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Probing the structure of the uncoupled nicotinic acetylcholine receptor

Jiayin Sun, J. Frederique Comeau, John E. Baenziger *

Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, 451 Smyth Rd, K1H 8M5 Ottawa, ON, Canada.

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ABSTRACT

In the absence of activating anionic lipids and cholesterol, the nicotinic acetylcholine receptor (nAChR) from *Torpedo* adopts an uncoupled conformation that does not usually gate open in response to agonist. The uncoupled conformation binds both agonists and non-competitive channel blockers with a lower affinity than the desensitized state, consistent with both the extracellular agonist-binding and transmembrane channel-gating domains individually adopting resting-state like conformations. To test this hypothesis, we characterized the binding of the agonist, acetylcholine, and two fluorescent channel blockers, ethidium and crystal violet, to resting, desensitized and uncoupled nAChRs in reconstituted membranes. The measured K_d for acetylcholine binding to the uncoupled nAChR is similar to that for the resting state, confirming that the agonist binding site adopts a resting-state like conformation. Although both ethidium and crystal violet bind to the resting and desensitized channel pores with distinct affinities, no binding of either probe was detected to the uncoupled nAChR. Our data suggest that the transmembrane domain of the uncoupled nAChR adopts a conformation distinct from that of the resting and desensitized states. The lack of binding is consistent with a more constricted channel pore, possibly along the lines of what is observed in crystal structures of the prokaryotic homolog, ELIC.

1. Introduction

The ability of the nicotinic acetylcholine receptor (nAChR) to undergo agonist-induced conformational transitions is sensitive to its membrane environment [1,2]. Increasing levels of anionic lipids and/or cholesterol in a reconstituted phosphocholine membrane stabilize an increasing proportion of agonist-responsive nAChRs [3,4]. In phosphocholine membranes lacking these activating lipids, the nAChR adopts an uncoupled conformation that binds agonist with resting state-like affinity, but one where agonist binding is "uncoupled" from channel gating [5]. Lipid-dependent uncoupling is accompanied by the solvent-exposure of peptide backbone hydrogens that are normally buried from solvent in the resting and desensitized states [5,6].

The nAChR exhibits two main functional domains, a predominantly β-sheet (β1–β10) extracellular agonist-binding domain (ECD) and an α-helical (M1–M4) transmembrane channel-gating domain (TMD). These two domains meet at a broad interface; with noncovalent interactions between loops at this interface playing an important role translating agonist binding into gating [7–9]. The uncoupling of binding-to-gating in phosphatidylcholine membranes lacking activing lipids could result from weakened interactions between loops at the ECD/TMD interface, thus leading to the enhanced backbone solvent exposure noted above [5]. One model proposes that lipids influence ECD/TMD interactions via the TMD α-helix, M4 [10–12], which is on the periphery of each subunit and thus exposed to the lipid environment [13,14]. M4 extends beyond the lipid bilayer to interact with the functionally important Cys-loop (β6–β7 loop) at the ECD/TMD interface. Activating lipids could enhance M4-Cys-loop interactions, altering the Cys-loop conformation to promote channel function [5,15,16].

The prokaryotic homolog ELIC shares a common topology with the nAChR [14,17], but its crystal structures exhibit several features consistent with those proposed for the uncoupled state (Fig. 1). First, the final five residues at the M4 C-terminus are not resolved, suggesting weak, if any, interactions between the M4 C-terminus and the β6–β7 loop. Second, there are few direct contacts between the β1–β2/β6–β7 loops and the M2–M3 linker, and thus likely weak ECD/TMD interactions [18]. Finally, no changes in the orientation of the pore-lining M2 α-helices are observed between structures crystallized in the presence versus absence of agonist, suggesting a functional uncoupling of binding and gating [19,20]. Intriguingly, the channel pore of crystallized ELIC is constricted relative to that of other closed pLGICs, raising the possibility that a more constricted pore may be a structural feature of the uncoupled state (Fig. 1c).

