Water-soluble models of ligand-gated ion channels would be advantageous for structural studies. We investigated the suitability of three versions of the N-terminal extracellular domain (ECD) of the α7 subunit of the nicotinic acetylcholine receptor (AChR) family for this purpose by examining their ligand-binding and assembly properties. Two versions included the first transmembrane domain and were solubilized with detergent after expression in Xenopus oocytes. The third was truncated before the first transmembrane domain and was soluble without detergent. For all three, their equilibrium binding affinities for α-bungarotoxin, nicotine, and acetylcholine, combined with their velocity sedimentation profiles, were consistent with the formation of native-like AChRs. These characteristics imply that the α7 ECD can form a water-soluble AChR that is a model of the ECD of the full-length α7 AChR.

Nicotinic acetylcholine receptors (AChRs) are integral membrane, pentameric ion channels in the central and peripheral nervous systems that participate in signal transmission associated with the release of acetylcholine (ACh). A considerable collection of studies of their cell biology, electrophysiology, and structure makes them the best characterized family of a superfamily of homologous neurotransmitter-gated channels that includes glycine, γ-aminobutyric acid, and 5-hydroxytryptamine3 receptors (1–3). Muscle-type AChRs are composed of four different subunits with the subunit composition (α1)2(β1)2δγ or α or β1)2δγ or α or β and bind the snake venom toxin α-bungarotoxin (αBgt). Neuronal AChRs that do not bind αBgt are formed from combinations of α2, α3, α4, or α6 subunits with β2, β3, β4, and/or α5 subunits. Neuronal AChRs that do bind αBgt are formed from α7, α8, and α9 subunits, perhaps in combination with unknown subunits. When heterologously expressed, α7, α8, and α9 form functional homomeric AChRs that appear to contain five identical subunits.

AChRs are composed of five homologous membrane-spanning subunits that are ordered around a central, cation-selective channel. The topology of AChRs that is predicted by hydrophobicity plots has received substantial experimental support (4, 5). The approximately 200 residues at the N-terminal half of each AChR subunit are extracellular, are N-glycosylated, contain sites for agonist and antagonist binding, and form the vestibule through which cations reach the transmembrane channel. Relatively little of the remainder of the primary sequence is extracellular. Three of the four transmembrane domains (M1–M3) that form the channel are grouped together following the N-terminal extracellular domain (ECD) and are separated from M4 by a large cytoplasmic loop. For the muscle-type AChR, three distinct regions of the primary sequence around amino acid residues 86–93, 149, and 190–198 of α1 subunits and peptide loops around residues 34, 55–59, 113–119, and 174–180 of the γ or δ subunit contribute to the ACh binding sites at the interfaces between α and δ and between α and γ (or ε) subunits, based on photoaffinity labeling and site-directed mutagenesis (6, 7). Because of primary sequence and topological similarity, homologous residues at subunit-subunit interfaces in other neuronal AChR subunits are expected to have similar roles in the agonist binding site. For example, residues of the α7 subunit homologous to those of α1 and to those around γ55 and δ57 have been shown to contribute to the agonist binding in homomeric α7 AChRs (8, 9).

Our knowledge of the molecular structure of AChRs, however, is far from complete. Electron diffraction methods using two-dimensional, tubular arrays of AChRs from Torpedo californica have successfully yielded structural details at 9 Å resolution in three dimensions (10–12) and at 7.5 Å in two-dimensional projection (13). Achieving higher resolution, however, has been elusive with membrane-bound or detergent-solubilized AChRs. No member of this superfamily of integral-membrane receptors has been crystallized, and the intact AChRs are too large (more than 200 kDa) for nuclear magnetic resonance spectroscopy.

An AChR formed from the ECD may be a suitable structural model of a full-length AChR, if the ECD can both fold and oligomerize in the absence of the remainder of the subunit. Several lines of evidence suggest that the ECD meets these requirements. First, the specific interactions between subunits that are important in assembly of the muscle-type AChR and for formation of mature acetylcholine binding sites appear to depend primarily on the ECD (14–21). The long cytoplasmic loop of α1 participates in assembly subsequent to the formation of heterodimers (22). Second, membrane-tethered ECDs of...
mouse muscle α1 and δ form heterodimers with ligand binding sites that reflect properties of a full-length AChR (23–25). Third, sequences of α7 that affect homomeric assembly have also been localized to the first half of the ECD and an area around M1 based on chimeras of α7 and α3 (26). According to this report, the long cytoplasmic loop of α7 is not essential for oligomerization.

