Biochimica et Biophysica Acta 1838 (2014) 1389–1395

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

NMR structures of the human α7 nAChR transmembrane domain and associated anesthetic binding sites

Vasyl Bondarenko [a], David D. Mowrey [a,c], Tommy S. Tillman [a], Edom Seyoum [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 & 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 29 August 2013
Received in revised form 19 November 2013
Accepted 23 December 2013
Available online 31 December 2013

Keywords:
α7 nAChR structure
Halothane
Ketamine
General anesthetics
NMR
Protein dynamics

ABSTRACT

The α7 nicotinic acetylcholine receptor (nAChR), assembled as homomeric pentameric ligand-gated ion channels, is one of the most abundant nAChR subtypes in the brain. Despite its importance in memory, learning and cognition, no structure has been determined for the α7 nAChR TM domain, a target for allosteric modulators. Using solution state NMR, we determined the structure of the human α7 nAChR TM domain (PDB ID: 2MAW) and demonstrated that the α7 TM domain formed functional channels in *Xenopus* oocytes. We identified the associated binding sites for the anesthetics halothane and ketamine; the former cannot sensitively inhibit α7 function, but the latter can. The α7 TM domain folds into the expected four-helical bundle motif, but the intra-subunit cavity at the extracellular end of the α7 TM domain is smaller than the equivalent cavity in the α4β2 nAChRs (PDB IDs: 2LLY; 2LM2). Neither drug binds to the extracellular end of the α7 TM domain, but two halothane molecules or one ketamine molecule binds to the intracellular end of the α7 TM domain. Halothane and ketamine binding sites are partially overlapped. Ketamine, but not halothane, perturbed the α7 channel-gate residue L9′. Furthermore, halothane did not induce profound dynamics changes in the α7 channel as observed in α4β2. The study offers a novel high-resolution structure for the human α7 nAChR TM domain that is invaluable for developing α7-specific therapeutics. It also provides evidence to support the hypothesis: only when anesthetic binding perturbs the channel pore or alters the channel motion, can binding generate functional consequences.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) belong to a superfamily of pentameric ligand-gated ion channels (pLGICs), including $5HT_3$, $GABA_A$, and glycine receptors, that mediate fast synaptic transmission in the central and peripheral nervous systems. The α7 nAChR is one of the most abundant nAChR subtypes in the brain and assembles as homomeric functional pentamers [1]. High expression levels of the α7 nAChR have been observed in brain regions involved in learning, memory, and cognition [2,3]. Therefore, the α7 nAChR is a viable target for therapeutics to regulate processes impaired in schizophrenia, Alzheimer's disease, and other neurological disorders [4,5]. α7 nAChR is also a target for therapeutic modulation of angiogenesis and inflammation [6,7].

In order to rationally design therapeutics specifically targeting the α7 nAChR, a high-resolution structure of α7 is highly desired. However, no experimental structure for the full-length α7 nAChR currently exists. The highest degree of structural information for α7 nAChR has been achieved for the extracellular (EC) domain, which contains the orthosteric ligand-binding site. X-ray structures of chimeras that have systematically modified the sequence of acetylcholine binding proteins [8–10] toward the human α7 nAChR provide invaluable atomic details for the α7 EC domain [11,12]. The overall topology and structural information for the transmembrane (TM) domain and the intracellular (IC) domain of α7 nAChR have relied on the 4-Å resolution model of the *Torpedo marmarota* nAChR determined by cryo-electron microscopy [13]. Recent crystal structures of homologous bacterial pLGICs from *Erwinia chrysanthemi* (ELIC) [14] and *Gloebacter violaceous* (GLIC) [15,16] as well as the *Caenorhabditis elegans* glutamate-gated chloride channel (GluCl) [17], have also added valuable structural templates for modeling pLGICs.

