ELIC-α7 Nicotinic Acetylcholine Receptor (α7nAChR) Chimeras Reveal a Prominent Role of the Extracellular-Transmembrane Domain Interface in Allosteric Modulation*

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Background: Allosteric modulators bound to the transmembrane domain (TMD) of the α7 nicotinic acetylcholine receptor (α7nAChR) can potentiate channel function.

Results: ELIC-α7nAChR showed potentiation only when the extracellular-transmembrane domain (ECD-TMD) interface matched that of α7nAChR.

Conclusion: PAM modulation through the TMD requires a more specific ECD-TMD interface than agonist activation.

Significance: The study provides insight into the basis for positive allosteric modulation of α7nAChR.

The native α7 nicotinic acetylcholine receptor (α7nAChR) is a homopentameric ligand-gated ion channel mediating fast synaptic transmission and is of pharmaceutical interest for treatment of numerous disorders. The transmembrane domain (TMD) of α7nAChR has been identified as a target for positive allosteric modulators (PAMs), but it is unclear whether modulation occurs through changes entirely within the TMD or changes involving both the TMD and the extracellular domain (ECD)-TMD interface. In this study, we constructed multiple chimeras using the TMD of human α7nAChR and the ECD of a prokaryotic homolog, ELIC, which is not sensitive to these modulators, and for which a high resolution structure has been solved. Functional ELIC-α7nAChR (EA) chimeras were obtained when their ECD-TMD interfaces were modified to resemble either the ELIC interface (EAELIC) or α7nAChR interface (EAα7). Both EAα7 and EAELIC show similar activation response and desensitization characteristics, but only EAα7 retained the unique pharmacology of α7nAChR evoked by PAMs, including potentiation by ivermectin, PNU-120596, and TQS, as well as activation by 4BP-TQS. This study suggests that PAM modulation through the TMD has a more stringent requirement at the ECD-TMD interface than agonist activation.

The α7 nicotinic acetylcholine receptor (α7nAChR) is a homopentameric acetylcholine-gated cation channel mediating fast synaptic transmission in neuronal cells (1) and calcium signaling in nonneuronal cells (2). The diverse biological functions of α7nAChR have made it a promising therapeutic target for numerous medical conditions, including pain (3, 4), inflammation (5), cardiovascular disease (6), and a variety of psychiatric and neurological disorders (7–9). α7nAChR opens the channel gate in the transmembrane domain (TMD) upon agonist binding to the extracellular domain (ECD), but channel function can also be modulated by ligand binding to allosteric sites distinct from the orthosteric agonist-binding sites in the ECD. Positive allosteric modulators (PAMs), especially those specific for α7nAChR, such as PNU-120596 (10) and TQS (11), are particularly of therapeutic potential (8). Based upon mutational analysis, certain PAMs are thought to act at the TMD or ECD-TMD interface (12, 13), whereas others, such as PNU-120596 and TQS, are thought to act through binding to intrasubunit sites near the middle of the α7nAChR TMD (13–17). The molecular mechanisms of allosteric modulation are not understood.

