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Molecular Interactions between Ligands and Nicotinic Acetylcholine Receptors Revealed by Studies with Acetylcholine Binding Proteins

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Abstract

Nicotinic acetylcholine receptors (AChRs) are the best characterized ion channels representing the Cys-loop ligand-gated ion channel superfamily. Studies using *Torpedo* AChRs in the closed and open states and acetylcholine binding proteins (AChBPs) from different origins have elucidated the most important structural and functional features of the agonist/competitive antagonist binding sites. The first step in recognizing the neurotransmitter ACh and other agonists is fundamental in the process of agonist-induced activation, including the opening of the intrinsic cation channel. The AChBP studies demonstrated that Loop C is an important structural feature that is modified by ligand binding. These studies defined important pharmacologic features of AChR ligands, including the differences between full and partial agonists, agonists and competitive antagonists, peptidic and non-peptidic ligands, and between high affinity and high selectivity. The studies showing the structural mechanisms by which specific ligands can activate, inhibit, and potentiate different AChR subtypes could be of therapeutic importance.

Keywords: Cys-loop ligand-gated ion channels; Nicotinic acetylcholine receptors; Acetylcholine binding proteins; Loop C; Crystallography

Abbreviations: AChR: Nicotinic acetylcholine receptor; AChBP: Acetylcholine bound protein; 5-HT: 5-Hydroxytryptamine (serotonin); ACh: Acetylcholine; GABA: γ-aminobutyric acid; DMXBA: 3-(2,4 dimethoxybenzylidene)-anabaseine; α-BTx: α-bungarotoxin; κ-BTx: κ-bungarotoxin; α-CbTx: α-cobrotoxin; MLA: Methyllycaconitine; d-TC: d-tubocurarine; DHβE: Dihydro-β-erythroidine; SPX: 13-desmethyl spirolide C; GYM: Gymnodimine A; α-CTx: α-conotoxin; $MTSET^+$: 2-(trimethylammonium) ethylmethanethiosulfonate; MMTS: methylmethanethiosulfonate; Ls: Lymnaeastagnalis; Ac: *Aplysia californica*; Bt: *Bulinus truncates*; Bg: *Biomphalaria glabrata*; *Ct: Capitella teleta;* GLIC: *Gloebacter violaceus;* ELIC: *Erwinia chrysanthemi*

Introduction

A vast amount of evidence indicates that nicotinic acetylcholine receptors (AChRs) are important for the homeostasis and function of our body. AChRs are cation channels members of the Cys-loop ligand gated ion channel superfamily, including type 3 serotonin (5-hydroxytryptamine; 5-HT) cation channels as well as anion channels such as type A and C γ-aminobutyric acid (GABA) and glycine receptors [1-3]. A large number of subunits have been cloned for all members of the Cys-loop super family from vertebrates and invertebrates (Ligand-gated ion channel database, http://www.ebi.ac.uk/compneur-srv/LGICdb/cys-loop.php) [4]. In vertebrates, AChR subunits are classified in two types, α (i.e., α1-α10) and non-α (i.e., β1-β4, γ, δ, and ε), where α subunits contain a disulphide bridge in the binding site, whereas non-α subunits do not. AChRs can be formed by the same subunit comprising homomeric receptors [e.g., α7, α8 (expressed only in chicks), and α9] or by different subunits comprising heteromeric receptors (e.g., α4β2, α3β4) [5,6]. However, not all possible subunit arrangements are functional. For example, the neuronal AChR subunits α2-α4, β2, and β4 can co-assemble in pair wise combinations forming functional AChRs (e.g., α4β2, α3β4). Although the α9 subunit is actually expressed in the cochlea, the functional receptor in hear cells is the α9α10 AChR subtype. Although the homomeric α7 AChR is functional, α7 AChRs containing other subunits (e.g., α7β2) have been characterized endogenously and probably these are the native AChRs. Each α5, α6, and β3 subunit does not form functional AChRs by binary combinations with another subunit, instead they prefer ternary combinations [e.g., $(\alpha4)2\alpha5(\beta2)_2$]. AChRs can even be formed by assembling four different subunits [e.g., $\alpha4\alpha6\alpha5(\beta2)_2$], but there is no evidence of a receptor subtype formed by five different subunits. In addition, two different stoichiometries of the α4β2 AChR (and probably for the α3β4 AChR as well), $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$, have been found in heterologous [7-10] and endogenous [11] cells. The $(\alpha4)_2(\beta2)_3$ stoichiometry is more sensitive to the action of agonists including agonist induced up-regulation, desensitizes less rapidly, and has lower Ca^{2+} permeability compared to that for the $(\alpha4)_3(\beta2)_2$ stoichiometry. The use of subunit concatemers might be useful in determining the subunit composition and stoichiometry of native AChRs [5].

