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Cockroach $GABA_B$ receptor subtypes: Molecular characterization, pharmacological properties and tissue distribution

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ABSTRACT

γ-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the central nervous system (CNS). Its effects are mediated by either ionotropic $GABA_A$ receptors or metabotropic $GABA_B$ receptors. $GABA_B$ receptors regulate, *via* $G_{i/o}$ G-proteins, ion channels, and adenylyl cyclases. In humans, $GABA_B$ receptor subtypes are involved in the etiology of neurologic and psychiatric disorders. In arthropods, however, these members of the G-protein-coupled receptor family are only inadequately characterized. Interestingly, physiological data have revealed important functions of $GABA_B$ receptors in the American cockroach, *Periplaneta americana*. We have cloned cDNAs coding for putative $GABA_B$ receptor subtypes 1 and 2 of *P. americana* (PeaGB1 and PeaGB2). When both receptor proteins are co-expressed in mammalian cells, activation of the receptor heteromer with GABA leads to a dose-dependent decrease in cAMP production. The pharmacological profile differs from that of mammalian and *Drosophila* $GABA_B$ receptors. Western blot analyses with polyclonal antibodies have revealed the expression of PeaGB1 and PeaGB2 in the CNS of the American cockroach. In addition to the widespread distribution in the brain, PeaGB1 is expressed in salivary glands and male accessory glands. Notably, PeaGB1-like immunoreactivity has been detected in the GABAergic salivary neuron 2, suggesting that $GABA_B$ receptors act as autoreceptors in this neuron.

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1. Introduction

The non-proteinogenic amino acid γ-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the central nervous system (CNS) of both vertebrates and invertebrates. GABA mediates its action *via* ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. Ionotropic $GABA_A$ receptors are ligand-gated Cl^- channels belonging to the Cys-loop ion channel superfamily (for a review, see: Olsen and Sieghart (2008)). Functional $GABA_A$ receptors are composed of five subunits that originate from different though homologous genes. The receptors are targets of important drugs with sedative and CNS-depressant activity. In both vertebrates and invertebrates, $GABA_A$ receptor subunits and their splice variants differ in developmental expression and in their cellular and even subcellular distribution (Simon et al., 2004; Ffrench-Constant et al.,

Abbreviations: 3-APPA, 3-aminopropylphosphinic acid; CNG, Cyclic nucleotide-gated; CNS, Central nervous system; ECL2, Extracellular loop 2; GB, $GABA_B$ receptor subtype; IBMX, Isobutylmethyl-xanthine; LN, Local interneuron; Pea, *Periplaneta americana*; PN, Projection neuron; RACE, Rapid amplification of cDNA ends; RDL, Resistant to dieldrin; SDN, Salivary duct nerve; SN, Salivary neuron; TM, Transmembrane domain.

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1991; Gisselmann et al., 2004; for a review, see: Buckingham et al. (2005)).

In contrast to $GABA_A$ receptors, $GABA_B$ receptors are members of the superfamily of G-protein-coupled receptors (GPCRs). Based on structural properties, they are assigned as family 3 (or type C) GPCRs (Bockaert and Pin, 1999). To form a functional receptor, two $GABA_B$ receptor subtypes, GB1 and GB2, have to heterodimerize (Kaupmann et al., 1998; White et al., 1998; Ng et al., 1999). In the heterodimer, GB1 binds to the ligand, whereas GB2 provides coupling to a G protein (Galvez et al., 2000, 2001). Both subtypes interact with each other *via* the extracellularly exposed N-terminal domains (Margeta-Mitrovic et al., 2001), the transmembrane domains (Schwarz et al., 2000; Monnier et al., 2011), and the coiled-coil domains in the intracellularly located C-terminus (White et al., 1998; Kuner et al., 1999). Receptor heterodimers can form even larger entities of oligomers mainly driven by the interaction of GB1 subunits (Maurel et al., 2008; Comps-Agrar et al., 2011; 2012). Activated GB1/GB2 heteromers inhibit adenylyl cyclase activity *via* $G_{\alpha i}$-subunits of heterotrimeric G proteins; this results in a reduced level of intracellular cAMP ($[cAMP]_i$). The $G_{\beta\gamma}$-subunits might inhibit presynaptic voltage-gated Ca^{2+} channels (Chen and van den Pol, 1998; Vigot et al., 2006) or activate inwardly rectifying potassium (KIR) channels (White et al., 1998).

