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Circular dichroism studies of extracellular domains of human nicotinic acetylcholine receptors provide an insight into their structure

Marios Zouridakis [a,b], Kalliopi Kostelidou [a], Alexandros Sotiriadis [a], Christos Stergiou [a,b], Elias Eliopoulos [c], Konstantinos Poulas [b], Socrates J. Tzartos [a,b,*]

[a] *Department of Biochemistry, Hellenic Pasteur Institute, 127, Vas. Sofias Ave., GR11521 Athens, Greece*
[b] *Department of Pharmacy, University of Patras, Rio, GR26500, Patras, Greece*
[c] *Department of Agricultural Biotechnology, Agricultural University of Athens, 75, Iera Odos, Votanikos, GR11855, Athens, Greece*

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Abstract

The extracellular domains (ECDs) of human nicotinic acetylcholine receptors (nAChRs) are of major pharmacological interest as drug targets in the autoimmune disease myasthenia gravis and in various neurological disorders. We have previously expressed and purified the human muscle α1-, β1-, γ- and ε-nAChR-ECDs, as well as the wild type and a mutant of neuronal α7-ECD, in yeast *Pichia pastoris*. The far-UV circular dichroism (CD) studies of these ECDs, presented here, revealed a major prevalence of β-sheet (∼40%) and a small proportion of α-helical (∼5%) structure for all ECDs, in good agreement with the secondary structure composition of the *Torpedo* muscle-type nAChR-ECDs and in less, but considerable, agreement with that of the homologous invertebrate acetylcholine-binding proteins (AChBPs). The near-UV CD studies of these nAChR-ECDs indicated well-defined tertiary structures, as was previously suggested by biochemical and immunochemical studies. Furthermore, the binding of cholinergic ligands to the mutant of α7-ECD resulted in no changes in its secondary structure, but revealed significant local conformational changes. Our present studies probe the structure of human nAChR-ECDs for the first time and further suggest that our expressed proteins fold to a near-native conformation, thus being suitable for further structural studies.

Keywords: Human nicotinic acetylcholine receptor; Extracellular domain; Circular dichroism spectroscopy; Ligand-binding; Secondary and tertiary structure

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of the ligand-gated ion channels (LGICs), which form pentamers of related subunits organized around a central pore [1]. Each subunit consists of an N-terminal extracellular domain (ECD), containing a conserved pair of linked cysteines which forms the so-called Cys-loop, a transmembrane region and a cytoplasmic loop [2]. nAChR-ECDs are 210–220 amino acid residues long and form 2–5 binding sites for cholinergic ligands.

Based on different localization and pharmacology, the nAChRs are classified into muscle and neuronal types [3]. Muscle-type nAChRs are found in fish electric organs and at the vertebrate neuromuscular junctions, where they mediate neuromuscular transmission. They form heteropentamers with a stoichiometry $(\alpha1)_2\beta1\gamma\delta$ in both *Torpedo* and embryonic mammalian nAChR and $(\alpha1)_2\beta1\varepsilon\delta$ in adult mammalian nAChR. The ECD of the α1-subunit is involved in the pathology of the autoimmune disease, myasthenia gravis, as it bears the main immunogenic region (MIR) against which a large number of anti-nAChR antibodies are directed [4]. α1-ECDs also participate in the formation of the two ligand-binding sites; one is formed between α1- and γ- or ε-ECDs, while the other between the second α1- and δ-ECDs, rendering the two sites non-equivalent [5].

Abbreviations: nAChR, nicotinic acetylcholine receptor; ECD, extracellular domain; nAChR-ECD, extracellular domain of nicotinic acetylcholine receptor; AChBP, acetylcholine-binding protein; α7-dm-ECD, double mutant of α7-ECD; α-Bgtx, α-bungarotoxin; NRMSD, normalized mean square deviation; MRW, mean residue weight

* Corresponding author. Tel.: +30 210 6478844; fax: +30 210 6478842.
E-mail addresses: tzartos@pasteur.gr, tzartos@upatras.gr (S.J. Tzartos).

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Neuronal nAChRs are widely distributed in the central and peripheral nervous system and exist either as heteropentamers containing 2–3 α-subunits (selected from subtypes α2–6) plus 2–3 β-subunits (β2–4) or as homopentamers (α7–9), with α7 being the only human subunit known to form a homopentamer. They play key roles in various neuron-neuron interactions and in many pathological conditions and diseases (epilepsy, schizophrenia, Alzheimer's, Parkinson's, etc) [6]. The ligand-binding sites are formed between regions of either adjacent α-ECDs (in homopentameric receptors) or between α- and β-ECDs (in heteropentameric receptors).

Elucidation of human nAChR-ECD structure at high resolution is required for the design of successful therapeutic approaches towards relevant disorders. Our understanding of nAChR-ECDs has been increased by the crystal structures of several invertebrate acetylcholine-binding proteins (AChBPs) from snails [7–9] and by the 4 Å resolution electron microscopy structure of the *Torpedo* nAChR [10]. The former are homopentameric soluble proteins, sharing 20–24% sequence identity with the human nAChR-ECDs and are most similar to α7-ECD. Yet, high resolution studies on human nAChR structure are not available. Therefore, circular dichroism (CD) is a valuable method for studying their structure.