Vertebrate AChRs are expressed in neuronal and non-neuronal tissues. In the peripheral and central nervous systems, presynaptic AChRs modulate the release of several neurotransmitters, including ACh, 5-HT, GABA, dopamine, norepinephrine, and glutamate, whereas postsynaptic AChRs mediate rapid transmission by converting a chemical signal into membrane depolarization (i.e., electrical signal), or trigger cytoplasmic cascades. These combined actions modulate several important functions in our body, including cognition, memory, pain perception, auditory response, and muscle contraction. In non-neuronal tissues, AChRs are involved in angiogenesis and immune responses [12,13]. Since AChRs are involved in such important physiological functions, their improper activities (e.g., decreased number, mutations, or hypo/hyperactivity) can produce several diseases,

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including Alzheimer's disease, Parkinson's disease, schizophrenia, nocturnal frontal lobeepilepsy, attention deficit hyperactivity disorder, Tourette's syndrome, drug and nicotine addictions, depression and anxiety, myasthenia gravis, myasthenic syndromes, tumor growth, and decreased immune response [2,3,12-15].

Several AChR subunits have been also cloned in insects and invertebrates [16]. In invertebrates, AChRs have become key elements for the development of neuroactive pesticides. For example, several neonicotinoids (e.g., imidacloprid, clothianidin, and thiacloprid) have insecticide activities but extremely low mammalian toxicities due to their high affinity and specificity for insect AChRs [16].

Breakthroughs in the Study of AChRs

Three main breakthroughs in the last 10 years have helped in elucidating the functionally relevant structural features of AChRs and their cousins [1,17,18]: (1) the *Torpedo* AChR structures in the closed [19] and open [20] states, showing the main features at the extracellular, transmembrane, and intracellular domains (Figure 1), (2) the crystal structures of several acetylcholine binding proteins (AChBPs) showing details of the binding sites for agonists and competitive antagonists [21], and (3) the recently elucidated prokaryotic cation channels showing subtle differences between the open and closed conformations, as well as between activated (i.e., with several primary amines [22]), blocked (i.e., with several cations and open-channel blockers [23]), and inhibited (i.e., with several allosteric modulators [24]) states. Table 1 summarizes the different three dimensional structures obtained so far for *Torpedo* AChRs and different AChBPs bound to a variety of ligands.

Structurally, AChRs are pentameric proteins with an extracellular domain that carries the binding sites for agonists (e.g., the neurotransmitter ACh and nicotine) and competitive antagonists [e.g., methyllycaconitine (MLA)]. In addition to these orthosteric sites, several additional sites have been characterized in this domain for allosteric modulators [25,26]. The transmembrane domain, specifically the M2 transmembrane segments from each subunit, forms the ion channel that is essential for cation flux (i.e., Na^+ and Ca^{2+}influx and K^+ efflux), finally producing membrane depolarization, and in the case of Ca^{2+}, triggering different intracellular pathways.

The ion channel is the maindomain for the interaction with a very broad group of compounds called noncompetitive antagonists [14,15,27]. In addition, several binding sites for negative allosteric modulators have been characterized between the four M1-M4 transmembrane segments [25,26].

