Chapter 1

Identification of Cholinergic Synaptic Transmission in the Insect Nervous System

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Abstract

A major criteria initially used to localize cholinergic neuronal elements in nervous systems tissues that involve acetylcholine (ACh) as neurotransmitter is mainly based on immunochemical studies using choline acetyltransferase (ChAT), an enzyme which catalyzes ACh biosynthesis and the ACh degradative enzyme named acetylcholinesterase (AChE). Immunochemical studies using anti-ChAT monoclonal antibody have allowed the identification of neuronal processes and few types of cell somata that contain ChAT protein. In situ hybridization using cRNA probes to ChAT or AChE messenger RNA have brought new approaches to further identify cell bodies transcribing the ChAT or AChE genes. Combined application of all these techniques reveals a widespread expression of ChAT and AChE activities in the insect central nervous system and peripheral sensory neurons which implicates ACh as a key neurotransmitter.

The discovery of the snake toxin alpha-bungatoxin has helped to identify nicotinic acetylcholine receptors (nAChRs). In fact, nicotine when applied to insect neurons, resulted in the generation of an inward current through the activation of nicotinic receptors which were blocked by alpha-bungarotoxin. Thus, insect nAChRs have been divided into two categories, sensitive and insensitive to this snake toxin. Up to now, the recent characterization and distribution pattern of insect nAChR subunits and the biochemical evidence that the insect central nervous system contains different classes of cholinergic receptors indicated that ACh is involved in several sensory pathways.

Introduction

The cholinergic pathway is one of the most important excitatory neurotransmission system in insect nervous system. The neurotransmitter; acetylcholine (ACh), synthesized by cholinergic neurons, is widely distributed throughout the nervous system and plays a key role in the insect synaptic neurotransmission (Fig. 1).[1,2] Two major types of ACh receptors have been characterized in insects: muscarinic ACh receptors (mAChRs) and nicotinic ACh receptors (nAChRs). Numerous studies have revealed the pharmacological properties of mAChRs and nAChRs on

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Insect Nicotinic Acetylcholine Receptors, edited by Steeve Hervé Thany.