Both the ECD and TMD of the uncoupled nAChR in phosphatidylcholine membranes bind agonists and non-competitive antagonists, respectively, with lower affinity than the desensitized nAChR [5,21]. As

Abbreviations: ACh, acetylcholine; α-BTX, α-bungarotoxin; Carb, carbamylcholine; Chol, cholesterol; ECD, extracellular domain; ELIC, *Erwinia* ligand-gated ion channel; GLIC, *Gloebacter* ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; PA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; pLGIC, pentameric ligand-gated ion channel; TID, 3-trifluoromethyl-3-(m-[^{125}I]iodophenyl) diazirine; TMD, transmembrane domain.

* Corresponding author.
E-mail address: John.Baenziger@uottawa.ca (J.E. Baenziger).

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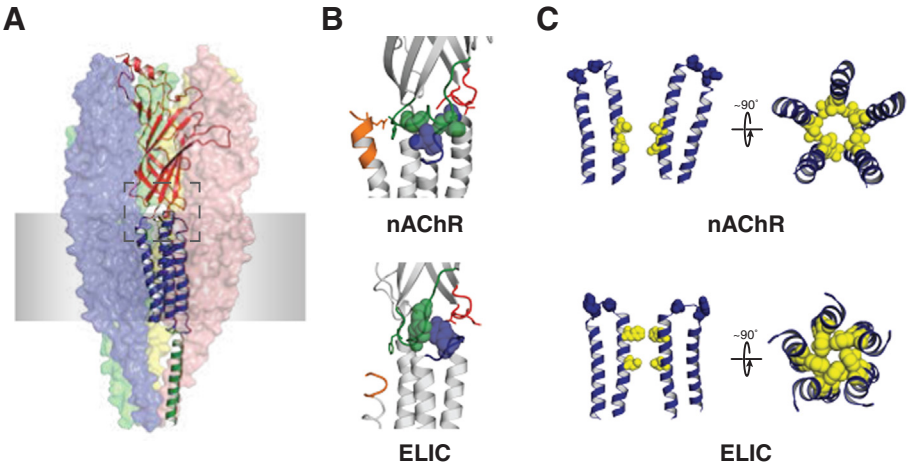

Fig. 1. Structural comparison of the nAChR and ELIC highlighting potential features of the uncoupled state. (A) The *Torpedo* nAChR structure (PDB ID: 2BG9) showing a single subunit in cartoon format. (B) The ECD-TMD coupling interface (dashed box in (A)) of a single subunit for the nAChR (top) and ELIC (bottom; PDB ID: 2VL0). The β6–β7 loop is in green, the M2–M3 linker in blue, the M4 C-terminus in orange, and the β1–β2 loop in red. In the nAChR, residues αV132 and αF135 of the β6–β7 loop and residues αP272 and αI274 of the M2–M3 linker are shown as spheres, as are the corresponding residues (D114 and R116 of the β6–β7 loop and P252 and Y256 of the M2–M3 linker) in ELIC. The interacting residues αF136 on the β6–β7 loop (F120 in ELIC) and αQ434 on the M4 C-terminus (no equivalent in ELIC structure) are shown in sticks. (C) A side view showing M2 and M3 from two nonadjacent subunits (left), with the gating residues αL251 and αV255 in the nAChR and L238 and F245 in ELIC highlighted as yellow spheres. The right panel shows a bird's-eye view of the pore-forming M2 α-helices from all subunits, illustrating the constricted diameter of the channel pore in ELIC.

the resting nAChR also binds both agonists and antagonists with relatively low affinity, the simplest interpretation is that each domain adopts a resting-state-like structure, with impaired communication between the two domains. The ELIC structure, however, raises the possibility that the TMD adopts a conformation distinct from both the resting and desensitized conformations. In fact, a distinct pore conformation could explain the apparently weak pore-binding of functional probes, such as ethidium bromide and 3-trifluoromethyl-3-(m-[-^{125}I]iodophenyl) diazirine (TID), to the uncoupled state [5,22] (see Discussion).