Agonist binding at the main domain domain triggers the opening of the ion channel, an intrinsic process called gating [1]. Determining how the agonist-induced structural changes, that start in the agonist binding pocket, are propagated through a distance of ~50 Å to the gate is central for the understanding of the receptor function. One of the AChR domains that have recently attracted attention among researchers is the extracellular–transmembrane interface. This is a very unique transitional zone where β-sheets from the extracellular domain merge with α-helices from the transmembrane domain, finally allowing functional communication between both domains [1,25,26].

Overall Structure of AChRs

AChRs are pentameric proteins where the subunits are arranged around an axis perpendicular to the membrane, and each subunit shares a basic scaffold composed of: (1) a large N-terminal extracellular domain of ~200 amino acids; (2) three transmembrane domains (M1-M3) separated by short loops; (3) a cytoplasmic loop of variable size and amino acid sequence; and (4) a fourth transmembrane domain (M4) with a relatively short and variable extracellular COOH-terminal sequence (Figure 1). AChRs are therefore built on a modular basis, with the extracellular domain containing the agonist binding sites, the transmembrane domain containing the pore, selectivity filter, and channel gate, and the cytoplasmic domain performing additional modulatory activities [19,20].

Recent structural studies have provided details of the three dimensional structure of AChRs and consequently for other members of this receptor superfamily. In particular, the structural model of the *Torpedo* AChR at 4 Å resolution [19] has revealed important information and has been invaluable in the interpretation of functional and pharmacological data. Although no structural information is available for any Cys-loop receptor at the atomic resolution level, the extracellular domain of the AChR α1 subunit has been resolved at 1.94 Å [28]. Additional high resolution structural information has become available from studies of proteins which show close structural similarity to AChRs, including soluble AChBPs from a variety of animals from the Mollusca [21,29] and Annelida [30] phyla [1,17], as well as prokaryotic proton-gated ion channels from the bacteria *Erwinia chrysanthemi* (ELIC) and *Gloebacter violaceus* (GLIC), respectively [1,18]. The characterization of GLIC showed that it forms a cation-selective channel that is activated by protons, where currents do not decay during activation, suggesting no or very slow desensitization [31]. Recent results using ELIC showed that different primary amines, including GABA, can also activate this channel [22]. The ELIC X-ray structure shows 16% sequence identity to αAChR subunits. In general, the extracellular domain is very similar to its eukaryotic counterpart and to AChBPs, but lacks the N-terminal α-helix. However, the putative binding site and several of the aromatic residues found in AChRs are conserved. The central part of the Cys-loop is also conserved but lacks the flanking disulfide-bridge.

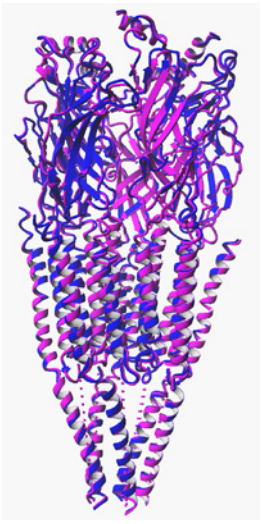

Figure 1: *Torpedo* AChR model showing the structural differences between the open (magenta) and closed (blue) states at 6.2 Å resolution (modified from [20]). Only subtle differences can be seen between both conformational states. AChRs are formed by three main domains: The extracellular domain shows the β-strands forming the β-sandwich structure.The transmembrane domain is formed by 20 α-helices, four α-helices per subunit. The cytoplasmic domain is the smallest domain.

