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Anesthetic effects on the structure and dynamics of the second transmembrane domains of nAChR α4β2

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ABSTRACT

Channel functions of the neuronal α4β2 nicotinic acetylcholine receptor (nAChR), one of the most widely expressed subtypes in the brain, can be inhibited by volatile anesthetics. Our Na^+ flux experiments confirmed that the second transmembrane domains (TM2) of α4 and β2 in 2:3 stoichiometry, $(\alpha4)_2(\beta2)_3$, could form pentameric channels, whereas the α4 TM2 alone could not. The structure, topology, and dynamics of the α4 TM2 and $(\alpha4)_2(\beta2)_3$ TM2 in magnetically aligned phospholipid bicelles were investigated using solid-state NMR spectroscopy in the absence and presence of halothane and isoflurane, two clinically used volatile anesthetics. ^2H NMR demonstrated that anesthetics increased lipid conformational heterogeneity. Such anesthetic effects on lipids became more profound in the presence of transmembrane proteins. PISEMA experiments on the selectively ^{15}N-labeled α4 TM2 showed that the TM2 formed transmembrane helices with tilt angles of $12°\pm1°$ and $16°\pm1°$ relative to the bicelle normal for the α4 and $(\alpha4)_2(\beta2)_3$ samples, respectively. Anesthetics changed the tilt angle of the α4 TM2 from $12°\pm1°$ to $14°\pm1°$, but had only a subtle effect on the tilt angle of the $(\alpha4)_2(\beta2)_3$ TM2. A small degree of wobbling motion of the helix axis occurred in the $(\alpha4)_2(\beta2)_3$ TM2. In addition, a subset of the $(\alpha4)_2(\beta2)_3$ TM2 exhibited counterclockwise rotational motion around the helix axis on a time scale slower than 10^{-4} s in the presence of anesthetics. Both helical tilting and rotational motions have been identified computationally as critical elements for ion channel functions. This study suggested that anesthetics could alter these motions to modulate channel functions.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) belong to a superfamily of ligand gated ion channels involving the rapid chemical transmission of nerve impulses at synapses. Previous studies found that some subtypes of nAChRs might be potential targets of general anesthetics and their normal channel functions could be inhibited by general anesthetics [1-6]. Neuronal α4β2 nAChR is one of the subtypes sensitive to general anesthetics [4,5,7]. It is also one of the most abundant nAChR subtypes in the brain. Despite ample evidence showing that general anesthetics could alter α4β2 nAChR functions, it remains largely unclear how anesthetics perturb the protein structures and dynamics that ultimately affect the protein functions. Therefore, the insights of anesthetic modulation on the structure and dynamics of the α4β2 nAChR are valuable to resolve a long time mystery of the molecular mechanisms of general anesthesia [8,9].

A comprehensive understanding of anesthetic action on α4β2 nAChR or other nAChR subtypes has often been restricted by limited available structural information of nAChRs in the past. The structural models of the closed- and open-channel α4β2 nAChR have been generated [10] recently via computations using the known structure of the *Torpedo* nAChR as a template [11]. More recent X-ray structures of pentameric ion channels in closed- and open-channel forms from *Erwinia chrysanthemi* and *Gloeobacter violaceus* provide high-resolution structural information relevant to nAChRs [12–14]. These new developments certainly facilitate the understanding of anesthetic action on α4β2 nAChR, but experimental studies of structural and dynamic effects of anesthetics on α4β2 nAChR may lead directly to insights into how anesthetics act on α4β2 nAChR and alter the protein function.