Previous molecular models for the α7 nAChR [18,19] were based on structures of the *Torpedo marmarota* nAChR [13]. Homology modeling can capture overall structural features that are likely sufficient for many purposes, but it may miss specific structural details that can differentiate functions and pharmacology of different nAChR subtypes. For example, the α7 and α4β2 nAChRs would have similar structural models, which cannot provide sufficient insights for reasoning why α7 is insensitive but α4β2 is hypersensitive to functional modulation by volatile anesthetics [20,21]. Reliable structures for individual subtypes of nAChRs, especially their TM domains, are also important for

* Corresponding author at: 2049 Biomedical Science Tower 3, 3501 Fifth Avenue, University of Pittsburgh, Pittsburgh, Pennsylvania 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 © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.12.018

the development of positive allosteric modulators with therapeutic potential, such as PNU-120596 [22–24] and TQS [25,26]. They are specific modulators for α7 nAChRs and have virtually no effect on other nAChR subtypes.

In the study reported here, we determined the structure of the human α7 nAChR TM domain using high-resolution solution state NMR. The structures newly determined for α7 and previously determined for α4β2 nAChRs (PDB codes: 2LLY; 2LM2) [27] offer an opportunity to make structural comparisons and to reveal a structural basis that differentiates function and pharmacology of different nAChR subtypes. In addition to the new structure for α7, we also determined binding sites in α7 for the volatile anesthetic halothane and the intravenous anesthetic ketamine. The identified structural and dynamics determinants from the study have general implication for anesthetic action in pLGICs.

2. Materials and methods

2.1. Sample preparations

The human α7 nAChR TM domain for the NMR study contained 137 residues (Fig. S1). In order to reduce complexity of the NMR spectra, the cytoplasmic loop between TM3 and TM4 was replaced with GGGEG, a sequence designed to avoid imposing structural constraints on interactions of the TM helices while providing a hydrophilic surface to enhance stability of the isolated TM domain. The TM3-4 loop of α7 nAChR is involved in receptor assembly and trafficking to the cell surface in eukaryotes [28,29], but studies with related pLGICs have established that the TM3-4 loop is not essential for channel function [30]. Glutamate mutations at the N- and C-termini (Fig. S1), designed to lower the pI of the construct, were necessary to secure protein stability for NMR measurements. Additional mutation of three hydrophobic residues to serine within the TM2–TM3 linker (Fig. S1) was also instrumental to prevent protein destabilization, similar to the previous observation on α4β2 TM domains [27]. Without these mutations, the isolated α7 TM domain had a tendency to aggregate on purification, most likely because hydrophobic residues normally shielded by the EC domain were exposed to solvent.

The same protocol as reported previously [27] was used for the α7 expression and purification. The protein was expressed in *Escherichia coli* Rosetta 2(DE3) pLysS (Novagen) at 15 °C for three days using the Marley protocol [31]. The protein was purified in LDAO using his-tag affinity column before and after cleavage of the his-tagged region. Each NMR sample contained 0.25–0.3 mM α7, 1–2% (40–80 mM) LDAO detergent, 5 mM sodium acetate at pH 4.7, 10 mM NaCl, and 20 mM 2-mercaptoethanol to prevent disulfide bond formation. 5% D_2O was added for deuterium lock in NMR experiments. The anesthetics ketamine (80–240 μM) or halothane (0.7–5.5 mM) were titrated into the samples using a micropipette or a gas-tight microsyringe, respectively. The concentration of the volatile anesthetic halothane was quantified based on ^{19}F NMR using the method reported previously [32].