The Extracellular Domain

The solution of the high-resolution structure of the AChBP from *Lymnaea stagnalis* (Ls-AChBP) was a giant step forward for our knowledge of the structure of the extracellular domain of AChRs [1,17,21]. Since then, several other AChBPs from mollusks and annelids have been characterized (Table 1). In general, AChBPs lack the transmembrane region but contain many of the structural features that give AChRs their unique signature and have therefore become functional and structural surrogates of the extracellular domain of the Cys-loop receptor superfamily. The AChR extracellular domain contains 210 amino acids and shares ~15-24% sequence identity to aligned sequences of the amino-terminal, extracellular halves of Cys-loop receptor subunits. Each AChBP monomer consists of an N-terminal α-helix, two short 3_{10} helices, and a core of 10 β-strands that form a β-sandwich structure. The inner β-sheet is formed by the β1, β2, β3, β5, β6 and β8 strands, and the outer β-sheet by the β4, β7, β9 and β10 strands. The N- and C- terminals are located at the top and bottom of the pentamer, respectively. In Cys-loop receptors, the end of β10 connects to the start of M1. Located at the bottom of the subunit; the linker between β6 and β7 strands is the signature Cys-loop found in all members of the superfamily, including bacterial ion channels.

Agonist binding sites are located at the subunit interfaces [1-3,17,18]. Each binding site isformed by two faces. One face, called the principal or "positive" face at the α-subunit, is formed by β-strands connected by three loops harboring key aromatic residues [i.e., Loop A (β4β5 loop), Loop B (β7β8 loop), and Loop C (β9β10 loop)]. The complementary or "negative" face at the non-α-subunit contributes with three β-strands clustered in segments by Loops D-F. Thus, key residues (corresponding to *Torpedo* α1-subunit) from the principal face come from Loop A (Trp86 and Tyr93), Loop B (Trp149 and Gly153) and Loop C (Tyr190, Cys192, Cys193 and Tyr198). The complementary face is formed by residues from Loop D (Trp55 and Asp57), Loop E (Leu109, Arg111, Thr117 and Leu119), and Loop F (Asp174 and Glu176) (residues from the *Torpedo* δ- or γ-subunit) [1,21,32].

The ancestral Cys-loop receptor was likely homomeric and contained five identical binding sites, similarly to present day homomeric receptors, such as α7 and 5-HT_{3A} receptors [32,33]. Evolution led to the appearance of new subunits which lost the ability to form agonist binding sites, giving rise to heteromeric receptors with fewer than five binding sites. The prototypic heteromeric receptors, muscle AChR and $GABA_A$Rs, contain only two agonist binding sites, which have to be both occupied to allow appropriate gating. Although homomeric receptors contain five identical binding sites, it was shown that occupancy of only three of the five sites is required for optimal activation [34].

Structural Changes of Loop C when Interacting with Agonists and Small Competitive Antagonists

Several lines of evidence indicate that ligands at the agonist binding site are stabilized by π-cation, dipole-cation, hydrogen bonding, and van der Waals interactions [35,36]. Agonist interaction produces activation (opening) of the AChR ion channel. The transition from the resting to the activated state is relatively fast, although the time regime is different among receptor subtypes. Molecular details about the activation process were determined by studies on AChBP- and AChR-bound ligand structures. For example, structural differences between the closed and open states permitted to determine that ACh elicits clockwise rotation of the inner β-sheet with respect to the outer β-sheet, causing tilting of the Cys-loop (Loop C) away from the five-fold axis [20].

The results using AChBPs with different ligands indicate that Loop C from the principal face is in an extended ("open") conformation in the resting AChR (no agonist), whereas in the presence of full agonists, Loop C is contracted ("closed") and caps the entrance to the binding cavity, trapping the agonist [36,37]. Cysteine substitution and subsequent oxidation studies on Loop C of muscle AChRs indicate that Loop C capping is involved in the transition of the closed receptor to an activated pre-open intermediate state [38].