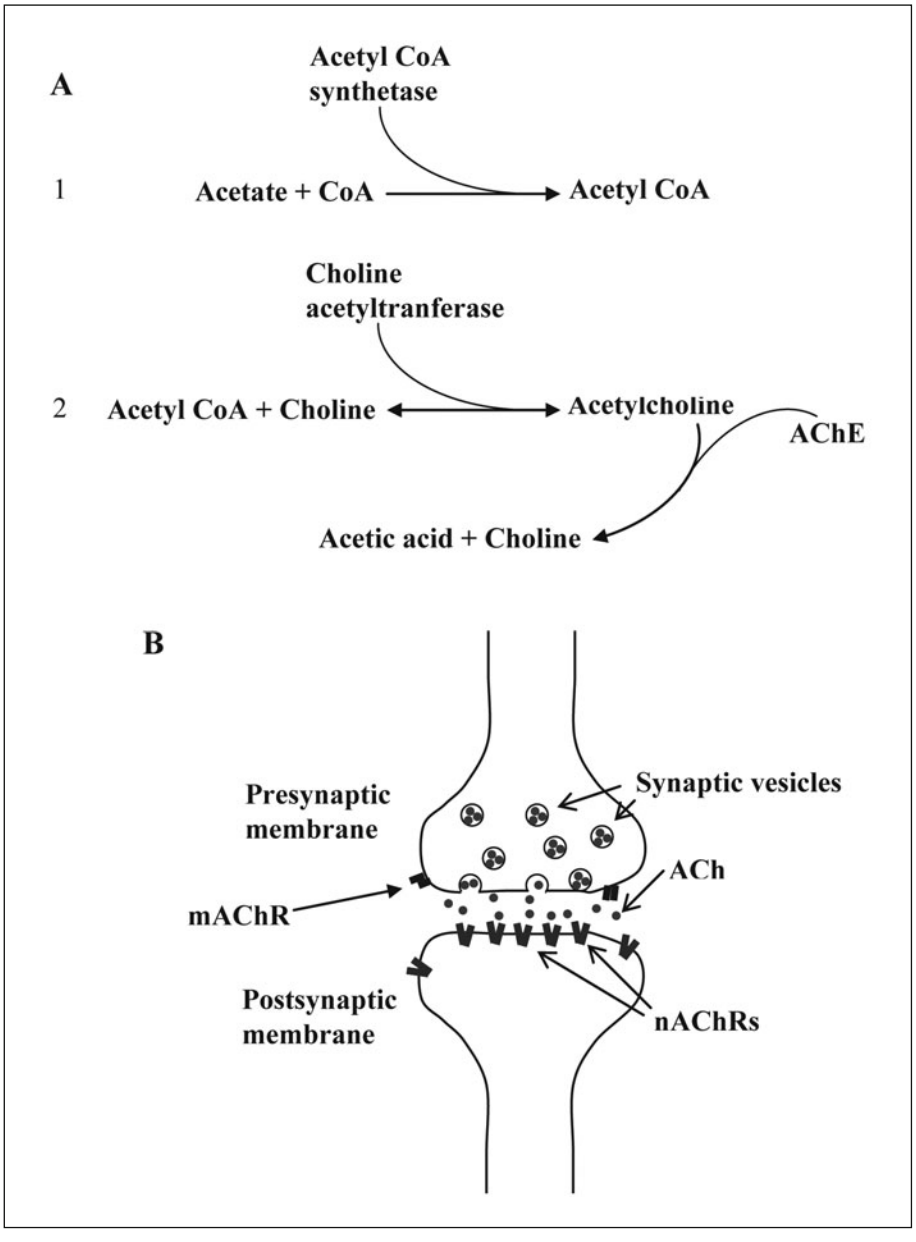

Figure 1. Schematic representation of the synthesis and hydrolysis of ACh.

synapse-free cell bodies as well as on the synapse-neuropile complex.[3-5] mAChRs have been reported as crucial element in the regulation of ACh release in insect synaptic neurotransmission. If it is well known that ACh interacts with postsynaptic nAChRs, the participation of mAChRs at presynaptic level has also been demonstrated electrophysiologically.[6,7] In this case, mAChRs sharing pharmacological properties very similar to vertebrate M2 mAChR-subtypes are involved

in the modulation of ACh release from presynaptic endings.[7] It has been demonstrated, particularly in cockroach central nervous system, that any means that increase ACh concentration within the synaptic cleft result in direct activation of presynaptic mAChRs involved in the negative feedback mechanism. At postsynaptic level, nAChRs are of particular interest because it has been shown that they are (1) involved in fast excitatory synaptic transmission and (2) are target sites for insecticides, particularly neonicotinoid insecticides.[8-10]

Additional complementary approaches including histochemical and immunocytochemical detection, electrophysiology and autoradiographic studies of choline uptake[11-15] have characterized the different physiological component of the cholinergic system corresponding to the ACh-synthesizing and –degrading enzymes such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), respectively. Their physiological implications have been clearly demonstrated since null mutations in either the ChAT or AChE genes result in the late embryonic lethality indicating the essential nature of ACh pathways in the insect.[16]

Because ChAT and AChE activities confirm the existence of both types of cholinergic receptor (i.e., mAChR and nAChR), alpha-bungarotoxin (α-Bgt)-binding studies have also been used to further identify, pharmacologically, insect nAChRs as sensitive- and insensitive-nAChR.[17] α-Bgt is the most useful tool in biochemical and pharmacological studies of putative nAChRs. This toxin is a component of the venom of the snake *Bungarus multicinctus*, which is a highly potent antagonist of nAChRs. Furthermore, it has been demonstrated that the insect nervous system contains high concentrations of α-Bgt binding sites.[18-20] Because α-Bgt blocks ACh-evocked excitatory postsynaptic potential (EPSP) in insect ganglia[3,21] the central role of nAChR subtypes in cholinergic synaptic transmission have been clearly established.[17,22] In the following sections we will discuss on the identification of cholinergic neurons and their distribution in the insect central nervous system (CNS).

