstrated that growth of octopaminergic arbors was controlled by a cAMP- and CREB-dependent positive-feedback mechanism that required DmOctβ2R octopamine autoreceptors. In addition, octopamine neurons regulated the expansion of excitatory glutamatergic neuromuscular arbors through DmOctβ2R on glutamatergic motor neurons (Koon et al., 2011). This indicated that this subtype of OctβR might play a crucial role in locomotion. Because CsOA2B2 is the ortholog of DmOctβ2R, we suppose that CsOA2B2 might also be involved in the locomotory behavior of the rice stem borer.

We have investigated the effects of potent CsOA2B2-receptor antagonists on the locomotion ability of rice stem borer larvae in behavioral experiments. This is a necessary first step in establishing the role of CsOA2B2 in the regulation of locomotor behaviors. The present study shows that mianserin and phentolamine affect the locomotor behavior of the rice stem borer. However, interpretation of present data should be made carefully. Because these antagonists are broad-spectrum, in consideration of multiple subtypes of octopamine receptor genes that have not yet been discovered in rice stem borers, it is probable that they can affect many octopamine receptor signaling pathways including CsOA2B2. For example, chlorpromazine has been shown to act as a high affinity antagonist on an OctαR (Huang et al., 2010) and an OctβR (Chen et al., 2010) in the silkworm, and it also has antagonistic effects on BmTAR2 (Huang et al., 2009). Phentolamine and mianserin, like chlopromzaine, can also block different types of octopamine receptors such as DmOctβ1R and DmOctβ3R in *Drosophila* (Bischof and Enan, 2004; Maqueira et al., 2005). Mianserin has also been shown to have a significant affinity for the *Drosophila* tyramine receptor, DmTyrR (Enan, 2005). Given that the treated ligands may act on multiple receptors, thereby regulating different signaling pathways, it is not unexpected that different antagonists exert effects with different time courses.

Taken together, topical application of the CsOA2B2 antagonists mianserin and phentolamine impaired the motor ability of individual rice stem borers. However, the molecular identities, tissue distribution and pharmacological properties of all members of the rice stem borer octopamine receptor family have so far not been unraveled. Hence we cannot exclude that additional octopamine receptor subtypes might have contributed to the observed effects. Additional work will be required to examine specific antagonist and other receptor subtypes to explore the possible functions of octopamine and octopamine receptors in locomotion activity and other biological processes in insects.

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