In mammals, $GABA_B$ receptors have been convincingly shown to have an impact on diverse neurological disorders, such as epilepsy, hyperalgesia, and autism (Prosser et al., 2001; Schuler et al., 2001; Fatemi et al., 2009). Moreover, these receptors contribute to the regulation of behavior and brain reward processes (for a review see: Vlachou and Markou, 2010). Surprisingly, current knowledge on arthropod $GABA_B$ receptors is rather fragmentary. In the American cockroach, *Periplaneta americana*, an established model organism for physiological and neurobiological experiments (Huber et al., 1990; Walz et al., 2006), application of the $GABA_B$ receptor agonist 3-aminopropylphosphinic acid (3-APPA) to the abdominal ganglion (A6) provided the first hint regarding the existence of $GABA_B$ receptors in insects (Hue, 1991). This treatment mimicked the GABA response and resulted in a hyperpolarization of the membrane potential that was resistant to picrotoxin, a $GABA_A$ receptor antagonist. Similarly, two $GABA_B$ receptor agonists, 3-APPA and SKF97541, induce hyperpolarization of the fast coxal depressor motor neuron (Bai and Sattelle, 1995). Pharmacological experiments have also provided evidence for an involvement of $GABA_B$ receptors in saliva production and/or secretion in *P. americana* (Rotte et al., 2009a). The acinar-type salivary glands of the cockroach are innervated *via* the salivary duct nerve (SDN) that originates from the subesophageal ganglion. In addition to several thin serotonergic fibers, SDN contains two relatively thick axons, the dopaminergic salivary neuron (SN)1 and the GABAergic SN2 (Elia et al., 1994; Davis, 1985; Baumann et al., 2002; Rotte et al., 2009a). When GABA is applied during the electrical stimulation of the SDN, it leads to an enhancement in the electrical and secretory response of the exocrine cells, whereas without electrical stimulation, GABA has no effect. The effects of GABA are mimicked by $GABA_B$ receptor agonists and blocked by $GABA_B$ receptor antagonists (Rotte et al., 2009a). Therefore, GABA has been suggested to act presynaptically *via* $GABA_B$ receptors on serotonergic and/or dopaminergic nerve fibers (Rotte et al., 2009a).

In *Drosophila melanogaster*, three $GABA_B$ receptor subtypes (GB1, GB2 and GB3) have been molecularly characterized (Mezler et al., 2001). Physiologically, *Drosophila* $GABA_B$ receptors have been shown to be required for normal development (Dzitoyeva et al., 2005), to regulate carbohydrate and lipid metabolism (Enell et al., 2010), to modulate olfactory processing (Olsen and Wilson, 2008; Lei et al., 2013), to regulate circadian activity (Hamasaka et al., 2005) and sleep maintenance (Gmeiner et al., 2013), and to participate in the behavior-impairing effects of ethanol (Dzitoyeva et al., 2003). Recently, a partial cDNA has been cloned from the tobacco budworm, *Heliothis virescens*, encoding an incomplete GB1 subunit (Pregitzer et al., 2013). In the present study, we have unraveled the molecular structure, pharmacological properties, and localization of two cockroach $GABA_B$ receptor subtypes, PeaGB1 and PeaGB2. Activation of the heterologously expressed GB1/GB2 heteromers results in the specific inhibition of adenylyl cyclase activity. Receptor expression in various organs and tissues has been examined by Western blot analyses and immunohistochemistry. Notably, GB1-like immunoreactivity has been detected in GABAergic fibers innervating the salivary glands suggesting that $GABA_B$ receptors act as autoreceptors in SN2 of *P. americana*.

2. Material and methods

2.1. Animals

American cockroaches (*P. americana*) were reared at 24–26 °C under a light–dark (LD12:12) cycle. Male and female adult cockroaches (4–6 weeks old) were used.

