Biochimica et Biophysica Acta 1818 (2012) 1261–1268

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

NMR structures of the transmembrane domains of the α4β2 nAChR

Vasyl Bondarenko [a], David Mowrey [a,c], Tommy Tillman [a], Tanxing Cui [a,b], Lu Tian Liu [a], Yan Xu [a,b,d], Pei Tang [a,c,d,*]

[a] Department of Anesthesiology, University of Pittsburgh, School of Medicine, USA
[b] Department of Structural Biology, University of Pittsburgh, School of Medicine, USA
[c] Department of Computational and Systems Biology, University of Pittsburgh, School of Medicine, USA
[d] Department of Pharmacology & Chemical Biology, University of Pittsburgh, School of Medicine, USA

ARTICLE INFO

Article history:
Received 20 December 2011
Received in revised form 30 January 2012
Accepted 7 February 2012
Available online 14 February 2012

Keywords:
Nicotinic acetylcholine receptor
nAChR
α4β2
NMR
Ion channel structure
Cys-loop receptor

ABSTRACT

The α4β2 nicotinic acetylcholine receptor (nAChR) is the predominant heteromeric subtype of nAChRs in the brain, which has been implicated in numerous neurological conditions. The structural information specifically for the α4β2 and other neuronal nAChRs is presently limited. In this study, we determined structures of the transmembrane (TM) domains of the α4 and β2 subunits in lauryldimethylamine-oxide (LDAO) micelles using solution NMR spectroscopy. NMR experiments and size exclusion chromatography-multi-angle light scattering (SEC-MALS) analysis demonstrated that the TM domains of α4 and β2 interacted with each other and spontaneously formed pentameric assemblies in the LDAO micelles. The Na^+ flux assay revealed that α4β2 formed Na^+ permeable channels in lipid vesicles. Efflux of Na^+ through the α4β2 channels reduced intra-vesicle Sodium Green™ fluorescence in a time-dependent manner that was not observed in vesicles without incorporating α4β2. The study provides structural insight into the TM domains of the α4β2 nAChR. It offers a valuable structural framework for rationalizing extensive biochemical data collected previously on the α4β2 nAChR and for designing new therapeutic modulators.

1. Introduction

The nicotinic acetylcholine receptor (nAChR) is a member of the Cys-loop superfamily that mediates fast synaptic transmission in the central and peripheral nervous systems. The α4β2 nAChR is the most abundant heteromeric nAChR subtype in the brain [1]. It has been implicated to play roles in Parkinson's disease [2], Alzheimer's disease [3], nicotine addiction [4,5], and general anesthesia [6–8].

Despite the importance of the α4β2 nAChR in various neurological conditions, experimental structural characterization of the α4β2 nAChR remains sparse [9–11]. Structural understanding of nAChRs has relied heavily on the cryo-electron microscopy structural model of the muscle type nAChR found in the electric ray *Torpedo marmorata* [12]. The structural model has only a resolution of 4 Å, but shows a pentameric scaffold and extracellular (EC), transmembrane (TM), and intracellular (IC) domains for each of the five subunits. More recently, homologous structures of the bacteria *Gloebacter violaceous* (GLIC) [13,14] and *Erwinia chrysanthemi* (ELIC) [15] and the *Caenorhabditis elegans* glutamate-gated chloride channel alpha (GluCl) [16] have been determined at resolutions of 2.9 Å, 3.3 Å, and 3.3 Å, respectively. Unlike mammalian Cys-loop receptors, GLIC and ELIC do not contain a large IC domain. Among the EC, TM and IC domains of Cys-loop receptors, the EC domain containing the orthosteric agonist-binding site has the richest structural information, mainly from crystal structures of the acetylcholine binding protein (AChBPs) [17–19], the EC domain of the mouse α1 subunit [20], and the chimera of the α7 nAChR-AChBP [21]. In contrast, the IC domain connecting TM3 and TM4 is the least studied domain. This domain is the least conserved among nAChR subunits and has the least structural data at present. The IC domain is known to modulate interaction with cytoskeleton components as well as channel desensitization [22]. However, it was demonstrated that fully functional channels could be obtained by replacing the IC domain with a short linker found in GLIC [23].

