Molecular Dissection of Cl$^-$-selective Cys-loop Receptor Points to Components That Are Dispensable or Essential for Channel Activity

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Cys-loop receptors are pentameric ligand-gated ion channels (pLGICs) that bind neurotransmitters to open an intrinsic transmembrane ion channel pore. The recent crystal structure of a prokaryotic pLGIC from the cyanobacterium Gloeobacter violaceus (GLIC) revealed that it naturally lacks an N-terminal extracellular α helix and an intracellular domain that are typical of eukaryotic pLGICs. GLIC does not respond to neurotransmitters acting at eukaryotic pLGICs but is activated by protons. To determine whether the structural differences account for functional differences, we used a eukaryotic chimeric acetylcholine-glutamate pLGIC that was modified to carry deletions corresponding to the sequences missing in the prokaryotic homolog GLIC. Deletions made in the N-terminal extracellular α helix did not prevent the expression of receptor subunits and the appearance of receptor assemblies on the cell surface but abolished the capability of the receptor to bind α-bungarotoxin (a competitive antagonist) and to respond to the neurotransmitter. Other truncated chimeric receptors that lacked the intracellular domain did bind ligands; displayed robust acetylcholine-elicited responses; and shared with the full-length chimeric receptor similar anionic selectivity, effective open pore diameter, and unitary conductance. We suggest that the integrity of the N-terminal α helix is crucial for ligand accommodation because it stabilizes the intersubunit interfaces adjacent to the neurotransmitter-binding pocket(s). We also conclude that the intracellular domain of the chimeric acetylcholine-glutamate receptor does not modulate the ion channel conductance and is not involved in positioning of the pore-lining helices in the conformation necessary for coordinating a Cl$^-$ ion within the intracellular vestibule of the ion channel pore.

Cys-loop receptors constitute a superfamily of cell surface oligomers that bind neurotransmitters such as acetylcholine (ACh),$^3$

nACHr, nicotinic acetylcholine receptor; pLGIC, pentameric ligand-gated ion channel; SHT, 5-hydroxytryptamine; MA, membrane-associated; NES, normal external solution; Rd-dTX, rhodaminylated α-bungarotoxin; SHTR, 5-hydroxytryptamine receptor; GlyR, glycine receptor; E$_{rev}$, reversal potential; FL, full-length α7-GluClβ chimeric subunit; FL-R, full-length receptor (α7-GluClβR); Δ1–Δ9, truncated α7-GluClβR subunits; Δ4–R, truncated α7-GluClβΔ4R; Δ5–R, truncated α7-GluClβΔ5R.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures and Figs. S1–S3.

2 Both authors made equal contributions to this work.

3 The abbreviations used are: ACh, acetylcholine; dBTX, α-bungarotoxin; GLIC, pLGIC from the cyanobacterium Gloeobacter violaceus; GluClR, glutamate-gated Cl$^-$-selective Cys-loop receptor; LigBD, ligand-binding domain; serotonin (5-hydroxytryptamine (5HT)), γ-aminobutyric acid (GABA), glycine (Gly), glutamate (Glu), and histamine to open an intrinsic transmembrane ion channel (for reviews, see Refs. 1–8). Hence, Cys-loop receptors mediate the rapid flow of ions such as Na$^+$, K$^+$, Ca$^{2+}$, and Cl$^-$ across the cell membrane down their electrochemical gradients to alter the membrane potential or enable Ca$^{2+}$ influxes and signaling. Cys-loop receptors are known also as pentameric ligand-gated ion channels (pLGICs). The subunits of pLGICs are radially aligned around an axis of 5-fold symmetry, which is the axis of the ion permeation pathway. Each subunit traverses the membrane four times. Upon receptor assembly, the N-terminal extracellular segment forms a ligand-binding domain (LigBD) having neurotransmitter-binding pockets at intersubunit interfaces. The ion channel domain is formed by 20 transmembrane helices, M1–M4 of each subunit (see Fig. 1, A and B, numbered 1–4). All eukaryotic pLGICs have a long intracellular sequence that connects M3 with M4. This sequence (the so-called M3-M4 loop or linker) forms an intracellular domain that plays a role in assembly (9–11), trafficking (12–17), targeting (18, 19), and clustering (20) of various pLGICs (for reviews, see Refs. 21–23). The intracellular domain is also a target for phosphorylation by various protein kinases that modulate channel desensitization and conductance (see Ref. 24 and references therein).

Recently, the three-dimensional structure of a prokaryotic pLGIC (GLIC) was determined at atomic resolution by x-ray crystallography (25, 26). It turns out that compared with GLIC eukaryotic pLGICs have extra amino acid sequences. These sequences are (i) an N-terminal extracellular sequence that by homology with acetylcholine-binding proteins (27–31) and eukaryotic pLGICs of known three-dimensional structure (32–34) probably folds into an α helix in all other eukaryotic pLGICs (e.g. α1 helix in Fig. 1, A and B, in blue) and (ii) an intracellular M3-M4 linker (e.g. Fig. 1A, uppermost scheme) that is much longer in eukaryotic pLGICs (~100–250 residues) (24) than in GLIC (a few residues) (25, 26). It is not known yet how the large intracellular M3-M4 linker of eukaryotic pLGICs folds apart from an amphipathic α helix that is located a few amino acids

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before M4 of the nicotinic acetylcholine receptor (nAChR) of Torpedo marmorata. This amphipathic α helix is termed the “membrane-associated (MA) helix.” The three-dimensional fold and the orientation of the intracellular MA helix have been brought into view by cryo-electron microscopy (cryo-EM) (32). It was hypothesized that charged residues of this helix line intersubunit portals through which cations laterally flow before or after they cross the transmembrane ion channel pore (depending on ion flow direction) (32, 35). Functional studies performed with a few vertebrate pLGICs showed that charged residues in the homologous MA helix of the cationic 5HT₃ (36–39) and αβ2 nACh (39) receptors modulate the ion channel conductance (for a review, see Ref. 40) (see sequences in Fig. 1C). Much less is known about conductance modulation by the MA helix in anionic pLGICs apart from a vertebrate Glyc1 receptor whose MA helix has residues implicated in modulation of the single channel conductance (41) (see sequence in Fig. 1, C and D, right, and supplemental Fig. S3A). No information regarding the functional role of such an intracellular MA helix in invertebrate Cys-loop receptors is available.