The interaction of several agonists (full and partial), competitive antagonists, and allosteric modulators with their binding sites was studied in minute detail by co-crystallization of various ligands with AChBPs from different species (Table 1). In addition to structural differences between ligands, diverse AChBP selectivity for several ligands was observed [17,39]. The structural basis to distinguish high affinity *vs* high selectivity for different ligands [e.g., d-tubocurarine (d-TC) and strychnine] was also described [40]. Another important structural difference is the interaction between full and partial agonists. In general, AChBP-agonist complexes show a fully contracted (closed) state, whereas AChBP-antagonist complexes show a more extended (open) state. However, additional experiments comparing the full agonist nicotine and the partial agonists cytisine and varenicline could not discriminate any variation in the Loop C closure [41]. In particular, varenicline interacts with highly conserved aromatic amino acids at the principal face of the binding site, and with less conserved hydrophobic residues at the complementary face (e.g., at Loop E) [30]. Interestingly, dihydro-β-erythroidine (DHβE), a potent competitive antagonist of β2-containing AChRs, imposes closure of the Loop C as agonists do, but also induces a structural change perpendicular to the observed Loop C movements [42]. To illustrate some important differences between agonists and competitive antagonists, AChBP structures complexed with the partial agonists varenicline and lobeline (Figure 2) and the competitive antagonist DHβE (Figure 3) are shown.

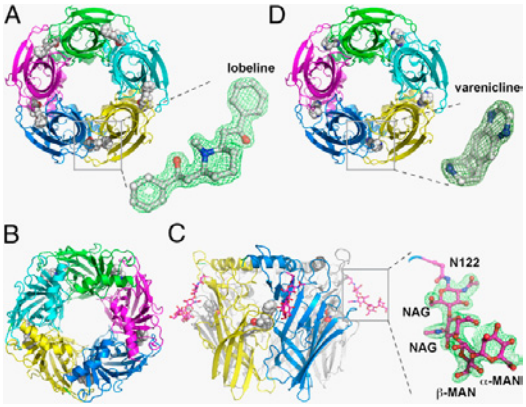

Figure 2: Structure of AChBP from *Capitella teleta* (Ct-AChBP) complexed with the partial agonists lobeline (A-C) and varenicline (D) respectively (modified from [30]). AChBP-lobeline structures showing the N-terminus pointing away (A) or toward (B) the viewer, and the subunit interface (principal face in yellow and complementary face in blue) towards the viewer (C). Each of the five subunits is displayed in a different color. Oxygen, red; nitrogen, blue; sulfur, green. The glycosyl chain N-linked to N122 is shown in magenta. The paucimannose chain is composed of N-acetylglucosamine (NAG), α-D-mannose (α-MAN), and β-D-mannose (β-MAN) (D) Ct-AChBP-varenicline complex. Lobeline and varenicline complexed with Ct-AChBP are shown as spheres, whereas the insets show the electron density of each molecule.