To determine whether AChRs formed from the ECD (residues 1–208) of α7 subunits are water-soluble structural models for the ECD of the full-length α7 AChR, we expressed two constructs of the ECD of the α7 subunit with M1 retained and one construct of the ECD without M1 in Xenopus oocytes. The constructs with M1 were included to explore the feasibility of removing M1 by in vitro processing subsequent to in vivo synthesis. We examined ligand binding properties and velocity sedimentation profiles as indicators of global structure and local structure at the agonist binding site of the resulting AChRs, which we have designated as α7 ECD AChRs. We found that each construct, including the water-soluble one without M1, assembles into an AChR with affinities for 125I-labeled αBgt (125I-αBgt), nicotine, and ACh that match those of the full-length α7 AChR. These properties demonstrate that the α7 ECD forms a water-soluble α7 ECD AChR that can be a starting point for structural studies of this superfamily of ion channels.

EXPERIMENTAL PROCEDURES

Design of α7M1, α7 Enterokinase (α7EK), and α7 Water-soluble (α7WS) Plasmids—The full-length cDNA sequence of chicken α7 (27) previously was cloned into a modified SP64T expression vector (28, 29). For α7M1, which encodes the ECD of α7 up to the start of M2, the α7 coding sequence from the beginning of M2 to past the native stop codon was removed by digestion with BglII and BamI. It was replaced by a double-stranded oligonucleotide cassette coding in-frame for the 19-amino acid sequence SQVTGEVIFQTPLIKNPRV and a stop signal. This sequence contained the epitope of mAb 142 from residues 2 to 17 amino acid sequence SQVTGEVIFQTPLIKNP followed by a stop codon was ligated between the BglII site of the N-terminal sequence and the RI site of a modified SP64T expression vector. Residues 1–6 of this sequence reconstructed the native sequence Arg203 to Thr209; residues 7–22 constituted the epitope for mAb 142. The oligonucleotides were synthesized using the phosphoramidite method on a Milligen oligonucleotide DNA synthesizer.

Protein Expression—In Xenopus oocytes—DNA from each of the three plasmids was purified on a 5% CTAB gradient gel and transcribed using an SP6 mMessage mMachine™ kit (Ambion, Austin, TX) and linearized DNA. The cytoplasm of each oocyte was injected with approximately 50 ng of cRNA and incubated at 18 °C for 3–5 days in 50% Leibovitz’s L-15 medium (Life Technologies, Inc.) in 10 mM Hepes, pH 7.5, containing 10 units/ml penicillin and 10 μg/ml streptomycin.

For α7M1 and α7EK, extraction of membrane-bound subunit protein with a buffer containing Triton X-100 was accomplished with a previously reported procedure (29). Oocytes were homogenized by hand in ice-cold buffer A (50 mM sodium phosphate, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzanilide, 15 mM iodoacetamide, pH 7.5). The membrane-containing fraction was separated by centrifugation, was washed twice with buffer A, and then was extracted with buffer B (40 mM sodium phosphate, 40 mM NaCl, 4 mM EDTA, 4 mM EGTA, 4 mM benzamid, 12 mM iodoacetamide, 2% Triton) during gentle agitation for 2 hours at 4 °C. The soluble fraction from this detergent extraction step was separated by centrifugation and was used for both Western blotting and assays of ligand binding.

For α7WS, the secreted fraction was defined as the subunit protein present in the L-15 medium following injection into oocytes in this fraction were removed by centrifugation. The cytoplasmic fraction of α7WS was defined as the subunit protein present in the soluble fraction following homogenization of the oocytes by hand in buffer A and centrifugation to sediment the insoluble, membrane component. The Triton-extracted fraction of α7WS was defined as the subunit protein present in the solvent following extraction in buffer B of the membrane component from the homogenization step during gentle agitation for 2 h at 4 °C.

Immunoblotting—Triton-extracted α7M1 and α7EK or secreted α7WS was incubated overnight at 4 °C with mAb 142 that had been coupled to Sepharose CL-4B (Pharmacia) with CNBr (32). After this concentration step, the protein was eluted at 55 °C with 2% SDS and 20 mM β-mercaptoethanol. Proteins were deglycosylated for 18 h at 37 °C with 1 unit of a mixture of endoglycosidase F and glycopeptidase F according to instructions of the manufacturer (Boehringer Mannheim). A sample without enzyme was run in parallel as the negative control.