Insect Acetylcholinesterase: Catalytic Properties and Tissue Distribution

Generally, it is suggested that the enzymes involved in ACh biosynthesis, ChAT and Acetyl CoA synthetase, are concentrated in the cytoplasm of cholinergic nerve terminals since ACh is thought to be synthesized mostly in these terminals. Conjugated choline is an indirect assay to identify cholinergic neurons because it is a metabolic product of ACh hydrolysis by AChE. The mode of ACh/AChE interaction is a matter of considerable interests. Generally, AChE is a type B carboxylesterase that rapidly hydrolyzes ACh at cholinergic synapses but also neuromuscular junctions. The AChE active sites are composed of an esteric region containing a nucleophilic serine residue that reacts covalently with acyl esters and an acid group interacting with ether the oxygen of ACh by hydrogen binding and an anionic site that interacts electrostatically with the onium head.[23,24] Hydrolysis of ACh involves an acetylation-deacetylation cycle. It has previously been demonstrated that the rate-limiting step is the deacetylation of the enzyme in vertebrates whereas it is the acetylation step in insect.[25] AChE occurs in several structurally distinct forms that can be differentiated by their number and type of subunits. Usually, in vertebrates, there are two acetylcholinesterases named AChE and butyrylcholinesterase (BuChE), both characterized by their capacity to hydrolyze very rapidly choline esters. Two main criteria allow to distinguish AChE from BuAChE. First, AChE and BuAChE seem to be typically blocked by different inhibitors such as carbamates and organophosphates. Second, vertebrates AChE hydrolyzes ACh or its analog acetylthiocholine (AcSCh) faster than propionylcholine (PrCh) or propionylthiocholine (PrSCh). Furthermore, it seems that AChE has not effect on butyrylcholine (BuCh) or butyrylthiocholine (BuSCh) while BuChE hydrolyzes BuCh or BuSCh.[24,26] In insects, despite the fact that AChE appeared to hydrolyze BuCh or BuSCh,[27] it is the only cholinesterase characterized today which is usually distinguished from others esters.

In vertebrates, the different forms of AChE correspond to different quaternary structures and exhibit different anchoring modes. AChE could exist as globular forms corresponding to the globular monomeric (G1), dimeric (G2) and tetrameric (G4) forms of catalytic subunits. These forms may be hydrophilic or amphiphilic. Asymmetric forms could also exist as one (A4) , two (A8) or three (A12) catalytic tetramers attached via a collagenous 'tail' (ColQ) or hydrophobic protein (P subunit) to the extracellular matrix.[28,29] In invertebrates, there are essentially globular forms. In insect, the amphiphilic form is generally prevalent. The mode of attachment of the amphiphilic forms in many insects is a glycophosphatidylinositol (GPI) anchor. This GPI anchor is covalently attached to a hydrophobic domain of the AChE-terminus. It has also been demonstrated that AChE possessed a molecular polymorphism with regard to the existence of amphiphilic and hydrophilic dimers of catalytic subunits which is present at all developmental stages.[29] Studies from *Musca* and *Drosophila* have suggested that the major form of AChE is an amphiphilic dimer which can be converted into a hydrophilic dimer and a hydrophilic monomer.[27,30,31] It is widely distributed in the brain, the thoracic and abdominal segments and the abdominal ganglia. The preferential distributions included the neuropiles of the CNS such as protocerebral and tritocerebral neuropiles, the lobula and medulla of the optic lobes, the central body, the internal of the calyces and the antennal glomeruli.[14,32-34]

Identification and Tissue Distributions of Choline Acetyltransferase in Insects

The most reliable marker of cholinergic neurons is the presence of ChAT, the enzyme catalyzing the synthesis of ACh. Cholinergic neurons can be detected by (1) using antibodies against ChAT, (2) *in situ* hybridization to detect the transcript and (3) staining for a reporter gene fused to the regulatory sequence of the ChAT gene.[35-37] In this case, ChAT has been first identified in the larval neuropil, including the larval antennal and optic lobes and in the gustatory target regions, which appeared to be due to the terminals of cholinergic afferents.[38-44] In *Drosophila melanogaster*, ChAT exists in cholinergic nerve terminals in at least two forms; a soluble and membrane-bound form that exhibit the same apparent molecular weight (75 kDa).[35] It should be noted that purification of locust ChAT results in a band at 65 kDa[45] very similar to that of reported in the spider *Cupiennius salei*.[46] The main reason that explains such difference may be that different forms of the enzyme exist.[41]