The GluClR is a glutamate-gated Cl⁻-selective Cys-loop receptor expressed as an α/β heteropentamer uniquely in invertebrates (42–44). Here, we used a eukaryotic chimeric receptor that consists of an extracellular acetylcholine-binding domain fused to the ion channel and intracellular domains of the GluClR. We removed segments of this chimeric receptor that do not have counterpart sequences in the prokaryotic homolog GLIC (i.e. the α1 helix and/or the M3-M4 linker) as well as sequences that include the M3 and/or M4 helices. Functional studies performed with the truncated receptors shed light on the involvement of the deleted elements in cell surface expression, ligand binding capacity, and various channel properties.

**EXPERIMENTAL PROCEDURES**

**Generation of cDNA Constructs**—The cDNA encoding the full-length α7-GluClβ chimeric subunit (FL) was generated as in Ref. 45 and was cloned into pcDNA3 and pMT3 vectors. Deletions were made by PCR performed principally in the QuikChange site-directed mutagenesis kit (Stratagene) where the entire plasmid is synthesized. We used primers that hybridize to 17–23 bases on each side of the open reading frame (ORF) sequence to be deleted (i.e. primers never contained the sequence to be deleted). This strategy yielded the truncated α7-GluClβ Δ1–Δ4 and Δ6–Δ9 subunits (Fig. 1A). To produce the Δ5 subunit, the primers designed for deleting the M3-M4 linker contained an additional sequence that encodes the Ala-Gly-Gly-Gly-Gly short stretch to introduce further flexibility compared with the Δ4 truncated receptor. To insert the hemagglutinin (HA) tag N-terminally to the first residue of the mature proteins, we used primers that contained the extra sequence flanked on both sides by 14–16 bases that hybridize with the ORF sequence around the point of insertion. The entire ORF of all PCR products was sequenced and subcloned into the original pcDNA3 and/or pMT3 vector.

**Confocal Microscopy**—Cells were seeded on a 35-mm glass bottom dish (MatTek, Corp., Ashland, MA) that was precoated with 20 μg/ml poly-l-lysine (Sigma-Aldrich). Twenty-four hours later, transfections were performed with calcium phosphate as described in the supplemental Experimental Procedures using 850 ng of pMT3 plasmid carrying a cDNA insert that encodes the chimeric subunit of interest. Three days after transfection, the cells were washed three times with 3 ml of normal external solution (NES; which was also used for patch clamp recordings; see composition below). Cells were incubated with a blocking solution containing 1 ml of NES and 0.02% BSA for 30 min at 4 °C and then incubated overnight at 4 °C with rhodaminylated α-bungarotoxin (Rd-αBTx; Invitrogen-Molecular Probes) at a final concentration of 270 nM. After several washes with 3 ml of NES, cells were visualized under a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging GmbH). To test whether Rd-αBTx binds nonspecifically, experiments were repeated with 5 mM nicotine.

Cells transfected with a cDNA encoding a given HA-tagged chimeric subunit were quenched as described above and then incubated for 1 h at 4 °C with 2 μg/ml rabbit anti-HA antibodies (Y-11; Santa Cruz Biotechnology, Inc.). After a few washes with 2 ml of NES containing 0.02% BSA, fluorescent goat anti-rabbit IgG antibodies (Alexa Fluor 488; Invitrogen-Molecular Probes) were added to a final concentration of 0.5 μg/ml, and the cells were incubated again for 1 h at 4 °C. Prior to the visualization by confocal microscopy, the glass bottom dishes were washed several times with 3 ml of NES to reduce nonspecific adsorption of antibodies by the cells. To test the specificity of the antibodies, experiments were repeated with untagged chimeric subunits.

**Ligand Binding Assays**—Ligand binding experiments were performed as in Pittel et al. (46). Curves of [³H]αBTx binding were plotted as a function of [³H]αBTx concentrations by fitting the curves to the data points with a nonlinear regression using Equation 1,

\[
B = \frac{B_{\text{max}}[\text{[³H]}\alpha\text{BTx}]}{K_D + \text{[[³H]}\alpha\text{BTx}]}
\]  
(Eq. 1)

where \(B\) is the bound [³H]αBTx, \(B_{\text{max}}\) is the maximal binding of [³H]αBTx, \([\text{[³H]}\alpha\text{BTx}]\) is the concentration of the radiolabeled toxin, and \(K_D\) is the αBTx dissociation constant.

For inhibition of αBTx binding by nicotine, curves of [³H]αBTx binding were plotted as a function of nicotine concentrations by fitting the curves to the data points with a nonlinear regression using the Hill equation (Equation 2),

\[
\frac{B}{B_{\text{max}}} = 1 - \frac{1}{1 + 10^{n_{H}\text{log}_{K_I} - \log_{[\text{Ag}]}^m}}
\]  
(Eq. 2)

where \(B\) is the bound [³H]αBTx, \(B_{\text{max}}\) is the maximal binding of [³H]αBTx, \(K_I\) is the agonist inhibition constant, \([\text{Ag}]\) is the concentration of the agonist (nicotine), and \(n_H\) is the Hill coefficient. Further technical details are provided in the supplemental Experimental Procedures.

**Whole-cell and Single Channel Patch Clamp Recordings**—Recordings were performed in either human embryonic kidney (HEK293T) cells or Chinese hamster ovary (CHO) cells, which were prepared as described in the supplemental Experimental Procedures. For whole-cell patch clamp experiments, the NES contained 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES adjusted to pH 7.35 with
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NaOH (310 mosM). For the determination of ionic permeability ratios, we also used external solutions with reduced NaCl concentrations of 70, 35, 17.5, and 0 mM. The equimolar conditions of these solutions were maintained by the addition of mannitol. We also used external solutions containing 140 mM sodium acetate instead of NaCl. The pipette solution in experiments using HEK293T cells contained 130 mM CsCl, 4 mM MgCl₂, 4 mM Na₂ATP, 1 mM EGTA, and 10 mM HEPES adjusted to pH 7.35 with CsOH (290 mosM). When using CHO cells, CsCl and CsOH were replaced by KCl and KOH, respectively. The electrode resistance was 6–10 megohms when filled with the pipette solution. External solutions were applied onto the cell by using the VC-77SP fast step system (Warner Instruments) combined with an N₂ pressure of 3–4 p.s.i.

