Insect Biochemistry and Molecular Biology 39 (2009) 842–849

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Molecular cloning and pharmacological characterization of a *Bombyx mori* tyramine receptor selectively coupled to intracellular calcium mobilization

Jia Huang [1], Hiroto Ohta [2], Noriko Inoue, Haruka Takao, Tomo Kita, Fumiyo Ozoe, Yoshihisa Ozoe*

Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690-8504, Japan

ARTICLE INFO

Article history:
Received 2 April 2009
Received in revised form
27 September 2009
Accepted 1 October 2009

Keywords:
Bombyx mori
Tyramine
Octopamine
G protein-coupled receptor
Cloning
Ca^{2+}
Agonist
Antagonist

ABSTRACT

Tyramine (TA) is a biogenic amine in invertebrates. cDNA encoding the TA receptor (TAR) BmTAR2 was cloned from the nerve tissue of the silkworm *Bombyx mori*. The receptor's functional and pharmacological properties were examined in *BmTAR2*-transfected HEK-293 cells. In [^{3}H]TA binding assays, BmTAR2 showed considerably higher affinity for TA than for other biogenic amines, with an IC_{50} value of 57.5 nM. Moreover, TA induced a dose-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cells, with an EC_{50} value of 11.6 nM, whereas octopamine and dopamine increased $[Ca^{2+}]_i$ only at concentrations above 100 μM. A few antagonists were found to inhibit the TA-induced increases in $[Ca^{2+}]_i$; the rank order of potency was yohimbine > chlorpromazine > mianserin. TA showed no effect on intracellular cAMP concentration. The data indicate that BmTAR2 belongs to the second class of TARs, which are selectively coupled to intracellular Ca^{2+} mobilization. RT-PCR analysis revealed that *BmTAR2* was expressed predominantly in the nervous tissue of *B. mori* larvae, suggesting that TA has neurotransmitter and neuromodulatory roles that are mediated by BmTAR2.

1. Introduction

Insects secrete serotonin (5-HT), dopamine (DA), histamine, octopamine (OA), and tyramine (TA) as physiologically important biogenic amines; OA and TA are trace amines in vertebrates. The structures of OA and TA differ only in the respective presence or absence of a hydroxyl group at the β-position in their side chains. OA has been shown to act as a neurotransmitter, neuromodulator, or neurohormone. It regulates various physiological functions in insects, such as circadian rhythms, endocrine secretion, fight and flight behaviors, and learning and memory (Roeder, 1999). TA was assumed to serve as a biosynthetic precursor of OA, rather than as a neuroactive substance. Therefore, not much is known about the physiological role of TA in insects.

In the past decade, increasingly more reports have supported the hypothesis that TA is not only a precursor but also a genuine signaling molecule in a variety of physiological processes (Lange, 2008). Studies of the *Drosophila* mutant *hono*, which contains a transposon upstream of the TA receptor (TAR) gene, indicate that TA has a functional role in the olfactory system as a neurotransmitter or neuromodulator (Kutsukake et al., 2000). In addition, some neurons that contained TA, but not OA, were identified (Nagaya et al., 2002), suggesting that TA may be a neuroactive compound. It was shown that the application of TA at nanomolar concentrations stimulated chloride conductance and urine secretion in the *Drosophila* Malpighian tubule (Blumenthal, 2003). In locust oviducts, TA attenuated cAMP levels and increased the amplitude of excitatory junction potentials (Donini and Lange, 2004). TA, but not OA, affected the appetite of the blowfly *Phormia regina* (Nisimura et al., 2005). Studies on *Drosophila* mutants with altered levels of TA and OA differentially implicated these amines in the regulation of locomotion and flight (Saraswati et al., 2004; Hardie et al., 2007; Brembs et al., 2007). In the silkworm *Bombyx mori*, increased levels of TA, but not OA or DA, suppressed post-mating pheromone production (Hirashima et al., 2007). The most cogent evidence in support of

Abbreviations: BmTAR2, *Bombyx mori* tyramine receptor 2; *BmTAR2*, gene encoding BmTAR2; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[cAMP]_i$, intracellular cAMP concentration; DMCDM, demethylchlordimeform or *N*′-(4-chloro-*o*-tolyl)-*N*-methylformamidine; ICL, intracellular loop; DA, dopamine; 5-HT, serotonin; NC-5, 2-(2,6-diethylphenylimino)imidazolidine; OA, octopamine; OAR, octopamine receptor; ORF, open reading frame; PEA, 2-phenylethylamine; PO-8, 5-(4-hydroxyphenyl)oxazole; PCR, polymerase chain reaction; RT, reverse transcription; TA, tyramine; TAR, tyramine receptor; TM, transmembrane domain.