Although structural understanding of the TM domain is not as inadequate as that on the IC domain, more thorough structural characterizations on the TM domain for individual Cys-loop receptors are required in order to satisfy the demands for rational design of therapeutic drugs and for discovery of molecular mechanisms of drug action [24,25]. The TM domain contains the channel gate. Hence, it is the critical region for controlling the flow of ions across the membrane [26]. Positive and negative allosteric modulators acting at the TM domains of nAChRs [27–29] have been implicated as useful therapeutics for neurological diseases. The TM domain also provides binding sites for general anesthetics. The intravenous anesthetic etomidate binds to the TM domain of the *Torpedo* nAChR [30]. The inhalational anesthetic halothane shows binding to the TM domain both experimentally in the *Torpedo* nAChR [31] and computationally

* Corresponding author at: 2049 Biomedical Science Tower 3, 3501 Fifth Avenue, University of Pittsburgh, Pittsburgh, PA 15260, USA. Tel.: +1 412 383 9798; fax: +1 412 648 8998.
E-mail address: tangp@upmc.edu (P. Tang).

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2012.02.008

in the α4β2 and α7 nAChRs [32–34]. High-resolution structural information of nAChR TM domains is therefore important both for characterizing mechanisms of action for existing drugs and identifying plausible binding sites for new drugs.

While the interplay between the EC and TM domains is critical for transducing ligand-binding signals to the channel gate, the intrinsic folding of the TM domain seems to be independent of the existence of the EC domain. Incorporation of the TM2 helix into a lipid environment was found to produce ion specific channels [35,36]. Furthermore, we have previously demonstrated faithful folding of the TM domain of the β2 subunit in membrane mimetic microclusters formed by a hexafluoroisopropanol and water mixture (1:1) [9]. Thus, it is reasonable and practical to solve structures of the TM domains in the absence of the EC and IC domains.

In the present study, we used lauryldimethylamine-oxide (LDAO) micelles as a membrane mimetic and solved the NMR structures for the TM domains of the α4 and β2 nAChR subunits. We found that the α4 and β2 TM domains form pentameric assemblies in LDAO micelles. When reconstituted into lipid vesicles, the α4β2 assemblies are capable of transporting Na^+ ions. High-resolution structures of the individual α4 and β2 TM domains and the assembled pentameric structural model for the α4β2 nAChR TM domain provide valuable templates for understanding mechanisms of channel function and drug action as well as for rational drug discoveries.

2. Materials and methods

2.1. Sample preparations

The method to obtain the α4 and β2 TM domains of the human nAChR was reported previously [9]. Proteins were expressed in *E. coli* Rosetta 2(DE3) pLysS (Novagen) at 15 °C for ~3 days using the Marley protocol [37]. The EC domain at the N-terminus was replaced with a TEV protease recognition site and a histidine tag. A short synthetic linker of five glycines replaced the IC domain. Each α4 or β2 TM domain contains 137 residues with an approximate molecular weight of 15 kDa. Glutamate mutations at the N- and C-termini, designed to lower the pI, were necessary to secure protein stability for NMR measurements. Mutation of three hydrophobic residues to serine within the TM2–TM3 linker of α4 or β2 was also instrumental to prevent protein destabilization. Direct exposure of hydrophobic residues to the aqueous phase in the absence of the EC domain resulted in protein precipitation in a short time period. Amino acid sequences showing mutations of the α4 and β2 TM domains are provided in the Supplementary Material (Fig. S1). The expressed proteins were purified by Ni-NTA (GE Healthcare) chromatography before and after overnight cleavage of the his-tagged region at 4 °C. The purification buffer contained 50 mM Tris, 150 mM NaCl, and 0.5% LDAO, and proteins were eluted with imidazole. Each NMR sample had 0.25–0.3 mM protein, 1–2% (40–80 mM) LDAO, 5 mM phosphate acetate pH 4.7, 10 mM NaCl, and 20 mM 2-mercaptoethanol to prevent disulfide bond formation. 5% D_2O was added to the NMR samples for deuterium lock in NMR measurements. In terms of the α4 and β2 contents, we prepared four types of samples: (1) pure α4; (2) pure β2; (3) α4:β2 = 2:3; and (4) α4:β2 = 3:2.

