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AmTAR2: Functional characterization of a honeybee tyramine receptor stimulating adenylyl cyclase activity

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ABSTRACT

The biogenic monoamines norepinephrine and epinephrine regulate important physiological functions in vertebrates. Insects such as honeybees do not synthesize these neuroactive substances. Instead, they employ octopamine and tyramine for comparable physiological functions. These biogenic amines activate specific guanine nucleotide-binding (G) protein-coupled receptors (GPCRs).

Based on pharmacological data obtained on heterologously expressed receptors, α- and β-adrenergic-like octopamine receptors are better activated by octopamine than by tyramine. Conversely, GPCRs forming the type 1 tyramine receptor clade (synonymous to octopamine/tyramine receptors) are better activated by tyramine than by octopamine. More recently, receptors were characterized which are almost exclusively activated by tyramine, thus forming an independent type 2 tyramine receptor clade. Functionally, type 1 tyramine receptors inhibit adenylyl cyclase activity, leading to a decrease in intracellular cAMP concentration ($[cAMP]_i$). Type 2 tyramine receptors can mediate Ca^{2+} signals or both Ca^{2+} signals and effects on $[cAMP]_i$. We here provide evidence that the honeybee tyramine receptor 2 (AmTAR2), when heterologously expressed in flpTM cells, exclusively causes an increase in $[cAMP]_i$. The receptor displays a pronounced preference for tyramine over octopamine. Its activity can be blocked by a series of established antagonists, of which mianserin and yohimbine are most efficient.

The functional characterization of two tyramine receptors from the honeybee, AmTAR1 (previously named AmTYR1) and AmTAR2, which respond to tyramine by changing cAMP levels in opposite direction, is an important step towards understanding the actions of tyramine in honeybee behavior and physiology, particularly in comparison to the effects of octopamine.

1. Introduction

Information processing in the central nervous system relies on the precise interplay of electrical and chemical signals. Whereas the velocity of signal propagation is determined by the activity of ion channels, the transfer of information between neurons and their target cells is usually achieved by chemical messenger substances, e.g. when released as neurotransmitters into the synaptic cleft. In addition, neurotransmitters and neuromodulators can exert fundamental modulatory functions on their target cells. In particular, the group of biogenic amines is known to evoke cellular signaling plasticity by inducing transient changes in intracellular second messengers (Blenau and Baumann, 2001). These control enzymatic reactions, which, in turn, lead to transient functional changes of target proteins or regulate transcription in the cell nucleus (Mellor and Parker, 1998; Shemarova, 2009).

Two biogenic amines, octopamine and tyramine, serve as the protostome's complement of the vertebrate adrenergic system. Various biochemical, pharmacological, and molecular studies have shown that octopamine and tyramine have a large impact on the physiology and behavior of protostomes (Blenau and Baumann, 2016; Chase and Koelle, 2007; Huang et al., 2016; Lange, 2009; Roeder, 2005; Scheiner et al., 2006; Stevenson and Rillich, 2012;

Abbreviations: HA, hemagglutinin A; GPCR, G protein-coupled receptor; n.a., not applicable; RFU, relative fluorescence units; TM, transmembrane.

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Verlinden et al., 2010). Both biogenic amines exert their activity by binding to members of the superfamily of G protein-coupled receptors (GPCRs). In the honeybee genome, five genes encoding receptors for octopamine and two genes coding for tyramine receptors have been identified (Cazzamali et al., 2005; Hauser et al., 2006). The family of octopamine receptors has been characterized comprehensively in recent years (Balfanz et al., 2014; Grohmann et al., 2003). At the cellular level, these receptors evoke Ca^{2+} release from intracellular stores (AmOctαR, previously named AmOA1 (Grohmann et al., 2003)) or activate adenylyl cyclases, thereby increasing intracellular cAMP concentrations ($[cAMP]_i$; AmOctβR1-4; Balfanz et al., 2014). In contrast to these receptors, only one tyramine receptor from the honeybee has been examined functionally so far. The AmTAR1 receptor (previously named AmTYR1; Blenau et al., 2000) inhibits adenylyl cyclase activity and thus leads to a reduction in $[cAMP]_i$ (Beggs et al., 2011; Blenau et al., 2000; Mustard et al., 2005). The functional and pharmacological properties of a second tyramine receptor from the honeybee (AmTAR2) are still elusive.

Previous studies, however, found that AmTAR2 phylogenetically clusters with a clade of tyramine receptors that is clearly set apart from the AmTAR1-containing group (Bayliss et al., 2013; Cazzamali et al., 2005; Hauser et al., 2006). Amongst the orthologues of AmTAR2 is a *Drosophila* receptor (DmTAR2; CG7431) that was studied in detail (Bayliss et al., 2013; Cazzamali et al., 2005). Most notably, this receptor seems to bind exclusively to tyramine. It is activated by sub-micromolar concentrations of tyramine and mediates Ca^{2+} signals when expressed in CHO cells or *Xenopus* oocytes (Cazzamali et al., 2005). The same observation was reported in an independent study (Bayliss et al., 2013). Similar to the *Drosophila* receptor and with a pronounced selectivity for tyramine over octopamine, a tyramine receptor from *Bombyx mori* (BmTAR2) causes an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$; Huang et al., 2009). However, in addition to tyramine receptors causing reduction in $[cAMP]_i$ (TAR1 clade) and receptors causing Ca^{2+} signals (TAR2 clade), there is a third clade of tyramine receptors (Bayliss et al., 2013). From this group, only one protein has been studied in detail, so far. When expressed in CHO cells, the *Drosophila* DmTAR3 receptor (CG16766) causes a decrease in $[cAMP]_i$ as well as Ca^{2+} signals (Bayliss et al., 2013). Compared to the other tyramine receptor subtypes, however, DmTAR3 showed a rather broad ligand spectrum (Bayliss et al., 2013).