α7nAChR is a member of the pentameric ligand-gated ion channel (pLGIC) superfamily, including different subtypes of nAChRs, 5-HT2A receptors (5-HT2ARs), glycine receptors (GlyRs), and GABA A receptors (GABA ARs). α7nAChR is also homologous to GluCl, and the prokaryotic pLGICs ELIC and GLIC, whose crystal structures have been determined in the absence and presence of ligands (18–28). A wealth of data from decades of biochemical characterization and more recent structural characterization of pLGICs provide a broad understanding of the events relating to agonist binding and channel activation (28–35). Agonist binding to orthosteric sites in the ECD leads to conformational transitions that propagate through the ECD-TMD interface to the TMD and open the channel gate. Upon prolonged exposure to agonist, the channel desensitizes and is no longer sensitive to agonist binding until it returns to the resting state. Among pLGICs, α7nAChR demonstrates particularly rapid desensitization. PAMs such as ivermectin, PNU-120596, and TQS can significantly slow down the desensitization of α7nAChR (10, 11, 36, 37). Several studies have demonstrated involvement of the ECD-TMD interface in desensitization (38–41), but the role of the ECD-TMD interface in allosteric modulation through the TMD is less known.
Chimeras joining the ECD from one pLGIC with the TMD from another provide valuable opportunities to understand how the interplay among different domains/regions underlies pLGIC function. Previous studies of GLIC-α1GlyR (42), α7nAChR-α1GlyR (43), α4β2nAChR-5HT3R (44), and α7nAChR-5HT3R (45) found that functional chimeras could be obtained by splicing the ECD prior to the first transmembrane helix of the TMD, although kinetic parameters could be improved by optimization of the ECD-TMD interface. In contrast, it has proven difficult to express a functional chimera of an α7nAChR TMD (13). A 5HT3R-α7nAChR chimera expressed a small current in only a few oocytes, which was sufficient to show that potentiation by the PAM PNU-120596 could be conferred by the α7nAChR TMD when activated by a suboptimal agonist (13). However, given the high homology between loop 7 and the TM2-3 linker of α7nAChR and 5HT3R (approximately 78% similarity), it is unclear what role the ECD-TMD interface might have played in the observed potentiation.

In this study, we constructed multiple chimeras combining the TMD of human α7nAChR and the ECD of the prokaryotic homolog ELIC (Fig. 1). ELIC is a cation channel activated by a group of primary amines, including cysteamine (46). In contrast to α7nAChR, ELIC is inhibited by the general anesthetic propofol but unaffected by PAMS like ivermectin (21, 47). We show here that ELIC is also affected by the α7nAChR-specific PAMS PNU-120596 and TQS. We examined the effect of the ECD-TMD interface on allosteric modulation through the TMD by engineering two classes of ELIC-α7nAChR (EA) chimeras: EAELIC, with an ECD-TMD interface resembling ELIC, and EAα7, with an α7nAChR ECD-TMD interface. Both classes of EA chimeras resulted in functional channels with similar activation and desensitization kinetics. However, only EAα7 retained sensitivity to the PAMS ivermectin, PNU, and TQS as well as the ability to be activated by 4BP-TQS. Thus, allosteric modulation through the TMD requires a specific ECD-TMD interface similar to that of α7nAChR, a more stringent requirement than that needed to activate the channel by agonists.

**EXPERIMENTAL PROCEDURES**

**Construction of EA Chimeras**—EA chimeras were constructed by fusing the ECD of ELIC ending after pre-TM1 (ELIC-S201) with the beginning of the human α7nAChR TMD (α7-Y210) using overlapping PCR (48). The resulting construct was subcloned into a Xenopus expression vector, pOTV (49) for expression of RNA. Mutations to match the sequence of interface elements between ELIC and α7nAChR were introduced using QuickChange Lightning Site-directed Mutagenesis kits (Agilent). All constructs were confirmed by sequencing in full.

**Electrophysiological Recordings in Xenopus Oocytes**—Channel function was measured by two-electrode voltage clamp experiments (50) using Xenopus laevis oocytes injected with RNA encoding the indicated constructs as described previously (20). Capped complementary RNA was synthesized with the mMessage mMMachine T7 kit (Ambion), purified with the RNaseasy kit (Qiagen) and injected (25–50 ng) into X. laevis oocytes (stages 5–6). Oocytes were maintained at 18 °C in modified Barth’s solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM HEPES, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamicin sulfate, pH 6.7. After 1–3 days expression, oocytes were clamped to a holding potential of −60 mV with an OC-725C Amplifier (Warner Instruments) in a 20-μl oocyte recording chamber (Automate Scientific). Currents were elicited using cysteamine as an agonist. The recording solutions contained 130 mM NaCl, 0.1 mM CaCl2, 10 mM HEPES, pH 7.0, with the indicated concentrations of cysteamine and other modulators. Data were collected and processed using Clampex 10 software (Molecular Devices). Nonlinear regressions were performed using Prism software (GraphPad).