Histochemical detection of esterase activity and immunohistochemical localization of ChAT revealed that both enzymes are widely expressed in the insect nervous system. In fact, anti-ChAT immunoreactivity is well correlated to regions expressing high esterase activity.[14] In the locust ocellar visual system for instance, ChAT is also localized within synaptic clefts of L-neurons in both of the brain regions where L-neurons make excitatory and inhibitory output synapses. This suggests that ACh released by ocellar L-neurons can evoke fast excitatory and inhibitory postsynaptic potentials in different postsynaptic neurons.[40,41] Nevertheless, caution is needed in interpreting the significance of the presence of ChAT and AChE activities because staining of AChE has also been found in noncholinergic regions such as glial cells.[24,44] It seems that ACh in these cells could be involved in the axon guidance and elongation during development.[43,47]

Identification of Native Neuronal Nicotinic Acetylcholine Receptors

To obtain a first indication of the nAChR subtypes present in the insect CNS, certain α-neurotoxins such as α-Bgt was used as tool to identify specific high affinity binding sites. Thus, membrane extracts prepared from the insect CNS are rich in specific [^{125}I]-α-Bgt sites, exceeding the number of sites probed by the radiolabelled mAChR ligand quinuclidinyl benzilate.[20,48-50] Specific binding of radiolabelled α-Bgt has also been demonstrated in specific neuropiles of *Drosophila melanogaster*, *Manduca sexta*, *Acheta domesticus* and *Periplaneta americana*.[3,51-54] In fact, Schmidt-Nielsen and its colleagues have detected [^{125}I]-α-Bgt binding

in the head and thorax of *Drosophila*.[51] In the honeybee brain, binding sites are confined to neuropil areas, particular to those with primary and higher order sensory projections.[15] The distribution of [^{125}I]-α-Bgt binding resembles to that reported in D. *melanogaster* and *M. sexta*. In addition, similar distribution has also been identified in the abdominal ganglia of *P. americana* and *A. domesticus* at sites of synaptic connexion.[3,55] In some insects, a single high affinity α-Bgt binding site (with similar pharmacological profile) has been identified. These results differ from recent findings showing the presence of both high and low affinity binding sites in *Myzus persicae* membranes.[56] In fact, data for [^{125}I]-α-Bgt saturable binding are consistent with two binding components, one of high affinity (K_d = 1.2 nM) and one of low affinity (K_d = 33 nM). This multiple α-Bgt binding sites suggest the presence of either multiple receptor subtypes or the cooperativity between nicotinic binding sites on the same receptor[56]. Because of these potential multiple α-Bgt sites, other studies have used additional radioligand binding studies with the norditerpenoid alkaloid methyllicaconitine (MLA) isolated from species of *Delphinium spp* (larkspurs) to confirm α-Bgt high affinity binding sites. MLA is a competitive nicotinic antagonist which is more discriminating than α-Bgt.[57] The distribution of [^{3}H]MLA and [^{125}I]-α-Bgt bindings sites have been compared in the brain of *Manduca sexta*. The patterns of labelling with both ligands are similar in the neuropil areas of the brain with a specific [^{125}I]-α-Bgt binding in an external area of the lamina and adjacent to the tapetum, a reflective tracheal structure at the base of the retina.[58] It is interesting to note that the isotopic dissociation of [^{3}H]-α-Bgt from *Myzus persicae* membranes is accelerated when initiated by MLA. These dissociation kinetics seem to be consistent with a model in which at least the majority of [^{3}H]-α-Bgt high affinity binding sites have two different ligand binding pocket (or domain) at each nicotinic receptor.[56]

Recently, neonicotinoid insecticides such as imidacloprid and azidonicotinoid have been used as ligand in order to identify specific nAChR subtypes. In *D. melanogaster*, the [^{3}H] IMI binding site is distinct from that for either [^{3}H]EPI and [^{3}H]α-Bgt. Saturable binding of [^{3}H]IMI to membranes of the peach potato aphid *Myzus persicae* revealed the presence of both high- and low-affinity [^{3}H]IMI binding sites having distinct dissociation constants and maximal binding capacities. Moreover, saturable [^{3}H]IMI binding analyzed on different insects, namely, *Nephotellix cincticeps* (Hemiptera: Cicadellidae), *Periplaneta americana* (Dictyoptera), *Lucilia sericata* (Diptera: Calliphoridae), *Drosophila melanogaster* (Canton S) (Diptera: Drosophiloidea), *Manduca sexta* (Lepidoptera: Sphingidae), *Heliothis Virescens* (Lepidoptera: Noctuidae) and *Ctenocephalides felis* (Siphonaptera: Pulicidae), have demonstrated that only aphids and green leaf hoppers (both hemiptera) have very high-affinity binding sites for [^{3}H] IMI.[59] This may indicate that only a single class of binding site is present and/or that there is no cooperativity between binding sites.