In this paper, we describe far- and near-UV CD studies of the glycosylated forms of the human muscle α1-, β1-, γ- and ε-nAChR-ECDs, and of a mutant form of human neuronal α7-ECD, which we have previously expressed in yeast *Pichia pastoris* [11–13]. The near-UV CD studies of these ECDs suggested well defined tertiary structures for all of them and revealed special local conformational changes in the mutant of α7-ECD, upon binding of several cholinergic ligands. The far-UV CD data demonstrated that high β-sheet structure (∼40%) is the dominant structural feature of all ECDs with a small contribution of α-helical content (∼5%), and that binding of cholinergic ligands to the mutant of α7-ECD does not alter its secondary structure. This is the first time that direct experimental evidence appears for the secondary structure composition of human nAChR-ECDs, which seems to be in very good agreement with that of the similar *Torpedo* muscle-type nAChR-ECDs [10] and in considerable, though lower, agreement with that of the AChBPs which are of lower sequence identity [7–9]. The present results, in addition to previous biochemical and immunochemical data implying considerable folding [11–14], strongly suggest that these ECDs fold to a near-native conformation and are suitable for more detailed structural studies.

2. Materials and methods

2.1. Constructs, protein expression and purification

cDNA cassettes encoding human muscle β1-, γ- and ε-nAChR-ECDs were cloned into a modified pPIC9 vector (Invitrogen), called pPIC9-FLAG [12]. The expressed ECDs carried an N-terminal FLAG peptide and a C-terminal polyhistidine (6-His) tag (Fig. 1A). The cDNA fragments for human muscle α1- and neuronal α7-dm-ECDs were cloned into the pPICZαA vector (Invitrogen), so that the C-terminals of the expressed ECDs were fused to the c-myc epitope and to a subsequent 6-His tag (Fig. 1B) [11,13]. The α7-dm-ECD is a double mutant carrying the cysteine (Cys) mutation to serine (Ser) at position 116 ($Cys^{116}Ser$) and the replacement of its Cys-loop (Cys^{128}-Cys^{142}) by the corresponding and more hydrophilic loop of *Lymnaea stagnalis* AChBP (Ls-AChBP) [13]. α7-dm ECD proved to be more soluble and with higher affinity for the cholinergic competitive antagonist α-bungarotoxin (α-Bgtx) compared to α7-wt-ECD [13]. All ECDs were expressed, purified and biochemically characterized as described in [11–13]. α1-ECD was produced as a monomer of ∼34 kDa, β1-ECD had an apparent dimeric form of 60–65 kDa and γ-, ε- and α7-dm-ECDs were mainly eluted in an oligomeric state (150–210 kDa) [11–13]. All ECDs used for CD were more than 95% pure, based on Coomassie Brilliant Blue-stained SDS/polyacrylamide gels. The protein concentration was determined based on the absorbance at 280 nm [15].

2.2. CD spectra

The CD spectra were measured at 20 °C using a Jasco Model J-715 spectropolarimeter (Japan Spectroscopic Co) in semi-automatic slit adjustment mode. The scan speed was set at 50 nm min^{-1}, the bandwidth at 1 nm, the response time at 2 s, and the scan ranges were 190–260 nm (far-UV) and 250–320 nm (near-UV) at 0.2 nm resolution. Optical activity was expressed as mean residue ellipticity $\{\Theta\}$, in degrees cm^2 $dmol^{-1}$, based on the calculated mean residue weight (MRW) of each ECD fragment. All ECDs were in 10 mM potassium phosphate buffer, 50 mM sodium fluoride, pH 7.4. The quartz cell path length was either 1 or 10 mm for far- and near-UV CD studies, respectively. Each spectrum represents the average of ten scans after being corrected by subtraction of a buffer blank. The upper limit of high tension voltage was 600 V for far-UV measurements. Secondary structures were calculated from the far-UV CD data,

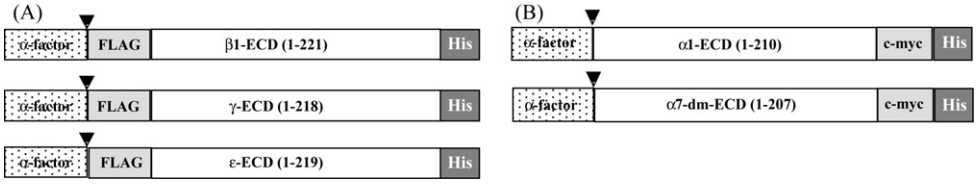

Fig. 1. Schematic representation of the human nAChR-ECDs used for CD analysis. (A) β1-, γ- and ε-ECDs expressed from pPIC9-FLAG vector [12]. (B) α1- and α7-dm-ECDs expressed from pPICZαA [11,13]. The α7-dm-ECD carries the single-point mutation $Cys^{116}Ser$ and its Cys-loop (Cys^{128}-Cys^{142}) has been replaced by the corresponding region of Ls-AChBP [13]. All ECDs were led by a signal peptide α-factor, which is cleaved at arrowheads positions by host enzymes and carried a 6-His tag and a FLAG tag or a c-myc epitope at their terminals. In parentheses are the numbers of amino acid residues of each ECD.