**Modeling of Chimeras**—Homology models for EA chimeras were generated using Modeler 9v8 (51, 52) based on the crystal structure of ELIC (Protein Data Bank (PDB) ID code 3RQW) and the NMR structure of the α7nAChR TMD (PDB ID code 2MAW). Five independent models were generated, and the model with the lowest discrete optimized potential energy was selected for presentation.

**RESULTS**

**Engineering Functional EA Chimeras**—Multiple EA chimeras were constructed to assess the requirements for efficient coupling at the ECD-TMD interface. The function of each chimera was tested in Xenopus oocytes by two-electrode voltage clamp electrophysiology. The data for agonist response are summarized in Table 1.

The original ECD-TMD interfaces for ELIC and α7nAChR are not compatible. A chimera (EAELIC) simply connecting the ELIC ECD with the α7nAChR TMD at the beginning of TM1 did not show any response to the ELIC agonist cysteamine when the chimera was expressed in Xenopus oocytes (Table 1). This was unexpected. Chimeras between most members of the pLGIC family were found to have at least some degree of function when joined in this manner (13, 42–45), with the exception of the AChBP-5HT3A chimeras (53). To rule out interference...
by the extended C terminus of α7nAChR, we replaced the last 18 residues of EAELIC compared with ELIC. This construct EAELIC also failed to exhibit current in response to cysteamine (Table 1).

The sequence, length, and position of the TM2-3 linker vary among the known pLGIC structures. The TM2-3 linker comprises 8 residues beginning at 22’ in ELIC (PDB ID code 3RQU) (20) and 10 residues beginning at 22’ in the NMR structure for the TMD of α7nAChR (PDB ID code 2MAW) (54). The TM2-3 linker in the cryo-EM structure of Torpedo nAChR has only 7 residues in the flexible loop beginning at 29’. If one counts the TM2-3 linker of Torpedo nAChR starting from 22’, it has a total of 14 residues (PDB ID code 2BG9) (55). A recent disulfide trapping experiment with the α subunit of mouse muscle nAChR showed a shift in register between TM2 and TM3, which could not be reconciled with 2BG9, but was consistent with the α7 NMR structure (54).

To improve coupling of the α7nAChr TM2-3 linker to the ELIC ECD without disrupting TMD interactions, we substituted varied lengths of the TM2-3 linker in α7nAChR with their counterparts in ELIC. Substitution of only 4 residues in the middle of the TM2-3 linker (EAELIC)5 or more extensive substitution from 19’ to 28’ (EAELIC)5 was not functional (Table 1).

A functional EA chimera (EAELIC)5 was obtained by replacing all 14 residues from 22’ to 35’ of the TM2-3 region with the 8-residue ELIC TM2-3 linker. A functional EA chimera (EAELIC)5 was also obtained by substituting the α7nAChR TM2-3 linker from 20’ to 26’ with the ELIC TM2-3 linker from 23’ to 29’. Both EAELIC5 and EAELIC responded efficiently to the ELIC agonist cysteamine, with EC50 values of 0.4 and 1.0 mm, respectively (Table 1 and Fig. 2), comparable with that for ELIC (20, 46). The efficient activation obtained with both EAELIC5 and EAELIC compared with EAELIC7 and EAELIC4 suggests that it is not the length of the TM2-3 linker, but its position at the ECD-TMD interface that is critical for efficient coupling.

To engineer a chimera with a more native α7nAChR ECD-TMD interface, we kept the α7nAChR TM2-3 linker intact but mutated the ELIC ECD to match the sequence of α7nAChR at loops 2, 7, and 9, and the pre-TM1 region. This construct, EAα7, is functional with an EC50 of 1.3 mm for cysteamine (Table 1 and Fig. 2). EA chimeras with mismatches at loop 2 (EAα71) or loop 9 (EAα72) still expressed as functional channels, but with a rightward shift in EC50 for cysteamine, suggesting a lower coupling efficiency (Table 1 and Fig. 2c). Mismatches at both loop 2 and loop 9 (EAα73), or at loop 7 alone (EAα74), resulted in chimeras that did not exhibit current in response to cysteamine (Table 1). Taken together, these results suggest that an ensemble of coupling among loop 2, loop 7, loop 9, the pre-TM1 region and the TM2-3 linker is essential for obtaining functional channels. Similar modifications were also needed for the functional AChBP-5HT3 chimera (53).