2.2. NMR spectroscopy

NMR spectra were acquired on Bruker Avance 600 and 800 MHz spectrometers at 45 °C using triple-resonance inverse-detection cryoprobes (Bruker Instruments, Billerica, MA). For ^{1}H, ^{15}N, and ^{13}C chemical shift assignment and the protein structure determination, a suite of NMR experiments were performed: HNCA (1024 × 28 × 72) and HN(CO)CA (1024 × 28 × 54), both with spectral windows of ^{1}H-12 ppm, ^{15}N-20.5 ppm, ^{13}C-19 ppm; HNCO (1024 × 32 × 40) with spectral widths of ^{1}H-11 ppm, ^{15}N-22 ppm, and ^{13}C-10 ppm; ^{15}N-edited NOESY (1024 × 36 × 104) with spectral windows of ^{1}H-11 ppm and ^{15}N-22 ppm, and a mixing time of 120 ms; ^{1}H–^{13}C HSQC (1024 × 256) with spectral windows of ^{1}H-11 ppm and ^{13}C-64 ppm; and ^{1}H–^{15}N TROSY–HSQC (1024 × 128) with spectral windows of ^{1}H-11 ppm and ^{15}N-22 ppm. HSQC spectra showing temperature dependence of amide proton chemical shifts were collected at 35, 40, and 45 °C. Residues of temperature coefficients below 4.5 ppb/K were considered to be in helical structure and involved in hydrogen bonds [33].

^{1}H–^{15}N TROSY–HSQC spectra were acquired at 600 MHz in the absence and presence of the anesthetics halothane or ketamine. Direct contacts of halothane with the α7 TM domain were determined by saturation transfer difference (STD) spectra [34]. A series of 1D STD spectra with different saturation times were collected in an interleaved fashion with on- and off-resonance frequencies of 0.4 ppm and 25 ppm, respectively. A recycle delay of 12 s and 64 scans were used for each STD spectrum. 2D saturation transfer spectra [35] were acquired in the presence of 3.2 mM halothane in an interleaved fashion with on- and off-^{1}H resonance frequencies of 6.48 ppm (the halothane proton frequency) and 25 ppm (blank), respectively. The selective saturation was achieved using an IBURP2 pulse train (50 ms Gaus1.1000-shaped with an interpulse delay of 4 μs). The total saturation time was 2 s and a recycle delay was 3 s. The ^{1}H chemical shifts were referenced to the DSS resonance at 0 ppm and the ^{15}N and ^{13}C chemical shifts were referenced indirectly [36].

2.3. Structure calculation and analysis

NMR data were processed using NMRPipe 4.1 and NMRDraw 1.8 [37] and analyzed using Sparky 3.10 [38]. ^{1}H, ^{15}N, and ^{13}C chemical shift assignments were performed manually. NOE cross-peak assignment was initially carried out manually and more cross-peaks were assigned later by CYANA 2.1 [39]. CYANA 3.0 was used for structural calculations. A total of 100 structures were calculated based on NOE and hydrogen-bonding restraints as well as TALOS dihedral angle restraints derived from the chemical shifts [40]. Of the 100 structures, 25 structures with the lowest target function were used for further refinement in CYANA 3.0. The 20 structures with the lowest target function after refinement were analyzed using VMD [41] and Molmol [42].

Contact map analysis (CMA) [43] was used for comparison of the α7 TM tertiary structures with structures of other homologous proteins. Internal cavities in the α7 TM domain were determined for each of the 20 NMR structures using the POVME algorithm [44]. Grids for cavities at the EC and IC ends of the nAChR TM domains were generated with 0.5 Å grid spacing. The mean ± standard error calculated based on cavity volumes for the 20 NMR structures is reported.

2.4. Visualization and molecular docking of anesthetics in the α7 nAChR

To assist with visualizing halothane- and ketamine-binding sites identified by NMR experiments, we performed targeted anesthetic docking to the α7 NMR structures. The targeted docking kept only those sites consistent with the NMR results. Docking was performed with Autodock4 [45] using a Lamarckian genetic algorithm with a grid spacing of 0.375 Å. For each binding site suggested by NMR, 250 independent anesthetic dockings were performed within a cube covering ~6600 $Å^3$ located at the IC end of the TM domain. Each docking calculation used an initial population size of 500.

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

Oligomerization states of the α7 TM domain in the NMR samples 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 molar mass of the protein-detergent complex was determined using ASTRA software (Wyatt Technology) [46]. The conjugate analysis module of ASTRA was used to differentiate contributions of the protein and detergent to the molecular weight. The specific refractive index (dn/dc) values of 0.185 and 0.148 were used for the protein and