Protein was denatured and reduced at 55 °C in SDS-polyacrylamide gel electrophoresis sample buffer containing 2% SDS, separated on a 13% acrylamide SDS-polyacrylamide gel electrophoresis gel, and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore). After being blocked in 5% powdered milk in phosphate-buffered saline (PBS, 100 mM NaCl, 10 mM sodium phosphate, pH 7.5) containing 0.5% Triton, the membrane was incubated with 2 μM 125I-αM142. Specific activities of the labeled antibodies ranged from 10^10 to 10^18 cpm/mol. Labeling was visualized by autoradiography.

Ligand Affinities—Immunol 4 plastic microwells (Dyntact Laborator, Chantilly, VA) with mAb 142 were coated at 236 for solid-phase assays (5). The wells were blocked with 3% bovine serum albumin in PBS. For α7M1 and α7EK, a sample of the Triton-solubilized protein from the equivalent of from one to three oocytes that had been injected with CRNA was added to each microwell. For the measurement of αBgt affinity, 1125I-αBgt was added to the Triton extract and incubated overnight at 4 °C. Total volume in each well was 100 μl. The wells were washed three times with ice-cold PBS containing 0.5% Triton, and the amount of radioactivity was measured using a γ counter. Each data point was measured in duplicate. For the competitive inhibition assays, the wells were washed free of the Triton solution after 24 h and loaded with 1125I-αBgt at 4 nM in the presence of 1-nicotine or ACh. The wells were incubated 8 h at 4 °C before washing and then measuring the amount of radioactivity. Each data point was measured in duplicate. Non-specific binding was measured with Triton-solubilized protein extracts from un.injected oocytes and generally was less than 5% of the specific binding.

For α7WS, the volume of L-15 medium above about six oocytes was added to mAb 142-coated microwells and incubated overnight at 4 °C for capture of the secreted α7 M1. The L-15 was removed with PBS. For the measurement of αBgt affinity in 0% Triton, 1125I-αBgt was added to buffer C (same composition as buffer B, except without Triton) and incubated overnight at 4 °C. Total volume in each well was 100 μl. The wells were washed three times with ice-cold PBS, and the amount of radioactivity was measured using a γ counter. Inhibition by nicotine and ACh in 0% Triton was measured after capture of the secreted α7WS, washing of L-15, and loading of each well with 0.6 mM 1125I-αBgt.
and the appropriate amount of inhibitor in buffer C. Total volume in each well was 100 μl. The wells were incubated 8 h at 4 °C before washing and measuring the amount of radioactivity. Data points were measured in duplicate. Nonspecific binding was measured with L-15 medium that was used to incubate un.injected oocytes and generally was less than 10% of the specific binding. Buffer B was substituted for buffer C at the step of loading the wells with 125I-αBgt or with 125I-Bgt and nicotine or ACh for the ligand affinity measurements of α7WS in 2% Triton.

The equilibrium dissociation constants $K_d$ for 125I-αBgt were determined by least-squares, nonlinear fitting to a Hill-type equation (Equation 1) using the graphing software KaleidaGraph (Synergy Software)

$$C = C_p + \frac{1}{1 + \left(\frac{L}{IC_{50}}\right)^n}$$

(Eq. 1)

where $C$ is the measured signal (counts/min), $C_p$ is the maximal signal (which corresponds to the maximum number of 125I-αBgt binding sites), $L$ is the concentration of 125I-αBgt, $n$ is the Hill coefficient. The half-maximal inhibition constants, $IC_{50}$, for nicotine and ACh in the presence of 125I-αBgt were determined by nonlinear fitting to Equation 2, where $L$ is the concentration of nicotine or ACh, $C_0$ is the maximal signal, and $C_1$ is a constant that represents signal that is not displaced by high concentrations of agonist. We used the Cheng-Prusoff equation (Equation 3) to estimate equilibrium dissociation constants from $IC_{50}$ values (33).

$$C = C_p + \frac{1}{1 + \left(\frac{L}{IC_{50}}\right) + C_1}$$

(Eq. 2)

$$K_d = \frac{IC_{50}}{aBgt}$$

(Eq. 3)

although other equations also have been described for that purpose (34). The $K_d$ values shown in Table I are the average and standard error of at least three independent assays of ligand affinity unless otherwise noted. Uncertainties shown in figures are standard errors.