To determine the macroscopic activation and desensitization time constants, the current rise and current decline were best fitted with a single exponential time course using equation 4, where

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + 10^{\log EC_{50} - \log [\text{ACh}] / V_{m}}} \]  

(Eq. 3)

where \( I \) is the current response, \( I_{\text{max}} \) is the maximal current response, \( EC_{50} \) is the agonist concentration that elicits 50% of the maximal current response, \([\text{ACh}]\) is the concentration of acetylcholine, and \( V_{m} \) is the Hill coefficient.

To determine the macroscopic activation and desensitization time constants, the current rise and current decline were best fitted with a single exponential time course using equation 4, where

\[ Y = A \cdot e^{-\frac{t}{\tau}} + C \]  

(Eq. 4)

where \( A \) is the current amplitude, \( t \) is the time, \( \tau \) is the time constant, and \( C \) is the constant \( y \)-offset.

To determine ionic permeability ratios, current-voltage relations were established in various external solutions (see compositions above). Currents were elicited by application of 200 \( \mu \text{M} \) ACh for 1 s. 0.3 s after the beginning of the ACh application, inverted 200-ms long voltage ramps from +80 mV to −100 mV were applied. The initial holding potential was −60 mV. Leak currents, which were obtained by the same protocol but in the absence of ACh, were subtracted from the ACh-elicited currents. Reversal potential ([\( E_{\text{rev}} \)]) values were corrected to account for the liquid junction potentials calculated by using the Junction Potential Calculator implemented in the pClamp 10 software (47). Anion to cation permeability ratios were calculated using the Goldman-Hodgkin-Katz equation (Equation 5),

\[ E_{\text{rev}} = \frac{RT}{F} \ln \frac{[\text{Cl}^-]_o + \alpha [\text{Na}^+]_o + \beta [\text{Cs}^+]_o}{[\text{Cl}^-]_o + \alpha [\text{Na}^+]_o + \beta [\text{Cs}^+]_o} \]  

(Eq. 5)

where \( E_{\text{rev}} \) is the reversal potential; \( R \), \( T \), and \( F \) are the gas constant, the absolute temperature, and the Faraday constant, respectively; subscript “o” and “i” are extra- and intracellular ion activities, respectively; and the permeability ratios are \( \alpha = P_{\text{Na}}/P_{\text{Cl}} \) and \( \beta = P_{\text{Cs}}/P_{\text{Cl}} \). Permeability ratios were analyzed using ion activities instead of ion concentrations. Ion activities were calculated on the basis of the Debye-Hückel theory (48) but with the corrections introduced in the Milero-Pitzer method for solutions having an ionic strength greater than 0.1 M (49) as implemented in the Electrolytes program of Aq-Solutions, a software package of programs for the quantitative treatment of equilibria in aqueous solutions (50).

For measurement of single channel currents from cell-attached membrane patches, electrodes were coated with Sylgard 184 (Dow Corning Corp.) to reduce pipette capacitance. The resistance of the recording electrodes was set on 10–15 megohms when filled with the pipette solution. Both the bath and pipette contained NES, and the pipette also contained 100 \( \mu \text{M} \) ACh. The acquisition of recorded data was performed at 10 kHz, and recordings were low-pass-filtered at 1 kHz. Single channel events were detected by the half-amplitude threshold criterion using the Clampfit 10 program implemented in pClamp 10. The mean value of the unitary current was obtained by curve fitting to the single channel current amplitudes using the Gaussian equation (Equation 6),

\[ f(x) = \sum_{i=1}^{n} A_i e^{-\frac{(x - \mu_i)^2}{2\sigma_i^2}} + C \]  

(Eq. 6)

where \( A \) is the amplitude; \( \mu \) is the Gaussian mean; \( \sigma \) is the Gaussian standard deviation; \( C \) is the constant \( y \)-offset for each component \( i \); and \( n = 2 \) components, \( i_1 \) and \( i_2 \) that correspond to closed and open channel states, respectively.

Computer-assisted Homology Modeling—The three-dimensional homology model was built as in Pittel et al. (46) with one major difference as follows. Previously, we connected the channel domain model of the 5HT₃A R (51) to the homology model of the α7-nAChR LigBD (46); here, we connected the channel domain model of the GluClβR (45) to the homology model of the α7-nAChR LigBD.

Statistical Analysis—\( p \) values correspond to one-way analysis of variance or unpaired, two-tailed \( t \) tests as detailed in the legends to figures and tables.

Other Methods—The following methods are provided in the supplemental Experimental Procedures: transfection of cultured cells and cell harvesting, further information regarding ligand binding assays, membrane preparation and polyacrylamide gel electrophoresis (PAGE), and preparation of cells for the electrophysiological experiment.

RESULTS

Structural Properties of Chimeric Receptors Used Here—Homomeric Cys-loop chimeric receptors, which have the LigBD of the α7-nAChR fused to the channel and intracellular domains of the cationic 5HT₃A receptor, have long been utilized for the characterization of various receptor-channel properties (e.g. Refs. 52–55). Here, we used a different chimeric receptor that also consists of five identical subunits, but each subunit is a
product of (i) the N-terminal extracellular part of the α7-nAChR subunit fused to (ii) the C-terminal transmembrane and intracellular part of the β subunit of an anionic GluClR (Fig. 1A, uppermost scheme) (45). This chimeric Cys-loop receptor is termed hereafter the α7-GluClβR or “full-length receptor” (FL–R). The α7-GluClβR responds to ACh that binds to the LigBD to open a Cl−-selective ion channel (45). As illustrated in Fig. 1A, the FL subunit was further used to produce truncated subunits (Δ1–Δ9). Supplemental Fig. S1 provides the sequence of the FL subunit. Supplemental Fig. S2 provides a sequence alignment of the chimeric subunits used here. Fig. 1B depicts a homology model of the chimeric α7-GluClβΔ4R, which lacks most of the M3-M4 linker.