ELIC (46) and α7nAChR (1) exhibit desensitization with sustained application of agonist, but α7nAChR (0.18 ± 0.05 min, n = 3) desensitizes an order of magnitude faster than ELIC (2.4 ± 0.2 min, n = 3) (Fig. 3). Measuring ensemble currents in Xenopus oocytes, both EAα7 and EAELIC exhibited initial desensitization (1.7 ± 0.2 and 1.6 ± 0.2 min, respectively, n = 3) comparable with ELIC, but then plateaued at 55 ± 11% and 74 ± 16% of the maximal current, respectively (Fig. 3).

EAα7 Shows Pharmacology Similar to α7nAChR, but EAELIC Does Not—ELIC and α7nAChR have different pharmacological profiles for allosteric modulators. Consistent with previous results (13–16, 57), ivermectin and two α7-specific PAMs, PNU-120596 and TQS, potentiated α7nAChR, but the intravenous general anesthetic propofol had no effects on α7nAChR (Fig. 4). In contrast, ELIC could be inhibited by propofol (21, 47).
but was insensitive to ivermectin, PNU-120596, or TQS (Fig. 4). The differences between ELIC and \( \alpha_7 \)nAChR present the opportunity to evaluate the role of the TMD and ECD-TMD interface in allosteric modulation. To this end, we compared the functional responses of \( \alpha_7 \)nAChR, ELIC, and EA\( \alpha_7 \) to the selected allosteric modulators (Fig. 4).

EA\( \alpha_7 \) responded to these modulators similarly to \( \alpha_7 \)nAChR, but distinctly different from ELIC. Resembling \( \alpha_7 \)nAChR, EA\( \alpha_7 \) was potentiated by ivermectin, PNU-120596, and TQS, and insensitive to propofol (Fig. 4). 4BP-TQS, an allosteric agonist and PAM for \( \alpha_7 \)nAChR (15), could also directly activate EA\( \alpha_7 \) and potentiate its channel response to agonist (Fig. 5).

**FIGURE 2. Activation of EA chimeras by cysteamine.** a and b, representative current traces for EA\( \alpha \text{ELIC} \) (a) and EA\( \alpha_7 \) (b). Bars over the trace indicate the length of application and cysteamine concentrations. Horizontal and vertical scale bars indicate 1 min and 0.1 \( \mu \)A current, respectively. c, cysteamine response curves for the functional EA chimeras. Current is expressed as a fraction of maximal current, \( n \approx 5 \) oocytes. Error bars indicate S.D. Data are fit to Hill equations with the following parameters: [EA\( \alpha \text{ELIC} \), EC\text{50} = 1.0 ± 0.02 mM]; [EA\( \alpha_7 \), EC\text{50} = 1.3 ± 0.1 mM]; [EA\( \alpha \text{ELIC} \), EC\text{50} = 0.4 ± 0.02 mM]; [EA\( \alpha_7 \), EC\text{50} = 2.2 ± 0.1 mM]. The corresponding Hill coefficients are 1.7 ± 0.1, 1.3 ± 0.1, 1.5 ± 0.1, 1.9 ± 0.1, and 2.4 ± 0.2, respectively.

**FIGURE 3. EA chimeras desensitize much more slowly than \( \alpha_7 \)nAChR.** Representative current traces show a 1.5-min agonist application to Xenopus oocytes injected with the indicated constructs. Acetylcholine at 100 \( \mu \)M was applied to \( \alpha_7 \)nAChR; 30 mM cysteamine was applied to ELIC and EA chimeras. Rate constants for the initial rate of desensitization were calculated (\( n = 3 \)): \( \alpha_7 \)nAChR = 0.18 ± 0.05; ELIC = 2.4 ± 0.2; EA\( \alpha \text{ELIC} \) = 1.6 ± 0.2; and EA\( \alpha_7 \) = 1.7 ± 0.2 min.