2.2. NMR spectroscopy

NMR spectra were acquired at 45 °C on Bruker Avance 600, 700, 800, and 900 MHz spectrometers equipped with a triple-resonance inverse-detection cryoprobe, TCI (Bruker Instruments, Billerica, MA). Spectral windows of 11 or 13 ppm (1024 data points) in the ^1H dimension and 22 or 24 ppm (128 data points) in ^{15}N dimension with a relaxation delay of 1 s (or 1.5 s at 900 MHz) were used for collecting ^1H–^{15}N TROSY–HSQC spectra. ^1H–^{13}C HSQC spectra were acquired as 1024 points in the ^1H dimension and 256 increments in the ^{13}C dimension with spectral windows of 11 ppm (^1H) and 64 ppm (^{13}C). For chemical shift assignment, we performed a suite of 3D experiments, including HNCA and HN(CO)CA (1024×36×80, 600 or 700 MHz) with a spectral window of 18 ppm in the ^{13}C dimension, HNCO (1024×36×40, 600 or 700 MHz) with a ^{13}C spectral width of 10 ppm, ^{15}N-edited NOESY (1024×36×160) with a mixing time of 120 ms at 900 MHz and 150 ms at 700 MHz, and ^{13}C-edited NOESY (1024×36×192, 700 MHz) with a mixing time of 150 ms. In addition, CBCA(CO)NH (1024×32×80, 700 MHz) with a ^{13}C spectral window of 60 ppm was acquired for α4. In order to evaluate the temperature dependence of individual residue chemical shifts, α4 and β2 ^1H–^{15}N TROSY–HSQC spectra were collected at 40, 43, 45, and 48 °C. The residues with temperature coefficients <4.5 ppb/K were considered to have hydrogen binding [38]. The observed ^1H chemical shifts were referenced to the DSS resonance at 0 ppm and the ^{15}N and ^{13}C chemical shifts were indirectly referenced [39].

2.3. Size exclusion chromatography-multi-angle light scattering (SEC-MALS) analysis

The molar masses of the protein-detergent complexes were determined using size exclusion chromatography (Superdex 200 10/300, GE Healthcare) coupled with multi-angle light scattering (HELEOS, Wyatt Technology), UV (Agilent 1100 Series; Agilent Technology), and differential refractive index (Optilab rEX; Wyatt Technology) detection. The measurements were performed on the samples that had been used for NMR in 10 mM sodium acetate pH 4.6, 100 mM NaCl, 0.05% LDAO at a flow rate of 0.5 mL/min at room temperature. HELEOS calibration constants were determined in the same buffer using chicken egg lysozyme (Affymetrix) as the standard. Light scattering data was analyzed and the molar mass of the protein–detergent complex was determined using ASTRA software (Wyatt Technology) [40]. The conjugate analysis module of ASTRA was used to differentiate contributions of the protein and detergent to the molecular weight of each complex. The specific refractive index (dn/dc) values of 0.185 and 0.148 were used for the protein and LDAO detergent, respectively [41]. The UV extinction coefficients of α4 and β2 were calculated from their sequences. A measured UV extinction coefficient of 0.06 for a 1% solution at 280 nm was used for LDAO.

2.4. The Na^+ flux assay for functional measurements

The Na^+ flux assay, as measured by the reduction of Sodium Green™ dye (Invitrogen, Carlsbad, CA) fluorescence due to Na^+ leaving the vesicles through open channels, is an effective way to macroscopically assess activity of the α4β2 TM channels. We prepared 25 mM vesicles with ~500 μM α4β2. The vesicles contained egg phosphatidylcholine (PC)/phosphatidylglycerol (PG) in a 3:1 molar ratio and lipid biotinyl-cap-PE (1 mol%). Lipids dissolved in chloroform were mixed with α4β2 and dried to a thin film by nitrogen gas. Residual organic solvent was removed by vacuum overnight. The lipid–protein mixture was hydrated overnight at 42 °C with a buffer solution containing 20 mM Tris, 100 mM NaCl, and 3 μM Sodium Green™ at pH 7.5. The vesicles were obtained by multiple subsequent cycles of freeze/thaw and sonication. Sodium Green™ dye outside the vesicles was removed by extensive dialysis.

The Na^+ flux assay was performed using an Olympus IX81 microscope (Olympus America, Center Valley, PA), equipped with a Sutter Lambda xenon exciter light source, various excitation and emission filters, and an ORCA-ER digital camera. For each measurement, vesicles containing α4β2 were added onto the streptavidin coated glass slide. The image acquisition started before vesicles were washed with a buffer solution (50 mM $CaCl_2$ 20 mM Tris at pH 7.5) to dilute the extra-vesicle Na^+ concentration. The resulting Na^+ concentration gradient drove Na^+ out of the vesicles when channels were formed. Consequently the fluorescence intensity resulting from Sodium