The rationale for using the chimeric α7-GluClβ receptor is primarily based on our interest to explore a pLGIC that has an intracellular domain of an invertebrate receptor. Secondary structure predictions suggest that only the β (but not the α) subunit of the invertebrate GluClR has an intracellular amphipathic α helix reminiscent of the MA helix of vertebrate pLGICs (Fig. 1D, left, and supplemental Fig. S3, B and C). To examine how this amphipathic α helix might affect channel activity, we decided to use at first the GluClβ homomeric receptor. However, unlike in Xenopus oocytes (42), HEK and CHO cells transfected with the GluClβ subunit alone rarely displayed current responses, which reached an amplitude of 250 pA at best. In contrast, the chimeric α7-GluClβ subunit is highly expressed in HEK and CHO cells and assembles as homomeric receptors that give reproducible robust macroscopic currents (Ref. 45 and herein). The high level expression also assisted us in getting cell-attached patches suitable for single channel recordings. Furthermore, the chimeric α7-GluClβ receptor has the LigBD of the α7-nAChR. The latter shares a very high homology with acetylcholine-binding proteins whose x-ray crystal structure was determined at atomic resolution (27–31). This homology helped us to predict the boundaries of the α1 helix, which was deleted here accordingly. Lastly, the chimeric α7-GluClβR was anticipated to display the ligand binding properties of the α7-nAChR and therefore to assist us in probing the folding, assembly, and cell surface expression of the chimeric receptors used here.

**Ligand Binding Properties of Chimeric Receptors—α-Bungarotoxin (αBTx) belongs to a family of α-neurotoxins that stabilize ACh receptors in a nonconducting state (56–58) by interacting with the ACh-binding pockets to sterically interfere with agonist binding (6, 29, 30, 59–62). αBTx binds to the brain α7-nAChR with nanomolar affinities (63). Intact and chimeric Cys-loop receptors that have the LigBD of the α7-nAChR bind small agonists, like ACh and nicotine, with low affinities (K<sub>D</sub> of tens or a few micromolar, respectively) (46, 52, 63, 64). Here, we initially screened for receptor cell surface expression by measuring the binding of [3H]αBTx (at 30 nm) to live cells transfected with the various chimeric subunits. Then, the receptors capable of binding were further explored in detail to determine their binding affinities for αBTx and nicotine.

Fig. 2 shows that αBTx specifically bound to live cells that were transfected with the FL subunit or with subunits whose M3-M4 linker had been considerably shortened (Δ4 and Δ5). In contrast, αBTx did not bind to cells transfected with chimeric subunits lacking either the N-terminal α1 helix (Δ1–Δ3), M3 together with the M3-M4 linker and M4 (Δ6), the M3-M4 linker together with M4 (Δ7), only M4 (Δ8), or both the α1 helix

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**Figure 1. Structural properties of pLGICs used here.** A, schematic representations of the FL and truncated (Δ1–Δ9) α7-GluClβ chimeric subunits. Blue and reddish segments belong to the N-terminal part (under "LigBD" in A), which forms the extracellular ligand-binding domain upon receptor assembly. The blue segment represents the α1 helix. The green and black segments (under "Pore" in A) belong to the C-terminal part whose helices M1–M4 form the transmembrane ion channel domain upon receptor assembly (numbered 1–4 in the uppermost scheme). The long intracellular M3-M4 linker is depicted as an elongated black box in the FL, Δ1–Δ3, and Δ8 subunits. B, side view of a homology model of the chimeric Δ4-R. For clarity, one of five subunits is colored as in A. Transmembrane helices are numbered 1–4 in one of the gray-colored subunits. M3-M4 linkers of two adjacent subunits are indicated. C, amino acid sequences N-terminal to M4. MA helices are underlined. Colored residues in the M3-M4 linkers were implicated previously in modulation of ion channel conductance. nAChRω1, ω1, ω4, and β2 are subunits of cationic nicotinic acetylcholine receptors; α1 (UniProtKB access number P02711) and β1 (Q6S310) are from Torpedo marmorata, and α4 (B7ZB7U) and β2 (Q9R291) are from mouse. STH3A and STH3B are subunits 3A (Q9UEP2) and 3B (O95264) of the cationic human serotonin receptor. GlyRα1 is the α1 subunit of an anionic human glycine receptor (Q14C71). GluClβ is the β subunit of a Cl−-selective glutamate receptor from Caenorhabditis elegans (Q17328). D, helical wheel representations of the predicted MA helices of the GluClβ (left) and GlyRα1 (right) subunits. Asterisks indicate the GlyRα1 residues that were implicated in modulation of ion channel conductance. Helical folds were predicted by SYMPRED (supplemental Fig. S3). Amino acids colored in blue, red, cyan, and orange (in C and D) correspond to basic, acidic, polar uncharged, and nonpolar residues, respectively.
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The receptors appear to form receptor assemblies at the cell surface. Fig. 3, R). The resulting dissociation constants (Table 1). The transfected with the FL and truncated unrelated transmembrane protein. HEK cells and 30 nM [3H] nicotine (presence of 2 mM nicotine) is depicted by the black columns, and nonspecific binding (in the presence of 2 mM nicotine) is depicted by the gray columns. “Ctrl” represents control binding to cells transfected with a plasmid encoding CD8, which is an error bars Table 1. The presence of 2 mM nicotine) is depicted by the black columns, and nonspecific binding (in the presence of 2 mM nicotine) is depicted by the gray columns. “Ctrl” represents control binding to cells transfected with a plasmid encoding CD8, which is an unrelated transmembrane protein. HEK cells and 30 nM [3H] nicotine were used. The error bars correspond to S.D. Student’s t tests, which compared toxin binding in the absence and presence of nicotine, gave p < 0.05 for cells transfected with the FL and truncated Δ4 and Δ5 chimeric subunits and p > 0.1 for cells transfected with an unrelated CD8 protein and the truncated Δ1–Δ3 and Δ6–Δ9 chimeric subunits.