**FIGURE 4. EA\( \alpha_7 \) resembles \( \alpha_7 \)nAChR pharmacology.** Representative traces of ELIC, \( \alpha_7 \)nAChR, and EA\( \alpha_7 \) show responses to the indicated allosteric modulators. Ivermectin (30 \( \mu \)M), TQS (100 \( \mu \)M), and propofol (100 \( \mu \)M) were applied with agonist cysteamine (ELIC, EA\( \alpha_7 \)) or acetylcholine (\( \alpha_7 \)nAChR) at the EC\text{20} for each construct; PNU-120596 (30 \( \mu \)M) was applied with agonist at the EC\text{70} for each construct. Bars over the traces indicate the length of application. Horizontal and vertical scale bars indicate 1 min and 0.1 \( \mu \)A current, respectively.

**FIGURE 5. EA\( \alpha_7 \) is activated and potentiated by 4BP-TQS.** Representative traces of ELIC and EA\( \alpha_7 \) show responses to cysteamine and 4BP-TQS. a, ELIC is insensitive to 4BP-TQS, but EA\( \alpha_7 \) is activated (b) and potentiated (c) by 4BP-TQS (100 \( \mu \)M). Cysteamine concentrations are at the EC\text{20} for each construct. Horizontal and vertical scale bars indicate 1 min and 0.1 \( \mu \)A current, respectively.
can still be modulated by these PAMs, suggesting that fast desensitization may not be required for potentiation by PNU-120596 or TQS. Because EA_{a7} and EA_{ELIC} show similar desensitization rates, desensitization cannot explain their distinct responses to the tested PAMs.

What may have contributed to the different responses of EA_{a7} and EA_{ELIC} to PAMs? The PAMs tested here bind to the TMD (13–15, 58). The differences between EA_{a7} and EA_{ELIC}/EA_{ELIC} are localized only to the ECD-TMD interface. One may suspect that lack of modulation in EA_{ELIC} results from interference with PAMs binding to a structurally distorted TMD due to a shortened TM2-3 linker. However, such a possibility is dismissed by EA_{ELIC}, which has a full-length TM2-3 linker that is unlikely to distort the TMD.

Because no high resolution structural data are available, the precise binding sites for ivermectin, PNU-120596, or TQS in α7nAChR are unknown. In the Caenorhabditis elegans GluCl crystal structure, ivermectin contacts both the TM2-3 linker and the residues toward the middle of the TMD (18). Mutational analysis on α7nAChR also suggests that ivermectin interacts with residues near the TM2-3 linker and residues near the middle of the TMD (16, 59). If direct contacts to specific residues in the TM2-3 linker are critical for ivermectin modulation, the ELIC TM2-3 linker may not provide proper contacts in EA_{ELIC} and EA_{ELIC}, thereby preventing ivermectin modulation.

In the case of PNU-120596 and TQS, they are much smaller than ivermectin and cannot simultaneously contact residues at both the ECD-TMD interface and near the middle of the TMD. If they directly contact the ECD-TMD interface, the role of the ECD-TMD interface in PAM modulation will be the same as discussed above for ivermectin. However, previous mutation studies suggest an intrasubunit binding site near the middle of the TMD for PNU-120596 and TQS (13–15, 58). In this case, the binding site is remote from the ECD-TMD interface. How can the interface still be important for potentiation? It is possible that the PAM binding to the proposed site near the middle of TMD allosterically induces changes to the ECD-TMD interface that are required for potentiation. These changes can be accommodated by the ECD-TMD interface in α7nAChR, but not in ELIC. This hypothesis is supported by a substituted cysteine accessibility study (60), which demonstrates that PNU-120596 binding can independently induce conformational changes to the ECD and ECD-TMD interface. The conformational changes induced by PNU-120596 are similar but not identical to those induced by the agonist acetylcholine (60).

Structural characterization of the precise binding sites for these PAMs is essential for understanding the mechanism of PAM modulation. Future high resolution structural studies of the EA chimeras in the absence and presence of modulators will provide insights into binding of PAMs to the TMD and the role of the ECD-TMD interface in allosteric modulation. Because of the prokaryotic origin of its ECD, the EA chimeras are good candidates for production in quantities suitable for high resolution structural studies (19, 20).

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