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Apisα2, Apisα7-1 and Apisα7-2: three new neuronal nicotinic acetylcholine receptor α-subunits in the honeybee brain

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Abstract

Acetylcholine is the principal excitatory neurotransmitter in the central nervous system of insects. Nicotinic acetylcholine receptors, which belong to the ligand-gated ion channel family, constitute important targets for insecticides. In the honeybee *Apis mellifera*, pharmacological evidence supports the existence of several nicotinic acetylcholine receptors. In this paper, we report the identification of three new genes that encode nicotinic acetylcholine receptor α-subunits in the honeybee. Phylogenetic comparisons with other ligand-gated ion channel subunit sequences support their classification as Apisα2, Apisα7-1 and Apisα7-2 subunits. Based on in situ hybridization experiments, we determined their expression patterns in the different brain regions of pupae and adult honeybees. Our results show that these nicotinic acetylcholine receptor subunits are differently expressed among the brain regions and that they appear at different stages of honeybee development.

Keywords: Nicotinic acetylcholine receptors; α-subunits; In situ hybridization; Honeybee

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are important excitatory neurotransmitter receptors in the central nervous system (CNS) of insects. Because of their central role, insect neuronal nAChRs have become important targets for pesticides. Over the last few years, nAChR subunits have been cloned from several insect species including fruit fly *Drosophila melanogaster*, aphid *Myzus persicae*, tobacco hornworm *Manduca sexta*, locusts *Schistocerca gregaria* and *Locusta migratoria* (for review, see Gundelfinger and Schulz, 2000). As for their vertebrate orthologs, the subunits have been classified into α subunit, if they contain adjacent cysteine residues allowing a disulphide bond, and non-α subunit (β subunit) if they do not contain these cysteines (Galzi and Changeux, 1995). The functional architecture and diversity of the insect nAChRs are poorly understood compared to those of mammalian receptors (Tomizawa and Casida, 2001).

In the honeybee *Apis mellifera*, evidence suggests that the acetylcholine (ACh) is widely distributed in the brain. Histochemistry experiments showed that the inactivating ACh enzyme, acetylcholinesterase (AChE) is located in different brain regions including the antennal lobes, the α-lobes and the calyces of mushroom bodies (Kreissl and Bicker, 1989). Biochemical identification of nAChRs was

Abbreviations: nAChRs, nicotinic acetylcholine receptors; CNS, central nervous system; ACh, acetylcholine; AChE, acetylcholine esterase; α-Btx, alpha-bungarotoxin; TM, transmembrane domain; dl, dorsal lobe; la, lamina; me, medulla; ca, calice; al, antennal lobe; ich, inner chiasma; ncc, noncompact Kenyon cells; occ, outer compact Kenyon cells.

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previously performed on honeybee head homogenates, showing high-affinity binding sites for the nicotinic receptor antagonist α-Bungarotoxin (α-Btx) (Huang and Knowles, 1990). α-Btx-binding experiments and immunocytochemical studies demonstrated a co-localization of nAChRs and AChE in the same brain regions (Kreissl and Bicker, 1989; Scheidler et al., 1990).

Brain injections of the nicotinic antagonist mecamylamine have shown that the nicotinic system is involved in memory processes in honeybee and has different behavioural effects according to the injection sites (Cano Lozano et al., 1996; 2001). Mecamylamine induced a strong impairment of the olfactory learning when injected into the mushroom body calyce and a disturbance of the retrieval process when injected into the α-lobes of mushroom bodies, suggesting that nAChRs are implicated in the formation and the recall of memory (Cano Lozano et al., 2001). In addition, nicotinic cholinergic system is also involved in habituation of the proboscis extension reflex in honeybee (Guez et al., 2001; 2003). The latter study suggests the existence of two different subtypes of nAChRs which have different affinity to two metabolites of the insecticide imidacloprid (Guez et al., 2003). The possible existence of different nAChR subtypes was further proposed by patch-clamp electrophysiological experiments performed on neurons isolated from the adult antennal lobes, showing that depending on the neuron type, imidacloprid either acts as a full agonist or as a partial agonist (Nauen et al., 2001). Finally, the existence of nAChRs has also been demonstrated on Kenyon cells isolated from mushroom bodies of honeybee pupae that express nAChRs with functional profiles reminiscent of the vertebrate neuronal nAChR α7-subtype (Bicker, 1996; Goldberg et al., 1999). Indeed, the ACh-induced currents mediated by calcium ions were blocked by α-Btx (Bicker, 1996).