The loss of α1 helix abolish assembly or transport of the receptors indicated in the panels. Insets correspond to Scatchard curves. D, inhibition of [3H]αBTx binding to the receptors indicated inside the panel by nicotine. KD concentrations of [3H]αBTx were used in D. Curves were fitted to the data points by a nonlinear regression using either Equation 1 (A–C) or Equation 2 (D). Error bars correspond to S.D. B, bound; F, free.

together with the M3–M4 linker (that was shortened as in the Δ4 subunit) (A9) (Fig. 2). Hence, the FL, Δ4, and Δ5 subunits appear to form receptor assemblies at the cell surface. Fig. 3, A–C, shows saturation curves of [3H]αBTx binding to the FL-R and the truncated α7-GluClβ4Δ4R (Δ4-R) and GluClβΔ5R (Δ5-R). The resulting dissociation constants (KD) are provided in Table 1. The KD values indicate that the affinity of the FL-R for αBTx (KD ≈ 10 nM) is lower than that previously determined for the homomeric brain and recombinant α7-nAChRs (KD ≈ 2 nM in Ref. 63) and the chimeric α7–5HT3AR (KD ≈ 4 and 1 nM in Refs. 46 and 52, respectively). Both truncated chimeric receptors (Δ4-R and Δ5-R) bound αBTx with slightly higher affinity than FL-R (Table 1). The linear fits of the Scatchard plots (Fig. 3, A–C, insets) indicate that in each chimeric receptor the binding sites that do accommodate αBTx are identical. Note that the maximal binding sites (Bmax) of the FL-R was ~3–4 times higher than the Bmax of the truncated receptors Δ4-R and Δ5-R (Fig. 3, A–C, insets, and Table 1).

As a next step, equilibrium binding assays were used to determine the capacity of nicotine to competitively inhibit [3H]αBTx binding (nicotine Kt). Fig. 3D shows the binding of [3H]αBTx (at its KD concentrations) in the presence of increasing concentrations of nicotine. Evidently, FL-R, Δ4-R, and Δ5-R display similar nicotine Kt values (Fig. 3D and Table 1), indicating that these three chimeric receptors share similar affinities for the agonist nicotine. These affinities (Table 1) are slightly lower than those obtained previously for the chimeric α7–5HT3AR (Kt ≈ 3.5 μM in Refs. 46 and 52). The similar Hill coefficient of ~1, which was measured for FL-R, Δ4-R, and Δ5-R (Table 1), indicates that at equilibrium the binding sites that do accommodate nicotine represent a single population of identical sites. Taken together, the binding results clearly show that the N-terminal extracellular part of FL-R, Δ4-R, and Δ5-R properly folds in three dimensions and assembles as a quaternary structure that gives rise to the formation of neurotransmitter-binding pockets akin to those of the native α7-nAChR.

### Table 1

| Receptor | αBTx (nM) | Nicotine (μM) | nH |
|----------|-----------|---------------|-----|
| FL-R     | 9 ± 1.3   | 7 ± 1.4       | 0.9 ± 0.17 |
| Δ4-R     | 3.1 ± 1.1 | 1.7 ± 0.4     | 0.8 ± 0.05 |
| Δ5-R     | 4.0 ± 0.8 | 2.3 ± 0.2     | 0.9 ± 0.01 |

Subunit Integrity and Cell Surface Expression of Full-length and Truncated Chimeric Receptors—The loss of αBTx binding capability as observed in the initial toxin binding screen (Fig. 2) cannot stand as an absolute proof for impaired folding or faulty receptor assembly. The following question therefore emerges: does removal of the α1 helix abolish assembly or transport of the receptor to the cell surface? Because it was already suggested for the muscle nAChR that failure to assemble as pentamers leads to receptor degradation (65), we first examined the integrity of the various chimeric subunits. Then, we followed the receptor cell surface expression using confocal microscopy. To this end, an HA tag was fused N-terminally to each chimera, and membrane preparations of transfected cells were analyzed by PAGE and Western blotting. The HA-tagged full-length subunit migrated as a doublet band (~52 and ~54 kilodaltons (kDa)) more slowly than the predicted molecular mass (47.5 kDa based merely on amino acid composition) (Fig. 4, FL under HA-tagged, indicated by a black arrowhead). We attribute this
molecular weight aggregates were also seen on the blot in large amounts (Fig. 4, left panel, first left lane) (amounts for analysis were calculated based on $[^{3}H]$aBTx binding). This indicates that the detection of HA-tagged monomers and multisubunit aggregates is specific to the expressed chimeric receptors.

Fig. 4 (left panel) also shows that subunits lacking the N-terminal $\alpha_1$ helix ($\Delta 1–3$ and $\Delta 9$) migrated close to their predicted monomeric size and formed multisubunit aggregates similarly to those of the FL subunit. This aggregation phenomenon, which was not accompanied with degradation of the protein, hints that chimeric receptors lacking the N-terminal $\alpha_1$ helix might arrive at the cell surface as pentamers (further indications are discussed below). The subunits of receptors lacking the M3–M4 linker ($\Delta 4$ and $\Delta 5$) migrated close to their predicted molecular mass mostly as monomers accompanied with very limited degradation (Fig. 4, right panel, right of the gray arrowhead). In contrast, the HA-tagged $\Delta 6–8$ subunits underwent marked degradation during expression (Fig. 4, right panel, below the non-degraded bands located to the right of the asterisks).

The truncated $\Delta 1–3$ receptors, which did not undergo degradation and displayed the typical aggregation pattern of the full-length subunit, were further examined for cell surface expression using confocal microscopy. Fig. 5A shows that the HA-tagged FL subunit formed receptors (HA–FL–R) on the surface of a live HEK cell that readily bound Rd–aBTx (red) and antibodies directed against the HA tag (Anti–HA; green). Merging of the two images (Rd–aBTx + Anti–HA; yellow) indicates that the two labeling types took place at the same cell surface loci. No cell surface labeling was observed when cells were exposed to 5 mM nicotine prior to and during the incubation with Rd–aBTx. Additionally, no cell surface labeling with anti–HA antibodies was observed in cells transfected with untagged subunits. These results of control experiments, which were performed for all the receptors under investigation (see below), clearly show that the labeling was specific to the presence of receptor subunits on the cell surface. Fig. 5B shows that although the $\Delta 1–3$ subunits did not bind aBTx they did arrive at the cell surface, suggesting that they might properly assemble as pentamers (discussed below). Fig. 5C shows that live cells transfected with the untagged FL, $\Delta 4$, and $\Delta 5$ subunits expressed receptors (FL–R, $\Delta 4$–R, and $\Delta 5$–R, respectively) on their surface that were able to bind Rd–aBTx.