Insect Nicotinic Acetylcholine Receptors Subunit Localizations

Over the last few years, different type of nAChRs have been discriminated electro-pharmacologically and numerous nAChR subunits have been cloned from several insect species.[60-64] Their localization identified from some insect including fly *Drosophila melanogaster*,[65-68] house fly *Musca domestica*,[69] locusts *Locusta migratoria*[70] cockroach *Periplaneta americana*[21,22] and honeybee *Apis mellifera*.[63,71,72] Northern blot analysis and *in situ* hybridization from the *Drosophila* transcripts encoding ALS, Dα2, SBD and ARD subunits are detected from 10 to 12h embryos.[73-76] Similar staining of honeybee Apisα3 mRNA have been found in the late larvae around day 5 after egg laying[71] and in the *Locusta migratoria* Locα1, Locα2 and Locβ2 subunits around day 7,[70] showing that nAChR subunit expression begin in the earlier insect stage. Despite the fact that it is difficult to identify the precise stained structures, it appeared that nAChR subunit expressions are localized into specific regions. In fact, during the late embryogenesis, intense staining with an SBD probe is observed in the *D. melanogaster*

sub- and supraosoephageal ganglia and the ventral cord. Hybridization with an ALS-specific probe also results in staining of the same parts of the embryonic CNS.[77] Similar expression is also detected at day 8 after egg laying, in the honeybee suboesophageal ganglion, which is found at the origin of the mandibular, maxillar and labial nerves that supply the mouthparts.[71] In the adult, immunohistochemical studies performed with anti-Dα3, anti-ARD and anti-Dα7 antibodies show a strong staining into the neuropils of the lamina, the medulla, the lobula and the lobula plate.[66,67] Similarly, the SBD and ALS subunits have also a distribution in the adult optic lobes.[65] Because ALS, Dα2 and SBD can be copurified by α-Bgt affinity chromatography, this specific colocalization allowed to consider the existence of an nAChR subtype including these subunits in the optic lobes. It is interesting to note that similar expression pattern has been identified into the honeybee *Apis mellifera* optic lobes for Apisα2, Apisα3 and Apisα7-2.[72] Regarding the subunit expressions in the adult neuropil, it is tempting to consider that nAChRs including these subunits have important function in the visual system. This has been recently studied using Dα7 subunit mutant flies. In this case, mutant flies have a defect in the jump circuit. Mutation disrupts sensory input to the giant fibers which thereby alters the *D. melanogaster* escape behavior.[67]

Conclusion

nAChRs mediate synaptic signal transmission in insect CNS. ACh release from the presynaptic nerve terminal of cholinergic synapses binds to its recognition site at postsynaptic AChR and induces conformational changes of the ionotropic receptor proteins. Both AChE and ChAT activities expressed at cholinergic synapses demonstrate the key role of ACh in the central nervous system of insects, in which ACh is a major excitatory neurotransmitter. Nevertheless, the presence of AChE and ChAT in noncholinergic tissues raises the question of a possible role of AChE distinct from the classical hydrolysis of ACh. In fact, it has previously been reported that this enzyme presents a remarkably wide range of noncholinergic functions such as cell adhesion, proliferation and neurite outgrowth.[78-80] The use of tritiated nicotinic agonists (nicotine, cytisine, methylcarbamylcholine...) or antagonists (α-bungarotoxin and methyllicaconitine) have given new insights on the characterisation of high and low affinity of nicotinic agonist binding sites. Thus nAChR subtypes have been divided in two categories, sensitive and insensitive to the α-Bgt. The hypothesis that the major neuronal nAChR is a homomeric molecular structure is advocated by the concordance between [^{125}I]-α-Bgt binding sites and presumably vertebrate homomeric α7-like receptors. But, the differences in binding studies reported in some insects suggest that they may contain several types of nAChRs, as it is the case in vertebrates. Finally, the criteria for identifying nAChR subtypes and functional properties of cholinergic pathways will be the isolation of insect nAChR subtypes. Such studies are now in progress.

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