Structure	Ligand bound	Conformational state	PDB	Resolution (Å)	Reference
AChR	None	Closed (Resting)	2BG9	4.0	[19]
AChR	ACh ACh	Closed (Desensitized) Open	4AQ5 4AQ9	6.2 6.2	[20]
α1	α-BTx	Open	2QC1	1.94	[28]
α1-13-residue peptide	α-BTx	-	1HCB	1.8	[52]
Ls-AChBP	HEPES	Intermediate	1I9B	2.70	[21]
Ls-AChBP	HEPES (-)-Nicotine CCh	Intermediate Closed Closed	1UX2 1UW6 1UV6	2.2 2.2 2.5	[53]
Ls-AChBP	α-CbTx	Open	1YI5	4.20	[54]
Ls-AChBP	DHβE	Closed	4ALX	2.51	[42]
hα7/Ls-AChBP chimera	n-Acetyl-d-glucosamine (+)-Epibatidine	Apo Closed	3SQ9 3SQ6	3.10 2.80	[62]
Bt-AChBP	CAPS	Intermediate	2BJ0	2.0	[55]
Ac-AChBP	None (+)-Epibatidine α-Lobeline MLA α-CTx ImI	Apo Closed Closed; g-to-*t* Intermediate Open	2BYN 2BYQ 2BYS 2BYR 2BYP	2.02 3.40 2.05 2.45 2.07	[46]
Ac-AChBP	HEPES α-CTx PnIA(A10L/D14K)	Intermediate Open	2BR7 2BR8	3.0 2.4	[56]
Ac-AChBP	SPX GYM	Intermediate Intermediate	2WZY 2X00	2.51 2.40	[49]
Ac-AChBP	α-CTx ImI	Open	2C9T	2.2	[57]
Ac-AChBP	α-CTx TxIA(A10L)	Open	2UZ6	2.4	[50]
Ac-AChBP	Cocaine Galantamine	Unchanged Unchanged	2PGZ 2PH9	1.76 2.88	[43]
Ac-AChBP	Imidacloprid Thiacloprid	Closed Closed	3C79 3C84	2.48 1.94	[58]
Ac-AChBP	Clothianidin Imidacloprid	Closed Closed	2ZJV 2ZJU	2.70 2.58	[16]
Ac-AChBP	Sulfate	Coordinated with Lys residues	3GUA	3.10	[59]
Ac-AChBP	Anabaseine DMXBA 4OH-DMXBA Tropisetron	Closed Intermediate Intermediate Intermediate	2WNL 2WNJ 2WN9 2WNC	2.70 1.80 1.75 2.20	[45]
Ac-AChBP	None Compound 31 Compound 35	Apo; g Intermediate Intermediate	2Y7Y 2W8F 2W8G	1.89 2.7 2.8	[44]
Ac-AChBP	Metocurine d-TC	- -	3PEO 3PMZ	2.10 2.44	[60]
Ac-AChBP	Fragment 1 Compound 3 Compound 4 Compound 6	Open; g g-to-*t* g-to-*t* endo	2Y54 2Y56 2Y57 2Y58	3.65 3.59 3.30 3.25	[39]
Ac-AChBP (Y53C mutant)	$MTSET^+$ MMTS and ACh	Open Closed	2XZ6 2XZ5	3.14 2.80	[51]
Ac-AChBP	d-TC Strychnine	Open Closed	2XYT 2XYS	2.05 1.91	[40]
hα7/Ac-AChBP chimera	Mutant I Mutant II + MLA Mutant III + MLA	Apo - -	3T4M 3SH1 3SIO	3.00 2.90 2.32	[61]
Ac-AChBP	Compound 18 (complex I) Compound 6 (complex II) Compound 6 (complex III)	Open Open Open	2XNT 2XNU 2XNV	3.21 2.55 2.44	[63]
Ac-AChBP	Varenicline Cytisine	Closed Closed	4AFT 4AFO	3.20 2.88	[41]
Ac-AChBP	Triazole 18	-	4DBM	2.30	[64]
Bg-AChBP1 Bg-AChBP2	None None	Pentagonal dodecahedron	4AOD 4AOE	~6 ~6	[29]
Ct-AChBP	None	Apo	-	Low resolution	[65]
Ct-AChBP	Varenicline α-Lobeline	Intermediate; g-to-*t* Closed; g-to-*t*	4AFG 4AFH	2.0 1.9	[30]
Ct-AChBP	Compound 5	Intermediate	4B5D	2.3	[66]

AChRs are obtained from *Torpedo marmorata* electric fish.

AChBPs are obtained from *Lymnaea stagnalis* (Ls), *Aplysia californica* (Ac), *Bulinus truncates* (Bt), *Biomphalaria glabrata* (Bg), and *Capitella teleta* (Ct), respectively.

The close (i.e., contracted) and opened (i.e., extended) states of Loop C in the AChBP-ligand complexes indicate the active/desensitized (i.e., agonists) and resting (i.e., antagonists) states, respectively. Several ligands, including partial agonists, adopt an intermediate configuration between that for full agonists and competitive antagonists, whereas allosteric modulators (e.g., cocaine and galantamine) do not produce any apparent change on Loop C.