Basic Channel Activity of Full-length and Truncated Chimeric Receptors—Chimeric receptors that displayed ligand binding activity were examined for their capacity to form receptors having channel activity. To this end, we expressed the said chimeric receptors in HEK cells and measured electrophysiologi-
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FIGURE 6. Receptor-channel activity of full-length and two truncated \( \alpha 7 \)-GluCl\( \beta \) chimeric receptors. Representative traces of ACh-evoked currents in HEK cells expressing FL-R (A), \( \Delta 4-R \) (B), and \( \Delta 5-R \) (C) are shown. Current recordings were preformed in the whole-cell configuration of the patch clamp technique at \(-60\) mV and at room temperature (RT). ACh concentrations in micromolar are indicated above the application black bars. D, averaged dose-response curves for the currents elicited by ACh in cells expressing the chimeric receptors indicated inside the panel. Curves were fitted to the averaged data points with a non-linear regression using the Hill equation (Equation 3) \( (r^2 = 0.98, 0.99, \text{and } 0.99 \text{ for FL-R, } \Delta 4-R, \text{and } \Delta 5-R, \text{respectively.}) \) The error bars correspond to S.E.

However, no responses to 3 mM ACh or 0.5 mM nicotine were observed in HEK and CHO cells transfected with the \( \Delta 1-\Delta 3 \) and \( \Delta 9 \) subunits. The degradation of the truncated \( \Delta 6-\Delta 8 \) chimeras was significant but not complete (Fig. 4, right panel). Hence, HEK and CHO cells were also transfected with these chimeric subunits, but no specific current responses were detected upon exposure of the cells to 3 mM ACh or 0.5 mM nicotine as was the case for untransfected cells or cells transfected with a plasmid encoding an irrelevant integral membrane protein (CD8). Additionally, HEK and CHO cells transfected with the FL and the truncated \( \Delta 1-\Delta 3 \) and \( \Delta 6-\Delta 9 \) subunits did not respond to 3 mM ACh or 0.5 mM nicotine.

Effects of M3-M4 Linker Truncation on Channel Ionic Selectivity and Unitary Conductance—Secondary structure predictions performed for the M3-M4 linker of the GluCl\( \beta \) subunit assigned an amphipathic \( \alpha \) helical fold to a sequence close to M4 (Fig. 1C and supplemental Fig. S3B, underlined). This helix is reminiscent of the amphipathic MA helix previously recognized in various vertebrate Cys-loop receptors (36, 39–41). Helical wheel representation shows that this predicted amphipathic MA helix of the invertebrate GluCl\( \beta \) subunit has positively charged, negatively charged, and nonpolar faces that are formed by residues belonging to three consecutive helical turns (Fig. 1D, left). If a charged \( \alpha \) helical face is lining portals through which ions flow in the GluCl\( \beta \), then it could potentially modulate ionic selectivity and/or channel conductance, properties that are controlled by the pore-lining M2 helices and M1-M2 linkers (24, 51, 66–73), with a contribution of residues belonging to the extracellular (74–76) or intracellular (36, 39, 41) domains. We therefore compared the ionic selectivity and unitary conductance of the full-length receptor with those of a truncated receptor that lacks most of the M3-M4 linker.

Fig. 7, A and B (left panels), shows examples of current-voltage relations plotted for FL-R and the truncated \( \Delta 4-R \). The gray curves correspond to experiments performed with almost symmetrical internal and external Cl\(^-\) ions, and the black curves correspond to experiments where all the external NaCl was replaced by mannitol. Evidently, omission of almost all external Cl\(^-\) ions shifted the \( E_{rev} \) toward positive membrane voltages by \( \Delta V \) values of 55 \( \pm \) 1.0 and 56 \( \pm \) 0.5 mV (mean \( \pm \) S.E.) in FL-R \( (n = 9) \) and \( \Delta 4-R \) \( (n = 10) \), respectively (e.g. Fig. 7, left panels).
The extent of rightward shifts depended on the change in the external activity of Cl\(^-\); the lower the external Cl\(^-\) activity, the larger the shift (Fig. 7, A and B, right panels). Curve fitting to the \(E_{rev}\) values as a function of external Cl\(^-\) activities by using the Goldman-Hodgkin-Katz equation (Equation 5) (Fig. 7, right panels) provided the \(P_{Cl}/P_{Na}\) and \(P_{Cl}/P_{Cs}\) permeability ratios (Table 3). Clearly, both FL-R and the truncated Δ4-R are highly selective to Cl\(^-\) ions. Replacing the Cl\(^-\) ions by acetate resulted in a slight permeation to acetate, which corresponds to \(~10\%\) of the permeability to Cl\(^-\) ions (Table 3).

Recordings of single channel currents from cell-attached patches were performed for FL-R and Δ4-R with 100 μM ACh in the recording pipette at various membrane voltages (Fig. 8, A and B, traces on the left). We did not observe any current fluctuations in a stepwise fashion between different levels (steps) of equal size, indicating that only one channel was present in each patch. Subconducting states were not observed in both chimeric receptors. Gaussian fits to all-point amplitude histograms provided the mean amplitudes (Fig. 8, A and B, right panels). Linear curve fitting to the mean single channel current amplitudes as a function of holding membrane voltages is shown in Fig. 8C. The slope of these current-voltage curves provided the unitary conductance of the ion channel. Evidently, the unitary conductance of the FL-R is very close to that of the Δ4-R: 25 and 27 picoisemens for FL-R and Δ4-R, respectively. These values are slightly different from the unitary conductance of the heteromeric GluClα/βR (21 picoisemens) (77).