To probe the conformations of the individual functional domains, we characterize here the binding of acetylcholine (ACh) and two fluorescent channel blockers, ethidium and crystal violet (CrV), to the uncoupled nAChR. We confirm that the ECD binds acetylcholine with an affinity similar to that of the resting state. Although the binding of both ethidium and CrV were characterized for resting and desensitized nAChRs, no binding of either probe was detected to the uncoupled nAChR. Our data suggest that the TMD of the uncoupled nAChR adopts a distinct conformation. We discuss the implications of an altered channel pore conformation in the uncoupled state in the context of current models of pentameric ligand-gated ion channel (pLGIC) gating.

2. Materials and methods

2.1. Materials

Frozen *Torpedo californica* electroplax tissue was obtained from Aquatic Research Consultants (San Pedro, CA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (PA), and cholesterol (Chol) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Soybean asolectin (L-α-phosphatidylcholine, type II-S), carbamylcholine chloride (Carb), proadifen hydrochloride, dibucaine hydrochloride, ethidium bromide, crystal violet (CrV), and sodium cholate were from Sigma (St. Louis, MO). α-Bungarotoxin (α-BTX) was from Tocris Biosciences. [^{3}H]-Acetylcholine iodide (76 mCi/mmol) (^{3}H-ACh) was from Perkin Elmer Life Sciences (Waltham, MA).

2.2. Preparation of reconstituted nAChR

Crude membranes from *T. californica* electroplax tissue were solubilized in 1% cholate and then applied to a bromoacetylcholine column as described elsewhere [5]. The bound nAChR was washed extensively with lipids solubilized in the same cholate buffer, and then eluted with 10 mM Carb. The pooled eluate was dialyzed extensively to form proteo-liposomes at a molar lipid-to-protein ratio of roughly 500:1.

2.3. Fourier transform infrared spectroscopy

Infrared spectra were recorded, after exposure of each nAChR membrane to ^2H_2O for 72 h at 4 °C, on a Digilab (now Agilent Technologies; Santa Clara, CA) FTS7000 spectrometer [5]. Spectra were processed using GRAMS/AI software (Thermo Scientific, Wlatham, MA), with resolution enhancement performed between 1900 and 1300 cm^{-1} with $\gamma = 7$ and a Bessel smoothing function set at 70%.

2.4. Equilibrium ^{3}H-ACh binding

nAChR membranes (roughly 45 nM and 150 nM ACh sites for asolectin nAChR and PC-nAChR, respectively) were incubated with the indicated concentrations of ^{3}H-ACh for a minimum of 75 min at 4 °C. Bound versus free ^{3}H-ACh were then separated from each other by filtration under vacuum through 25 mm GF/F glass fibre filter paper (Whatman) [5]. Specific binding is the difference between total binding and non-specific binding, the latter observed in the presence of a 20-fold molar excess of α-BTX. Filter papers (bound ^{3}H-ACh) and filtrate (free ^{3}H-ACh) were quantified by liquid scintillation counting in Ecolite(+)™ (MP Biochemicals, Santa Ana, CA). To compare our binding data to previously published results, we fit the binding isotherms with a hyperbolic binding function ($Y = B_{max} * X(K_{eq} + X)$ using GraphPad Prism, where Y is specific binding and X is the concentration of ^{3}H-ACh). As with previously published data, ACh binding to the nAChR in either asolectin or PC/PA/chol membranes was well characterized by a single K_{eq} value (see Results).

2.5. Fluorescence spectroscopy

Spectra were acquired at 22.5 °C on a Cary Eclipse fluorescence spectrometer (Agilent Technologies) [5]. Except where noted, data was collected with excitation/emission wavelengths set to 530 nm/590 nm and excitation/emission slits at ±5/20 nm for ethidium (except where noted). For CrV, the excitation/emission wavelengths and excitation/emission slit widths were 590/630 nm and ±10/20 nm, respectively.