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A new *Drosophila* octopamine receptor responds to serotonin

Yi-xiang Qi [a, b, 1], Gang Xu [a, 1], Gui-xiang Gu [a], Fen Mao [a], Gong-yin Ye [a], Weiwei Liu [c], Jia Huang [a, *]

[a] Ministry of Agriculture Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou, China
[b] Department of Entomology, College of Agriculture, South China Agricultural University, Guangzhou, China
[c] Neuroscience Research Institute and Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA 93106, USA

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ABSTRACT

As the counterparts of the vertebrate adrenergic transmitters, octopamine and tyramine are important physiological regulators in invertebrates. They control and modulate many physiological and behavioral functions in insects. In this study, we reported the pharmacological properties of a new α2-adrenergic-like octopamine receptor (CG18208) from *Drosophila melanogaster*, named DmOctα2R. This new receptor gene encodes two transcripts by alternative splicing. The long isoform DmOctα2R-L differs from the short isoform DmOctα2R-S by the presence of an additional 29 amino acids within the third intracellular loop. When heterologously expressed in mammalian cell lines, both receptors were activated by octopamine, tyramine, epinephrine and norepinephrine, resulting in the inhibition of cAMP production in a dose-dependent manner. The long form is more sensitive to the above ligands than the short form. The adrenergic agonists naphazoline, tolazoline and clonidine can stimulate DmOctα2R as full agonists. Surprisingly, serotonin and serotoninergic agonists can also activate DmOctα2R. Several tested adrenergic antagonists and serotonin antagonists blocked the action of octopamine or serotonin on DmOctα2R. The data presented here reported an adrenergic-like G protein-coupled receptor activated by serotonin, suggesting that the neurotransmission and neuromodulation in the nervous system could be more complex than previously thought.

1. Introduction

Neurotransmitters and neuromodulators like epinephrine (also called adrenaline), norepinephrine (also called noradrenaline), dopamine (DA) and serotonin (5-HT) are important neuroactive molecules to control and regulate many aspects of behaviors and physiology in vertebrates. DA and 5-HT are also important neuroactive substances in invertebrates. However, epinephrine and norepinephrine have no known physiological relevance in protostomes and their roles are considered to be fulfilled by their invertebrate counterparts, octopamine (OA) and tyramine (TA) (Roeder, 2005). Norepinephrine, epinephrine, OA, TA and DA are all derived from tyrosine and their chemical structures are similar (Blenau and Baumann, 2001). 5-HT is derived from tryptophan and structurally quite different from other monoamine neurotransmitters.

Biogenic amines mainly exert their effects through G-protein-coupled receptors (GPCRs). Based on the structural and signaling similarities to vertebrate adrenergic receptors, insect octopamine receptors are grouped into three classes: α-adrenergic-like receptors (OctαR, also known as OAMB or OA1), β-adrenergic-like receptors (OctβR, also known as OA2) and octopamine/tyramine or tyramine receptors (Oct-TyrR or TAR1) (Evans and Maqueira, 2005). Activation of OctαR primarily leads to the elevation of $[Ca^{2+}]_i$ when expressed in cell lines (Balfanz et al., 2005). A total of three receptor genes have been characterized from *Drosophila* encoding β-type adrenergic-like receptors. Activation of this group of receptors, DmOctβ1R, DmOctβ2R and DmOctβ3R all leads to increased intracellular cAMP levels (Balfanz et al., 2005; Maqueira et al., 2005). Both OctαR and OctβR show high specificity to OA over other biogenic amines such as TA and DA. The TAR1 can be stimulated by both TA and OA to reduce forskolin-stimulated cAMP

Abbreviations: OA, Octopamine; TA, Tyramine; 5-HT, serotonin; DA, Dopamine; GPCR, G protein-coupled receptor; $[cAMP]_i$, intracellular cyclic adenosine monophosphate level; $[Ca^{2+}]_i$, intracellular calcium level; HEK 293, human embryonic kidney 293; CHO-K1, Chinese hamster ovary K1.

* Corresponding author. Institute of Insect Sciences, Zhejiang University, Yuhangtang Road 866, Hangzhou 310058, China.
E-mail address: huangj@zju.edu.cn (J. Huang).

[1] These authors contribute equally to this work.

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levels. Based on the pharmacological properties of CG7431 from *Drosophila melanogaster* (Cazzamali et al., 2005) and an orthologous receptor from *Bombyx mori* (Huang et al., 2009), a new class of tyramine receptors (TAR2) was added to the classification (Farooqui, 2012). The group of TAR2 is specifically activated by TA to increase $[Ca^{2+}]_i$ (Huang et al., 2009) or exclusively causes an increase in $[cAMP]_i$ (Reim et al., 2017). Bayliss et al. revealed that the receptor encoded by gene *CG16766* is activated by a number of biogenic amines, including TA, OA and DA. It is not only coupled to intracellular Ca^{2+} mobilization, but also reduces forskolin-stimulated cAMP level upon activation (Bayliss et al., 2013). Hence, CG16766 represents a new group of tyramine receptors which is designated the tyramine 3 receptors (TAR3).