**DISCUSSION**

In the present study, a eukaryotic chimeric ACh-Glu Cys-loop receptor (α7-GluClβR) was used to generate truncated variants reminiscent of the prokaryotic homolog GLIC. The first three truncated subunits (Δ1–Δ3) lacked N-terminal amino acids that by homology to acetylcholine-binding proteins (27–31), *Torpedo* and muscle nAChRs (32, 33), and GluClαR (34) belong to a putative N-terminal α helix (α1 helix; Fig. 1, A and B, and supplemental Fig. S1). In SDS-PAGE, these three truncated subunits (Δ1–Δ3) displayed their predicted size as well as multisubunit aggregates typical of the full-length α7-GluClβR (Fig. 4). As mentioned earlier, failure of muscle nAChRs to assemble as pentamers leads to their degradation (65). Here, the truncated Δ1–Δ3 subunits did not undergo degradation (Fig. 4), and they reached the plasma membrane as assessed by confocal microscopy (Fig. 5). As such, these truncated subunits (Δ1–Δ3) seem to properly fold and assemble as transmembrane complexes that might reflect pentameric assemblies. This possibility is supported by previous studies performed on the assembly process of various nAChRs that indicated that assemblies arriving at the cell surface correspond to mature pentameric receptors (*e.g.* 78–84). However, unlike the full-length α7-GluClβR, the chimeric receptors lacking the putative N-terminal α1 helix did not bind the competitive antagonist αBTx (Fig. 2) and could not be activated by ACh or nicotine. Careful inspection of our homology model (Fig. 1B) indicates that the α1 helix forms a few hydrogen bonds and multiple van der Waals interactions with several residues that belong to the adjacent subunit. As such, the deletion of the α1 helix could potentially cause rearrangements at the intersubunit interfaces and consequently could change the structure of the neurotransmitter-binding pockets. If so, this could lead to inability of the receptor to accommodate agonists and competitive antagonists. Although we do not know how removal of the α1 helix reshaped the neurotransmitter-binding pockets, we tried to activate the truncated chimeric receptors with protons, which were shown to activate GLIC, but with no success.

Truncated chimeric subunits that had a shortened M3-M4 linker assembled as functional receptors (Δ4-R and Δ5-R) that displayed the activity typical of the FL-R despite not having an intracellular domain. First, they bound αBTx and nicotine with affinities that were not less than the affinities displayed by the FL-R (Table 1). Second, the truncated receptors Δ4-R and Δ5-R formed functional channels having macroscopic activation properties (EC\(_{50}\) Hill coefficient, and activation rate) that did not differ from those of the FL-R (Fig. 6 and Table 2). These observations are in accordance with the study of Jansen *et al.* (85) who showed that replacement of the long M3-M4 linker in other eukaryotic pLGICs (5HT\(_{3A}\) and GABAp1 receptors) by the short M3-M4 linker of GLIC still provided functional cha-
channels with a slight or no change of the EC_{50} and with no apparent change in the slope of the dose-response curves (85).

In previous studies, GLIC was reported as a non-desensitizing (86) or slowly desensitizing (87) pLGIC. Another prokaryotic pLGIC from *Erwinia chrysanthemi* (ELIC) (88) was also shown to desensitize slowly (89). These observations raise the question whether slow desensitization rates are associated with the natural lack of an intracellular domain in the prokaryotic homologs. When whole-cell responses are measured in HEK cells, desensitization is detected in the FL-R but not in GLIC (86). This is when the recordings are performed at the same membrane voltage (−60 mV) under saturating agonist concentrations and the same external and internal ionic strengths using the same perfusion conditions (Fig. 6A here compared with Fig. 3c in Ref. 86). Our kinetic analysis of whole-cell currents measured for the truncated receptors Δ4-R and Δ5-R under the same conditions as for FL-R showed that the desensitization typical of FL-R did not disappear, and its rate (τ_{des}) did not change upon removal of the intracellular domain (Table 2). Taken together, it is evident that the intracellular domain of the chimeric ACh-Glu receptor does not modulate cooperativity and desensitization.

In a previous study, fluorescent proteins were inserted in the M3-M4 linker of the GluCl α and β subunits, and co-expression of both subunits in *Xenopus* oocytes gave agonist-induced currents that corresponded to 90% of the currents observed with the WT receptor (90). This suggests that the intracellular domain of the heteromeric GluClα/βR has flexible portions that can readily accommodate such insertions. However, the complete removal of the intracellular domain here (Δ4-R and Δ5-R) did reduce the expression level at the cell surface by 3–4 times (compared with FL-R) as indicated by the determinations of the maximal αBTx binding sites (B_{max} in Fig. 3, A–C, and Table 1). It is therefore apparent that the intracellular domain of the full-length chimeric ACh-Glu receptor facilitates a high level of cell surface expression by a yet unknown mechanism. In various other eukaryotic pLGICs, the M3-M4 linker was indeed implicated in modulating expression levels and subunit specificity by affecting receptor assembly (nAChRs (9, 10) and 5HT_{3}Rs (11)), targeting (nAChRs (18, 19)), trafficking (GABA_{A}Rs (14, 16), nAChRs (12, 13, 15), and glycine receptor (GlyR) (17)), and clustering (GlyR (20)).

Naturally occurring mutations in the human muscle nAChR that cause congenital myasthenic syndromes led to the recog-