2.2. Cloning of the PeaGB1 cDNA

The degenerate primer pair 5′-GARGCIGCIAARATGTGG-3′ (sense) and 5′-CCARTTRTCYTCRTACC-3′ (antisense) corresponding to the highly conserved amino acid sequences EAAKMW and WYEDNW, respectively, in the N-terminal domain of arthropod GB1 receptors were used for the amplification of PeaGB1 cDNA fragments. A second degenerate primer pair (5′-CCIGTITGYAAYACIATHATG-3′; 5′-TCRTTDATYTGYTTIAC-3′) corresponding to the amino acid sequences PVCNTIM and KVKQIND was used to amplify a cDNA fragment encoding transmembrane domains (TM)2–4. Poly(A)$^+$ RNA was isolated by using the Micro Fast-Track™ 2.0 Kit (Life Technologies, Karlsruhe, Germany). cDNA synthesis was performed with the Superscript™ First-strand cDNA Synthesis System for RT-PCR (Life Technologies) or with the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Waldbronn, Germany). The polymerase chain reaction (PCR) was carried out for 2.5 min at 94 °C (1 cycle), followed by 35 cycles of 40 s at 94 °C, 30–40 s at 42–50 °C, 60 s at 72 °C, and a final extension of 10 min at 72 °C. PCR products were sub-cloned into pGEM-T vector (Promega, Mannheim, Germany) and subsequently sequenced (AGOWA, Berlin, Germany or GATC, Konstanz, Germany). Based on this sequence information, a specific primer pair (sense 5′-GCGATCCGACTGACGCTGTGCG-3′; antisense 5′-CGTGCACACCCCCAGCAACATG-3′) was utilized to amplify the missing intermediary cDNA fragment. For rapid amplification of cDNA ends (RACE) PCR experiments, specific primers were designed. The missing 3′-region of the cDNA was obtained with the SMARTer™ RACE cDNA Amplification Kit (Clontech, Heidelberg, Germany) by using the sense primers 5′-CGGCCTGAACTCGAACATTGTG-3′ and 5′-GGTGTTCGGCTTGTTCCTGGCG-3′ (for nested PCR). Amplification of the cDNAs' 5′-region was performed with the antisense primers 5′-CGTGTCACTATTTCAATGCCGATCTC-3′ and 5′-CAACACGTGACCAACCAAACTTCTCC-3′ and by employing the 5′/3′ RACE Kit, 2nd Generation (Roche, Mannheim, Germany) with the antisense primer 5′-CTGTGGCAGATGGATGCGTTCGG-3′.

2.3. Cloning of the PeaGB2 cDNA

A degenerate primer pair (sense 5′-TTYTTYGAYATGATGC-3′; antisense 5′-GCCCADATICCRTC-3′) was used to amplify a cDNA fragment encoding part of the N-terminal region of the receptor. A second primer pair (sense 5′-ACICAYCCIATGTTYAC-3′; antisense 5′-CARTAYTCRTTYTC-3′) enabled the amplification of an overlapping cDNA fragment covering TM1-4. Amplification was carried out for 2.5 min at 94 °C (one cycle), followed by 35 cycles of 40 s at 94 °C, 40 s at 34 °C, 50–100 s at 72 °C, and a final extension of 10 min at 72 °C. The missing 5′-region was isolated with the 5′/3′ RACE Kit, 2nd Generation by using the specific antisense primer 5′-CGTAAGGTGCCAGCGTCGGGACG-3′. A cDNA fragment encoding TM5-6 was amplified with the specific sense primer 5′-GACCAAACAAATGGAGC-3′ and the degenerate antisense primer 5′-TAYTCIACDATRTCRCAYTT-3′.

To identify putative sequences encoding $GABA_B$ receptor subtype 2 of *P. americana* in the *Periplaneta* expressed sequence tag (EST) database, we screened the sequence reads produced by Hiseq2000 and the contigs generated by Trinity by using the TBLASTN algorithm (Altschul et al., 1997). The GB2 amino acid sequences of *D. melanogaster* (accession no.: NP_732658 and NP_524438) and *Pediculus humanus* (no. XP_002424838) were used as queries. Based on the EST fragment EST 71100, we used the specific primers 5′-GGCGGGGTTTTCGCTCGCTTTCGG-3′ (sense) and 5′-GAGACGAATGGAGGTGGTGTCAC-3′ (antisense) to amplify the missing 3′-end.

2.4. EST database construction

Cockroaches for constructing the EST database were kindly provided by M. Mizunami (Graduate School of Life Science, Hokkaido University). For RNA extraction, we collected six categories of developmental stages, namely (i) 12 egg cases, (ii)