The honeybee (*Apis mellifera*) is an important model organism for studying the action of biogenic amines on behavior (Blenau and Baumann, 2016, 2001; Blenau and Thamm, 2011; Ellen and Mercer, 2012; Giurfa, 2006; Scheiner et al., 2006). Physiologically, octopamine and tyramine are often considered to act similarly in an animal (Fussnecker et al., 2006; Kutsukake et al., 2000; Ormerod et al., 2013; Scheiner et al., 2002; Schulz and Robinson, 2001; Selcho et al., 2012). However, there is also evidence for different effects of these amines on behavior (Roeder et al., 2003; Saraswati et al., 2004; Scheiner et al., 2014b). Whether and how these effects can be traced back to the repertoire and the signaling capabilities of individual receptors is a challenging question. Here, we constitutively expressed the AmTAR2 receptor in a cell line and examined its coupling to intracellular second messengers and its pharmacological properties. For comparison, we re-examined the AmTAR1 receptor (Blenau et al., 2000) in the same experimental setup. We show that AmTAR2 has a strong preference for tyramine over octopamine (1,000-fold difference in EC_{50}). Intriguingly, this receptor specifically induces cAMP production upon activation. AmTAR1, in contrast, decreases intracellular cAMP upon activation (Blenau et al., 2000; this study). *In vivo*, the effects of their activity apparently depend on their tissue and/or cell-type specific expression pattern and eventually on their potential cross talk to octopamine receptor signaling.

2. Material and methods

2.1. Cloning of the honeybee tyramine receptor AmTAR2

Total RNA was extracted from 50 brains of foragers using TRIzol™ LS Reagent (Life Technologies GmbH, Darmstadt, Germany) and mRNA was purified using the Micro-FastTrack™ 2.0 Kit (Life Technologies GmbH). Synthesis of cDNA was carried out with the AccuScript™ High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Böblingen, Germany). For amplification of the entire coding region of Am*tar2*, specific primers were designed based on available sequence information (Cazzamali et al., 2005): sense primer 5′-GCGGAAGTCATCGTCAATGG-3'; antisense primer 5′-GGTCGTATCAACGTTCATTCCC-3′ (TIB Molbiol, Berlin, Germany). PCR was performed using the following protocol: 94 °C for 150 s, 35 cycles at 94 °C for 40 s, 66.5 °C for 40 s and 72 °C for 90 s and a final extension at 72 °C for 10 min. The PCR product was cloned into the pGEM-T vector (Promega, Mannheim, Germany) and sequenced on both strands (GATC Biotech AG, Konstanz, Germany). The nucleotide sequence is available via GenBank® (https://www.ncbi.nlm.nih.gov/genbank/, accession number KX881421).

2.2. Multiple sequence alignment and phylogenetic analysis

For phylogenetic analysis, we included amino acid sequences of biogenic amine receptors of various insects. Sequences were obtained from NCBI databases (NCBI, Bethesda, USA). Multiple amino acid sequence alignment was carried out with ClustalW and consequently trimmed to the regions from TM 1–5 and TM 6–7. Afterwards a Bayesian analysis (MrBayes v3.2.6; Ronquist et al., 2012) was performed with 1,000,000 generations, an initial burn in of 2,500, and the substation model LG + I + G, determined by Protest 3.4.2 (Darriba et al., 2011).

Sequence identity and similarity of tyramine receptors between *A. mellifera, B. mori* and *D. melanogaster* were determined with BLAST (NCBI, Bethesda, USA) after pairwise alignment.

2.3. Construction of pcAmtar2-HA expression vector

For heterologous expression of AmTAR2, receptor encoding cDNA was modified in a PCR with specific primers (sense primer 5′-TTTAAGCTTCCACCATGGCGAATCAAACGG-3′; antisense primer 5′-TTTGAATTCCGTTCTCGTTGTGTCTTTCTT-3′). In front of the start codon, a Hind*III* restriction site and a Kozak consensus motif (CCACC; Kozak, 1984) were inserted. The stop codon was replaced by an Eco*RI* recognition sequence. We reused the pcAm*5-ht1A*-HA construct (Thamm et al., 2010) and exchanged the Am*5-ht1A* cDNA for the Am*tar2* cDNA by ligation into the Hind*III* and Eco*RI* sites. The resulting construct is extended in frame at the 3′ end with a sequence encoding the hemagglutinin A (HA) tag (Am*tar2*-HA) which allowed us to monitor receptor protein expression using a specific anti-HA antibody (Roche Applied Science, Mannheim, Germany). Similarly, we used our previously cloned Am*tar1* cDNA (former name Am*tyr1*; Blenau et al., 2000) and fused the HA-tag in frame to the 3' end by PCR. Both constructs, Am*tar1*-HA and Am*tar2*-HA were cloned into pcDNA3.1 vector (Invitrogen, Karlsruhe, Germany) for expression in eukaryotic cells. All constructs were confirmed by sequencing.

2.4. Functional expression of the AmTAR1-HA and AmTAR2-HA receptors

For AmTAR1-HA and AmTAR2-HA expression and