* Corresponding author. Tel.: +81 852 32 6573; fax: +81 852 32 6092.
E-mail address: ozoe-y@life.shimane-u.ac.jp (Y. Ozoe).
[1] Present address: ‡Institute of Insect Sciences, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, China.
[2] Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan.

0965-1748/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2009.10.001

TA as a legitimate signaling molecule came from a genetic study of *Caenorhabditis elegans* (Alkema et al., 2005), suggesting that TA functions independently of OA in the inhibition of egg laying, the modulation of reversal behavior, and the suppression of head oscillations in response to anterior touch.

The first potential insect TAR was cloned from *Drosophila melanogaster* (Arakawa et al., 1990). This receptor was initially deemed an OA receptor (OAR), since OA reduced the intracellular cAMP concentration ($[cAMP]_i$) by activating the receptor when expressed in CHO-K1 cells. However, TA was found to be 13- to 33-fold more potent than OA in studies of both ligand binding and attenuation of adenylate cyclase activity. For that reason, this receptor was named Tyr-dro (Saudou et al., 1990). In a different study, OA activated this receptor, which increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) with a potency similar to TA, but with a shorter lag phase and time to the peak than for TA. Importantly, TA was almost two orders of magnitude more potent than OA with respect to binding affinity and attenuation of adenylate cyclase activity (Robb et al., 1994). Thus, the receptor was named OA/TAR and is probably coupled to multiple signaling pathways. With further cloning and characterization of orthologous genes from different insect species, all receptors, including a *B. mori* TAR (BmTAR1) that had been previously characterized by our group (Ohta et al., 2003), were shown to prefer TA over OA in the attenuation of adenylate cyclase activity; although, the elevations in $[Ca^{2+}]_i$ were not consistent among the studies. Consequently, receptors of this class may function as specific tyraminergic receptors in insects.

A member of the second class of TARs, DrmTR, that was specific for TA was cloned from *D. melanogaster* (Cazzamali et al., 2005). When the cDNA of this gene (CG7431) was expressed in CHO cells or *Xenopus* oocytes, the expressed receptor was specifically activated by TA, but not by other biogenic amines (up to 100 μM) or neuropeptides (up to 10 μM). Cazzamali et al. (2005) identified three other homologous genes from *D. melanogaster* (CG16766), *Anopheles gambiae*, and *Apis mellifera*, but functional characterization of the encoded receptors has not been reported. We cloned a cDNA encoding a homologous receptor from *B. mori*. We expressed it in HEK-293 cells in order to pharmacologically characterize this new family of TARs. The results of this work are reported here.

2. Materials and methods

2.1. Chemicals

OA, DA, and 2-phenylethylamine (PEA) were purchased from Nacalai Tesque (Kyoto, Japan). TA, *meta*-TA, tolazoline, and yohimbine were purchased from Sigma–Aldrich (St. Louis, MO). Clonidine, naphazoline, and histamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMCDM (*N*′-(4-chloro-*o*-tolyl)-*N*-methylformamidine), NC-5 (2-(2,6-diethylphenylimino)imidazolidine), and PO-8 (5-(4-hydroxyphenyl)oxazole) were synthesized in our laboratory, according to previously reported methods (Benezet and Knowles, 1976; Nathanson and Kaugars, 1989; Khan et al., 2003). Metoclopramide, mianserin, cyproheptadine, and chlorpromazine were obtained from RBI Research Biochemicals (Natick, MA). 5-HT, cinnamyl alcohol, eugenol, and *trans*-anethole were purchased from TCI Chemical (Tokyo, Japan). α-Terpineol was obtained from Chem Service (West Chester, PA). Terpinen-4-ol was obtained from Acros Organics (Morris Plains, NJ). Hinokitiol was donated by Asahi Kasei (Tokyo, Japan). [^{3}H]TA (20 Ci/mmol) and [^{3}H]cAMP (17 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Other general chemicals were purchased from Wako Pure Chemical Industries.