**FIGURE 8. Analysis of single channel conductance for FL-R and Δ4-R.** A and B, left, representative single channel currents recorded in cell-attached patches of the indicated chimeric receptors. Measurements were performed at the indicated holding voltages that correspond to the inverted voltage command (−V_{C}). The closed state level is indicated by C (left of each current trace), and openings are upward or downward deflections. Horizontal black bars correspond to 20 ms. A and B, right, event amplitude histograms for the single channel currents. The event amplitude histograms were fitted with two Gaussian functions (Equation 6); one represents the closed state, and the other represents the open state. C, mean currents obtained from the Gaussian fits are plotted as a function of holding voltages (error bars correspond to ±S.D.). Current (I)-voltage (V) curves represent linear fits to the data points (r^2 > 0.99 for both) with slopes corresponding to the single channel conductances, which are 25 and 27 picosiemens for FL-R and Δ4-R, respectively.
nition that (i) the intracellular domain imposes a single kinetic mode for the activation of the muscle receptor and stabilizes its open state (91), and (ii) the M3 helix of the muscle receptor plays a key role in the mechanism of channel gating (92). The M3 helices are connected to the intracellular domain, and they have multiple contacts with membrane lipids (93) as well as with the M2 helices (25, 26, 34, 88). Indeed, M3 was inferred to move together with M2 during gating of the Torpedo nAChR (94) and the prokaryotic pLGICs (25, 26). It is noteworthy that ionic selectivity in Cys-loop receptors greatly relies on precise movement and positioning of the intracellular end of the M2 helices (contributed by their dipole moment) and the interactions of a partially dehydrated Cl− ion with backbone amides at position −3′ (45). Such a mechanism of Cl− accommodation at the intracellular vestibule of the ion channel pore (45) has recently been suggested based on the first x-ray crystal structure of a eukaryotic pLGIC, the GluClβR, that shows iodide occupancies at the intracellular mouth of the ion channel pore (34). Hence, if the intracellular domain of the GluClβR is involved in the modulation of M2 motions, then it could potentially play a role in the aforementioned rearrangement of the narrow opening necessary for coordinating Cl− ions in the selectivity filter. However, removal of the intracellular domain (in Δ4-R and Δ5-R) did not affect the PCl/PNa ratio and slightly increased the PCl/PCa ratio (Fig. 7 and Table 3), indicating that in the GluClβR the intracellular domain does not modulate ionic selectivity. These observations are in line with the case of other pLGICs, the vertebrate 5HT3A and GABAβ1 receptors, where replacement of the M3-M4 linker by the short sequence of GLIC had no effect on ionic selectivity (85).

In addition, the mutational analysis performed previously in the putative selectivity filter of the α7-GluClβR (45) showed that mutations that led to a significant Cs+ leakage through the mutated ion channel pore also increased the permeability to acetate and 2-hydroxyethanesulfonate (isethionate), which are larger anions than Cl−. This effect of the mutations was attributed to an increase in the effective open pore diameter (45). Here, the deletion of the M3-M4 linker did not change the permeability to acetate (Table 3), which strongly indicates that in the GluClβR the intracellular domain is not involved in setting the effective open pore diameter.

Taken together, our current results provide evidence that the intracellular domain of the GluClβR does not play a role in positioning of the cytoplasmic ends of the five M2 helices when the selectivity filter comes into existence on channel opening. This conclusion is corroborated by the single channel analysis, which showed no change in the unitary conductance upon removal of the intracellular domain (Fig. 8).

The cryo-EM studies of the cationic Torpedo nAChR assign an amphipathic helical fold to a sequence located a few amino acids before M4 (32, 35) (Fig. 1C, two first lines). According to these cryo-EM studies, the five intracellular MA helices (one of each subunit) slant toward the axis of 5-fold symmetry and thereby shape a funnel-like structure that projects into the cytoplasm in continuation to the transmembrane channel domain (32). These MA helices of the Torpedo nAChR delineate two charged portals located at the α/γ and α/β subunit interfaces (32, 35). It was hypothesized that negatively charged residues that line these intersubunit portals facilitate ion transport by preventing anions and large cations from reaching the vicinity of the intracellular vestibule of the ion channel pore (32, 35).

The MA helix of the α subunit of the Torpedo nAChR was photolabeled with [3H]chlorpromazine (96) and [3H]azetomidate (97). The incorporation of [3H]azetomidate in the MA helix was found to be similar in both the open and desensitized states (97), suggesting that the MA helix is rather immobile. Although immobile, the MA helices appear to have a functional role in modulating the ion channel conductance of several vertebrate Cys-loop receptors (40). These include the cationic 5HT3A (36, 39) and α4β2-nACh (39) receptors as well as the anionic GlyRα1 (41) whose unitary conductances were changed following specific substitutions in their MA helices.

Secondary structure predictions of the M3-M4 linker of the invertebrate GluClβ subunit indicate that this linker has an amphipathic α helix close to M4 (Fig. 1C, last line, and supplemental Fig. S3B). The distribution of positive charges along this predicted amphipathic α helix (see Fig. 1D, left) hints that it might facilitate transport of Cl− ions. This is provided that the predicted GluClβ MA helix is (i) organized like the MA helix of the Torpedo nAChR and (ii) that its positively charged residues line portals like those described for the Torpedo nAChR (32, 35). On the other hand, if intracellular portals in the GluClβR were lined by negatively charged residues, then the flow of Cl− ions could be restricted. However, our single channel analysis showed that the Δ4-R, which lacked the entire intracellular domain, including the predicted amphipathic α helix near M4, shares similar single channel conductance with the FL-R (Fig. 8). We conclude that, unlike the case of the aforementioned vertebrate pLGICs, the predicted MA helix of the invertebrate GluClβ subunit does not play a role in modulating the ion channel conductance of the GluClβR. We argue that it is not likely that in the heteromeric GluClα/βR the α helix predicted before M4 of the α subunit is responsible for such modulation. This is because the predicted GluClα MA helix has only two positively charged residues and one negatively charged residue, and they are not organized on successive helical turns (supplemental Fig. S3C).

In conclusion, the current study highlights the importance of the N-terminal extracellular α1 helix for the function of the ACh-Glu receptor-channel complex; this is plausibly by stabilizing the intersubunit interfaces adjacent to the neurotransmitter-binding pockets. The intracellular domain of the GluClβR has no role in the modulation of the macroscopic...
activation and desensitization, ionic selectivity, effective open pore diameter, and conductance of the ion channel. We thus conclude that upon channel opening the intracellular domain of the GluClR is involved neither in the positioning of the pore-lining helices nor in the rearrangements necessary to shape the intracellular vestibule of the ion channel pore for the coordination of Cl\(^-\) ions. In addition, the predicted MA helices of the GluClR are not involved in the modulation of the ion channel conductance. Because the chimeric ACh-Glu receptors lacking the intracellular domain were expressed at lower levels than the full-length chimeric receptor, further studies are required to determine whether the negatively and positively charged faces of the predicted MA helix could be involved in mechanisms that facilitate high levels of cell surface expression.

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