Solid-state NMR spectroscopy is a powerful technique for the characterization of membrane protein structures and dynamics and for the investigation of ligand–protein interactions [15-23]. The polarization Inversion and Spin Exchange at the Magic Angle (PISEMA) experiment [24] is particularly useful for the determination of topological structures and dynamics of helical proteins in a well-oriented lipid environment [25-29]. Ligand binding to the *Torpedo* nAChR was comprehensively analyzed using static ^2H and cross polarization magic angle spinning (CPMAS) ^{13}C solid-state NMR

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experiments [30,31]. The structures of the transmembrane domains of the *Torpedo* nAChR were also examined by various solid-state NMR methods [32-34], including PISEMA [33]. However, no solid-state NMR study on neuronal nAChR has been reported previously. In the present study, we embedded the second transmembrane domains (TM2) of α4β2 nAChR in lipid bicelles, which served as membrane mimetic media and magnetically aligned the protein. The structural and dynamic properties of the TM2 α4β2 nAChR in the absence and presence of anesthetics halothane or isoflurane were investigated using solid state NMR, especially PISEMA experiments. Anesthetics were found to affect both helical tilting and rotational motions that have been identified computationally as critical elements for ion channel functions. This study suggested that anesthetics could alter these motions to modulate channel functions.

2. Materials and methods

2.1. Materials and sample preparation

The second transmembrane (TM2) domains of the human nAChR were obtained by solid phase synthesis [35,36]. The α_4 and β_2 TM2 domains have the sequences of EKIT**L**CISV**LL**S**L**TVF**LLL**ITE and EKMTLCISVLLALTVFLLLISK, respectively. In order to simplify the studies, only seven leucine residues in α_4 TM2 domain, as indicated in bold letters, were ^{15}N labeled.

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). A conventional protocol of bicelle preparation [37] was followed. 1,2-dimyristoyl-sn-glycero-3-phosphocoline (DMPC) and 1,2-dihexyl-sn-glycero-3-phosphocholine (DHPC) were mixed in a desired molar ratio ($q = 3.2$). Lipid concentration was 28% of sample volume (typically 220 μL). To extend sample stability for samples containing the nAChR TM2 domains, 1,2-O-ditetradecyl-sn-glycero-3-phosphocoline (14-O-PC)) and 1,2-O-dihexyl-sn-glycero-3-phosphocholine (6-O-PC) replaced DMPC and DHPC, respectively. A small amount of deuterated $DMPC_{d54}$ (~1 mg) was added to each sample for ^{2}H NMR. The α4 or β2 TM2 was dissolved in 100 μL trifluoroethanol and added to the 6-O-PC-chloroform solution. The organic solvents were removed under a stream of nitrogen gas, followed by further evaporation under high vacuum overnight. The aqueous solutions were prepared by adding 110 μL of deuterium-depleted water to dried 14-O-PC and 6-O-PC/peptide. The 14-O-PC suspension was vortexed extensively followed by three freeze/thaw cycles (liquid nitrogen/42 °C). The 14-O-PC suspension at 42 °C was then added to the 6-O-PC/peptide, followed by vortexing and three freeze/thaw cycles. Slow-speed centrifugation was sometimes necessary to remove air bubbles in the sample. A transparent solution was obtained that was viscous at 38 °C and fluid at 4 °C. Parallel-oriented peptide-containing bicelles were prepared by adding 100 mM $YbCl_3$ $6H_2O$ to the peptide-bicelle solution to reach a final lanthanide concentration of 3 mM. The sample was transferred to a 5-mm OD glass tube (New Era Enterprises, Newfield, NJ) using a pre-cooled pipet tip at 4 °C. The glass tube was sealed with a tight fitting rubber cap and further sealed with hard bees wax. The α4-TM2 concentration was 7.4 mM in the α4 samples and 3 mM in the α4β2 samples. The molar ratio of β2 to α4 was 1.5 in the α4β2 samples to ensure a formation of $(\alpha4)_2(\beta2)_3$. The peptide to lipid ratio is ~1:60. Anesthetics halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane) and isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) were added directly to the NMR tube (pre-cooled in an ice bath) and mixed thoroughly with the samples. The anesthetic concentrations were determined using ^{19}F NMR with 5 mM trifluoroacetic acid (TFA) as a reference.