So far, only one nAChR subunit called Apisα3 has been cloned and its expression characterized in honeybee. This subunit presents typical features of nAChR α-subunit and a high sequence homology to human α3 subunit and is expressed in the suboesophageal ganglia in larvae and further in the optic, dorsal and antennal lobes and the calyces of mushroom bodies in adult (Thany et al., 2003).

In the present study, we have identified and cloned three new genes that encode α2, α7-1 and α7-2 nAChR α-subunits in the honeybee brain. Their expression patterns were studied at pupal and adult stages to determine if the different subunits present the same localization in the brain and if they appear at the same developmental stage.

2. Materials and methods

2.1. Identification of Apis mellifera α-subunit cDNA sequences

The nAChR α-subunits sequences were identified by screening in silico a honeybee EST database (http://titan.biotec.uiuc.edu/honeybee_project.htm) and a partial genomic DNA library (Honeybee Genome Project at Baylor College of medicine, http://www.hgsc.bcm.tmc.edu/projects/honeybee/). Using the Apisα3 sequence and the BLAST algorithm on the honeybee EST database, we identified three ESTs corresponding to clones BI516654, BI516733 and BI513319. These clones were kindly provided by Prof. G. Robinson, (W.M. Keck Center for Comparative and Functional Genomics University of Illinois). Complete sequence of these clones were performed and compared to genomic database sequences. In each case, in silico chromosome walking was performed in order to identify the potential full-length open-reading frames. RT-PCR experiments using total RNA from honeybee brain and primers encompassing the hypothetical ATG initiation codons were performed in order to confirm the predicted 5′ end of the open-reading frame. Comparison of the complete sequences of these three genes with vertebrate nAChR α-subunit sequences suggested that the three genes corresponded to Apisα2, Apisα7-1 and Apisα7-2 subunits.

2.2. RT-PCR amplification

Total RNA was isolated from adult brains using the RNeasy Mini Kit (Qiagen). RT-PCR experiments were performed with the Taq PCR Master Mix Kit (Qiagen). The following sense primers were used: 5′ GCCTACTATTCAAAATGATACT (Apisα2), ATGAGACGTTGGACTCTCATGG (Apisα7-1), ATGAAGGGCAGATTACGTTGGT (Apisα7-2) and antisense primers GGCCGGTGCCTCGCAGAGAATG (Apisα2), CTTCACAACGACGTTCGTCGG (Apisα7-1) and CCATCGTACGTCCAGGAGGC (Apisα7-2). PCR products were cloned in pGem vector (pGEM T-easy vector system, Promega) and sequenced.

2.3. Nomenclature

Sequence similarity searches with *Homo sapiens* neuronal nAChRs showed most homology with two different neuronal α-subunits: Hsα2 (U62431) and Hsα7 (CAA49778).

2.4. Animals

Honeybee pupae (stage P6) were taken from the hive and kept in an incubator at a constant temperature (33 °C) for 2 days. Their age was determined in agreement with data obtained by Winston (1987), using pigmentations of eyes, joints and legs as criteria for identifying the developmental stages. Adult honeybees were collected at the entrance of the hive when flying back to the colony after foraging.

2.5. In situ hybridization

In situ hybridization was performed on cryostat frontal sections of 20 μm thickness with digoxigenin-labelled RNA