The g-to-*t* (also called Tyr-flip) conformational state corresponds to the opening of the lobeline pocket. The g state is the closed lobeline pocket, whereas in the endo configuration the ligand is unable to induce the opening of the lobeline pocket.

Table 1: Conformation states of AChRs and AChBPs in unbound and bound conditions.

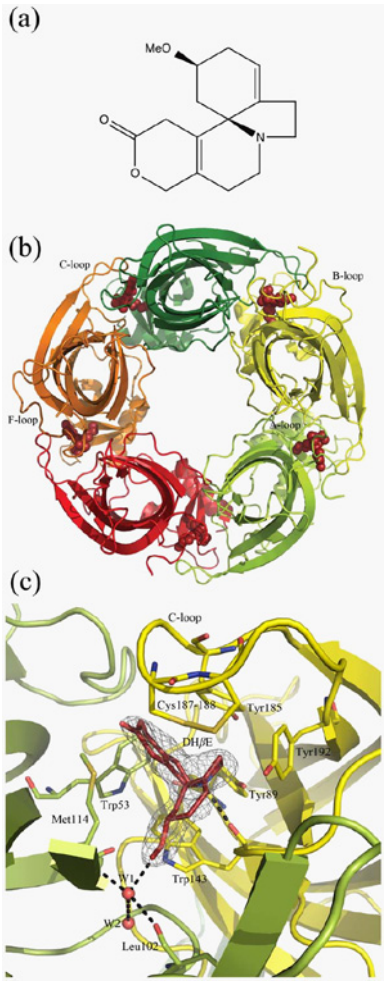

Figure 3: Structure of AChBP from *Lymnaea stagnalis* (Ls-AChBP) complexed with the competitive antagonist dihydro-β-erythroidine (DHβE) (modified from [42]). (a) Structure of DHβE. (b) Complex viewed along the five-fold symmetry axis. The five subunits are shown in different colors and DHβE in red spheres. (c) Interfacial binding pocket formed by the highly conserved aromatic residues Tyr89, Trp143, Tyr185, and Tyr192 from the principal side of the interface (yellow) and Trp53 from the complementary side (limon). DHβE is shown in red, and hydrogen bonds between DHβE and its surroundings are shown as stippled lines.

Some other ligands produce an intermediate state, whereas the allosteric modulators cocaine and galantamine do not produce any apparent conformational change [43]. For example, the dibenzosuberyl-atropine analogs, compounds 31 and 35, which pharmacologically act as mixed competitive/noncompetitive antagonists on AChRs [44], induce an intermediate open configuration of Loop C, suggesting an intermediate resting/activated state. Interestingly, partial agonists of the α7 AChR such as DMXBA [3-(2,4-dimethoxybenzylidene)-anabaseine], its hydroxyl metabolite 4OH-DMXBA, and tropisetron, display multiple orientations within the five binding sites and adopt an intermediate configuration between that for full agonists and competitive antagonists [45]. Nevertheless, the partial agonist α-lobeline induces a strong Loop C closure [30,46]. More specifically, the interaction of α-lobeline with the AChBP site opens a subpocket to accommodate the α-hydroxyphenetyl moiety, inducing the g-to-t (also called Tyr-flip) conformational state [39], where the g state is the closed lobeline pocket, whereas in the endo configuration the ligand is unable to induce the opening of the lobeline pocket. These results suggest that the different occupation and orientation between full and partial agonists may produce distinct conformations of Loop C. Interestingly, α-lobeline binds to Ct- and Ac-AChBPs at different configurations [30,46]. In Ct-AChBP, the α-lobeline piperidine ring adopts a chair conformation and its hydroxyl group is in the S-configuration, whereas in the Ac-AChBP the ring adopts a half-chair conformation and its hydroxyl group is in the R-configuration.

The AChBP structures in the absence of ligands or buffer molecules (i.e., Apo) show that the Loop C is more flexible than that in the presence of ligands, and that there are ordered water molecules filling the pocket. This structure is reminiscent to the *Torpedo* AChR model in the resting state [19]. Instead, buffer molecules (e.g., HEPES, CAPS) in the binding pocket move Loop C in a more open configuration, suggesting an intermediate state [46].