2.2. Insects

B. mori eggs (Kinshu × Showa strain) were purchased from Ueda Sanshu (Ueda, Japan). The larvae were reared at 25 °C on the artificial diet Silkmate 2S (Nihon Nosan Kogyo, Yokohama, Japan).

2.3. Cloning of BmTAR2

The silkworm KAIKObase of the National Institute of Agrobiological Sciences (http://sgp.dna.affrc.go.jp/) was searched with the *DrmTR* gene (Accession No. AY034617). Three genomic ramen-contig sequences with high similarity were found - 493 663, 70 113, and 763 818. Seven transmembrane domains (TMs) were predicted from these contigs and a set of primers, BmTAR2-F1 (5′-GTTGA TCGTGGTGACTGTCATTGG-3′) and BmTAR2-R1 (5′-CCTCCAGAA CGCTAGTCTGAAATCC-3′), were designed, based on the TM I and TM VII/C-terminus nucleotide sequences, respectively. Total RNA was extracted from the nerve tissue (brains and subesophageal ganglia) of 50 *B. mori* fifth instar larvae using the Isogen RNA extraction reagent (Nippon Gene, Tokyo, Japan). cDNA synthesis and 5′RACE were performed following the CapFishing Full-Length cDNA Premix Kit protocol (Seegene, Seoul, Korea). The 1.1 kb PCR product was obtained using the primers BmTAR2-F1 and BmTAR2-R1 and the cDNA as a template. This 1.1 kb PCR product was sequenced and confirmed to code for BmTAR2, distinct from BmTAR1 (Ohta et al., 2003). To obtain the full-length cDNA, 5′RACE analysis was performed with nested PCR, using two antisense primers, BmTAR2-raceR1 (5′-GGCCAGCTGGTGATGTCTTCTT-3′) based on the intracellular loop (ICL) 3 and BmTAR2-raceR2 (5′-GAAGATCCGCTACAGCAAGACTCG-3′) based on TM II, and a 5′RACE-sense primer included in the Seegene kit. The sequence of the 455 bp fragment obtained by 5′RACE corresponded to nucleotides −118 in the 5′ untranslated region (UTR) to 337 in the open reading frame (ORF) (See Fig. S1). The downstream region from TM VII to the 3′ UTR was identified by referring to the ramen-contig data without performing 3′RACE. Finally, a cDNA (1523 bp) fragment encoding the 5′ UTR (118 bp), ORF (1359 bp), and 3′ UTR (46 bp) was obtained by PCR using a set of primers, BmTAR2-full-F (5′-GTGA TAGTAACCGTTATGTGC-3′) and BmTAR2-full-R (5′-CCGTAAAAT CACTCTTTG-3′), and was confirmed to be a target gene (*BmTAR2*) by direct DNA sequence analysis. However, the data indicated the presence of mixed nucleotides at 16 sites in the DNA sequence. To clone a DNA fragment corresponding to the ORF, a slightly shorter region was re-amplified by PCR using the primers BmTAR2-KpnI-F (5′-TTGGTACCATGGCTAAATCACCATCCGAA-3′) and BmTAR2-EcoRI-R (5′-TTGAATTCCCGTAAAATCACTCTTTG-3′), and the 1523 bp PCR product as a template. The resulting PCR product was treated with restriction enzymes (Toyobo, Osaka, Japan) and inserted into similarly digested Bluescript vector [pBS-KS(−); Stratagene]. After DNA sequencing, 12 clones were classified into two types of polymorphic genes: *BmTAR2.1* (Accession No. AB462481, Fig. 1) and *BmTAR2.2* (Accession No. AB462482). Newly assembled scaffold data from KAIKObase identified contigs 16 765, 16 781, and 16 782, and yielded the BmTAR2 1523 bp sequence. KOD DNA polymerase (Toyobo) was used for PCR. DNA sequencing was performed with the ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) of BmTAR2 transcripts from body regions

Total RNA was isolated from the brains, nerve cords, silk glands, midguts, and Malpighian tubules of *B. mori* fifth instar larvae, using Isogen. The isolated RNA (1 μg) was reverse-transcribed using PrimeScript™ RT reagent (Takara) and mixtures of oligo(dT) and