Recently, a novel octopamine receptor (*CsOA3*) was cloned from the rice stem borer, *Chilo suppressalis* (Wu et al., 2014). In this study, we isolated the orthologous gene of *CsOA3* from *D. melanogaster* (*CG18208*), named *DmOctα2R*. The *DmOctα2R* gene gives rise to two transcripts by alternative splicing (*DmOctα2R-L* and *DmOctα2R-S*). In stably transfected human embryonic kidney 293 (HEK 293) cells or Chinese hamster ovary K1 (CHO-K1) cells, activation of either DmOctα2R-L or DmOctα2R-S resulted in inhibition of forskolin-stimulated cAMP synthesis. In addition to OA and TA, 5-HT also activates DmOctα2R in a dose dependent manner.

2. Methods

2.1. Chemicals

(±)-Octopamine hydrochloride (OA), tyramine hydrochloride (TA), dopamine hydrochloride (DA), epinephrine hydrochloride (E), norepinephrine hydrochloride (NE), serotonin hydrochloride (5-HT), 5-carboxamidotryptamine maleate salt (5-CT), 5-methoxytryptamine (5-MT), α-methylserotonin maleate salt (αm-5-HT), 8-Hydroxy-DPAT hydrobromide (8-OH-DPAT), lisuride, yohimbine (YH), epinastine (EP); chlorpromazine (CH); phentolamine (PA); Methiothepin mesylate salt (MT), Ketanserin (+)-tartrate salt (KS), G418 disulphate salt, forskolin, 3-isobutyl-1-methylxanthine (IBMX) were all obtained from Sigma-Aldrich (St Louis, MO, USA). Tolazoline hydrochloride, clonidine hydrochloride, naphazoline hydrochloride were purchased from Selleck Chemicals (Houston, TX, USA).

2.2. Insects

Flies were reared at 25 °C and ambient humidity under a 12:12 h light: dark photoperiod using standard cornmeal medium.

2.3. Cloning of CG18208

Total RNA was isolated from mixed whole bodies of male and female flies using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Single-strand cDNA synthesized from RNA using a ReverTra Ace-α-kit (Toyobo, Osaka, Japan) was used as a template for PCRs. Primers were designed based on the sequence data of *CG18208* published in FlyBase (http://www.flybase.org). The forward primer CG18208-compF, located upstream of the putative start codon and the reverse primer CG18208-compR, located downstream of the putative stop codon, were used to amplify the full-length coding sequence of the CG18208 cDNA. The PCR program started with a denaturation step for 30 s at 98 °C, followed by 38 repeats of the following cycle: one step for 10 s at 98 °C, one step for 30 s at 60 °C, one step for 1 min at 72 °C, and a final extension of 10 min at 72 °C using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). PCR products were separated to check the size 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 a 3730 XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA).

2.4. Multiple sequence alignment and phylogenetic analysis

The primary structure of the protein was deduced from the cDNA sequence and used for phylogenetic analysis. Sequences used for the alignments were identified by BLAST programs from the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments of the complete amino acid sequences were performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The results were displayed by BioEdit. The transmembrane segments were predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0). The phylogenetic tree and molecular evolutionary analyses were performed using MEGA 5.05 software with the neighbor joining method, using the divergent *Drosophila* FMRFamide receptor (DmFR) as out-group.

2.5. Construction of expression plasmids

An expression plasmid containing the Kozak consensus sequence (Kozak, 1987) was constructed by PCR with specific primers CG18208-*Hin*d III and CG18208-*Xho*I. The PCR product was digested with *Hin*d III and *Xho*I. The digested DNA fragments were purified using PCR Clean-Up Kit (Axygen, Union City, CA, USA) and then subcloned into hemagglutinin epitope-Tagged (HA-Tagged) pcDNA 3.0 vector (Invitrogen) yielding pcCG18208. The correct insertion was confirmed by DNA sequencing.

2.6. Cell culture, transfection, and creation of stable cell lines

Human Embryonic Kidney 293 (HEK 293) cells and Chinese hamster ovary K1 (CHO-K1) cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HEK 293 cells and CHO-K1 cells were grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco BRL, Gaithersburg, MD, USA) and Dulbecco's modified Eagle medium Nutrient Mixture F-12 (1:1) (DMEM/F-12) (Gibco BRL, Gaithersburg, MD, USA) respectively. Both cell lines were supplemented with 10% fetal bovine serum (Gibco BRL) at 37 °C and 5% CO_2. After transfection of the pcCG18208 plasmid into the cells using Lipofectamine 2000 (Invitrogen), the antibiotic G418 (0.8 mg/mL) was added to the medium to select for cells that constitutively expressed the receptor. After 2 weeks of G418 selection, G418-resistant colonies were trypsinized in cloning cylinders and transferred to 12-well plastic plates for expansion. Four to six individual cell lines were analyzed for determination of the receptor mRNA expression by RT-PCR and localization of the protein by immunofluorescence (Fig. S1). Cells were grown on Lab-Tek™ II Chambered Coverglass (Nunc) and fixed for 20 min in 4% paraformaldehyde. Fixed cells were incubated in phosphate buffer containing 5% normal goat serum for 2 h at 25 °C, followed by incubation for overnight with anti-HA tag mouse monoclonal antibodies (dilution 1:500, Abcam) at 4 °C. Samples were rinsed with PBS and incubated with goat anti-mouse secondary antibodies labeled with FITC (dilution 1:500, Abcam) for 2 h at 25 °C. After several rinses with PBS, the nucleus was stained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Sigma). Images were recorded using a Zeiss LSM 800 confocal microscope (Carl Zeiss SAS, Germany). The clonal cell line that most efficiently expressed pcCG18208 was chosen for this study.

2.7. cAMP determination

cAMP levels were measured as previously described (Qi et al.,