Structural Changes of Different Loops when Interacting with Large Competitive Antagonists

Competitive antagonists overlap the agonist sites and inhibit their pharmacological action. In addition to this competitive (steric) mechanism, there is information that some antagonists [e.g., α- and κ-bungarotoxin (α- and κ-BTx), α-cobrotoxin (α-CbTx), and methyllycaconitine (MLA)] maintain the AChR in the resting state [36,47,48], probably inhibiting the gating process. At the molecular level, MLA, the potent competitive antagonist of the α7 AChR, induces an intermediate open configuration of Loop C [46]. Although the Loop C conformation distinguishes agonists from competitive antagonists, additional results indicate that Loop F is another structural component responsible for ligand selectivity; especially for antagonists [45]. Although the toxins 13-desmethyl spirolide C (SPX) obtained from *Alexandrium ostenfeldii* and gymnodimine A (GYM) obtained from *Karenia selliformis* are among the most potent non-peptidic antagonists (i.e., binding affinities in the subnanomolar concentration range), they are nonselective antagonists, probably because they interact in less proportion with Loop F in the complementary face [49]. Crystallographic information supports the view that in general peptidic antagonists (i.e., snail and snake neurotoxins) maintain the AChBP in the resting state, whereas other nonpeptidic antagonists induce an intermediate state (Table 1). Different snail α-conotoxins (α-CTxs) also differ in their intrinsic orientations [50], probably resembling their receptor selectivities.

The Ac-AChBP-Y53C mutant binds to Cys-modifying agents by different manners [51]. In this regard, MTSET$^+$ [2-(trimethylammonium) ethylmethanethiosulfonate] produces a less constricted Loop C, similar to peptidic antagonists, whereas the conformation of Loop C in the presence of MMTS (methylmethanethiosulfonate) and ACh resembles that for agonists (Table 1). Since the location of the conserved amino acid Y53 is at Loop D, these results also emphasize the concept of concerted interactions between Loops D and C. Another important difference between agonists and antagonists is that cation–π interactions are formed between the conserved Trp residue in Loop B (i.e., W143 in Ls-AChBP) and agonists such as (-)-nicotine [53], whereas no cation–π interactions are formed with antagonists such as α-CTxs [46,50,56,57] and neonicotinoids [16,58]. Taking into account all studied ligand interactions, a good correlation (with some few exceptions) between Loop C closure and the type of ligand (i.e., full and partial agonists, competitive antagonists, and modulators) was found [1]).

In addition to details on ligand interactions, AChBP studies have also helped in finding an ion selectivity filter in the extracellular domain

of Cys-loop ligand-gated ion channels [59]. This selectivity filter is negatively charged in cation ion channels and positively charged in anion ion channels.

Concluding Remarks

AChRs mediate rapid transmission throughout the nervous system and also present functional roles in non-neuronal tissues. Structural and functional studies permitted to highlight the importance of the extracellular domain for the binding of agonists, competitive antagonists, and allosteric modulators. It is remarkable the advancement in our knowledge on ligand recognition and binding through the study of AChBPs from mollusks and annelids bound to a variety of ligands at atomic resolution. The current knowledge of the structural components of the AChR binding sites is paramount in differentiating agonists (full vs. partial) from competitive antagonists (small and large) and modulators. The crystallographic results in combination with mutagenesis, biochemical, electrophysiological, and animal behavior studies will help in the design of more selective agonists and competitive antagonists that can be used for the treatment of AChR-related diseases. For example, compound 5 (3-[(2(S)-azetidinyl)methoxy]-5-[(1S,2R)-2-(2-hydroxyethyl)cyclopropyl] pyridine) is a highly selective partial agonist of the α4β2 AChR with antidepressant properties that has been recently co-crystallized with an AChBP [60-66].

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