2.2. NMR spectroscopy

All solid state NMR experiments were performed at 40 °C on a Bruker Avance 600 MHz NMR spectrometer equipped with a ^{1}H/^{31}P/^{15}N(^{2}H) Bruker flat-coil probe. ^{19}F NMR experiments were performed on a Bruker Avance 600 spectrometer with a Bruker TXO probe. The 1-D ^{15}N cross-polarization experiments were conducted using a ^{1}H 90° pulse of 5.1 μs, a 49 kHz ^{1}H-decoupling field, 1 ms contact time, 3 s recycle delay, and 10,000 scans. The same cross-polarization parameters were used in the 2-D ^{15}N-^{1}H PISEMA [38]. Other parameters included ±35 kHz frequency jumps for the Lee-Goldberg condition, 400–1000 scans, and 6–8 s recycle delay. The ^{1}H carrier frequency was set at ~4.5 ppm on parallel oriented bicelle samples. The ^{15}N chemical shift frequencies were referenced to solid ammonium sulfate at 26.8 ppm (relative to liquid ammonia at 0 ppm). The ^{2}H NMR experiments were performed using a solid echo sequence $((\pi/2) - \tau - (\pi/2) - \tau)$. The 90° pulse length was 2 μs and τ values were ~40 μs (perpendicular bicelle) and ~20 μs (parallel bicelle). The recycle delay of 0.5 s and the scans of 256 to 10,000 were used.

2.3. NMR data processing

All NMR spectra were processed using NMRPipe [39] and analyzed using Sparky [40]. The data from PISEMA experiments were fitted to the PISA (Polarity Index Slant Angle) [41,42] wheels using the program developed by Veglia's group [26]. The tilt angle (θ) of the helix axis with respect to the bicelle normal, rotation angle (ρ) of the helix around its axis, the angle between the N-H bond and helix axis (δ), and the dipolar coupling constant (K_{DD}) were determined via fittings. The principal values of the ^{15}N chemical shift tensors [24] for non-glycine residues, $\sigma11 = 64$ ppm, $\sigma22 = 77$ ppm, and $\sigma33 = 217$ ppm, were adopted for the fittings. These values were multiplied uniformly by a factor of 0.95 to accommodate reduced ^{15}N chemical shift anisotropy of proteins in bicelles, as reflected in the reduction of spectral span of the same proteins in bicelles in comparison to those in mechanically aligned lipids.

The TM2 chemical shift assignments were accomplished by combining several sources of information, including the previous assignments of δ–TM2 nAChR [33], the solution NMR structure of β2-TM2 nAChR [36], and the best fitting of PISA wheel. L19 is distinctly separated from the rest of leucine residues in a top view of the NMR structure (pdb code: 2K59) [36]. L19 also has weaker NMR signal than other leucines, presumably because it is located at the lipid-water interface and has less protection from solvent exchange. The distinct location and intensity of L19 convinced us to assign L19 to the most downfield peak in the α4-TM2 PISEMA spectra. Once L19 is defined, the rest of peaks were assigned automatically by PISA wheel fitting. The final assignment of our α4-TM2 PISEMA spectra matches very well with a 2-D projection of the leucine distribution along helical axis from N- to C-terminus in the solution NMR structure [36]. The assignment also agrees well with that for δ–TM2 nAChR [33].

The orientational order parameter, S_i^{CD}, of the ith CD bond vector can relate to the residual quadrupolar splitting, $\Delta\nu^i_Q$, in a ^{2}H NMR spectrum using the equation [43]

$$\Delta\nu_Q^i = \frac{3}{2}A_Q\frac{3\cos^2\theta - 1}{2}S_i^{CD} \quad (1)$$

where $A_Q = e^2Qq/h = 167$ kHz is the static deuterium quadrupolar coupling constant for aliphatic CD bonds, θ is the angle between the bilayer normal and the magnetic field.

2.4. The Na^+ flux assay and confocal fluorescence microscopy

The Na^+ flux assay, as measured by the enhancement of Sodium Green™ dye (Invitrogen, Carlsbad, CA) fluorescence due to Na^+ entry into vesicles through open nAChR channels, is an effective way to assess nAChR activity macroscopically. We prepared 30 mM large lamellar vesicles with 250 μM α4β2. The vesicles contain phosphatidylcholine (PC) and phosphatidylglycerol (PG) in a 4:1 molar ratio