Sucrose Gradient Sedimentation—Membranes from 10–20 oocytes that had been injected with the α7M1, α7EK, or full-length α7 cRNA were extracted in buffer B. AChRs in membrane vesicles from T. californica and AChRs from the TE671 cell line (35) were solubilized in buffer B. About 200-μl aliquots of solubilized protein containing from 20 to 100 nmol of 125I-αBgt binding sites were layered onto 5-m sulfone gradients (5–20%) (w/v) in 0.5% Triton solution of 100 mM NaCl, 10 mM sodium phosphate, 5 mM EGTA, 5 mM EDTA, and 1 mM NaN3 at pH 7.5. The gradients were centrifuged 75 min at 70,000 rpm (approximately 400,000 g) in a Beckman NVT90 rotor. For determining a ligand binding profile, aliquots of 11 drops each (approximately 130 μl) from the gradient were collected into Immuno 4 plastic microtubes coated with mAb 142 for α7M1, mAb 236 for α7EK, or mAb 318 (a rat monoclonal antibody against an epitope in the cytoplasmic domain) (27) for the full-length α7. The entire gradient was collected in 40 fractions. After 24 h at 4 °C, the microtubes were washed and filled with 4 ml 12 125I-αBgt in buffer B for 6 h at 4 °C, followed by washing and quantitation of bound 125I-αBgt by γ counting.

Processing of α7WS was slightly more involved because of the low concentration of the secreted protein in the incubation medium. The protein was concentrated by binding overnight at 4 °C to α-toxin that had been isolated from the venom of Naja naja siamensis with ion exchange chromatography (36) and then coupled to Sepharose CL-4B with CNBr (32). The bound protein was eluted from the α-toxin with 200 μl of 100 mM nicotine and was layered onto 5-m sulfone gradients (5–20%) without Triton in centrifuge tubes. The gradient was centrifuged 75 min at 70,000 rpm (approximately 340,000 × g) and 4 °C, as was done for α7M1 and α7EK. The entire gradient was collected in 39 fractions of 6 drops each, because the drop size was larger in the absence of detergent. After 24 h at 4 °C, the microtubes were washed and filled with 1 nm 125I-αBgt in buffer C for 6 h at 4 °C, followed by washing and quantitation of bound 125I-αBgt by γ counting.

Comparison of AChR Yields—The yield was defined as the theoretical maximal amount of bound 125I-αBgt, which was the value of $C_p$ in Equation 1 from 125I-αBgt-binding assays. The AChR yield was calculated in terms of 125I-αBgt binding sites per oocyte.

RESULTS

Expression of α7 ECD Proteins in Xenopus Oocytes—The three variations of the ECD sequence of the α7 subunit were studied (Fig. 1). The first construct, α7M1, includes the N-terminal ECD, M1, and the portion of α7 between M1 and M2 up to residue Ile240. It was intended to demonstrate the pharmacological properties and oligomerization behavior of the α7 subunit proximal to M2 and tethered to the membrane through M1. The second construct, designated α7EK, contains a peptide spacer of 27 amino acid residues that is spliced between Thr208 and Leu211 at the junction of the ECD and M1. α7EK also contains M1 and terminates at Ile236, as well as an EK-specific protease site between the extracellular domain and M1. In contrast to the two membrane-tethered proteins, α7WS does not contain M1; the mAb 142 epitope tag of the α7WS protein follows directly after the ECD.

![Diagram](image-url)

**FIG. 1. Designs of the three α7 ECD subunit proteins α7M1, α7EK, and α7WS.** The ECD, which extends from residues 1 to 208 of the mature chicken α7 AChR (27) is included in each protein. In α7M1 and α7EK, the first transmembrane domain, M1, extends from residues 209 to 233. The α7 sequence from residues 234 to 240, which extends to the beginning of M2, is included after M1 in these two proteins. The segment labeled 1425 on the C-terminal side of M1 is the epitope tag for mAb 142. Besides the mAb 142 epitope, α7EK also contains an epitope tag for mAb 236, designated 236v, as well as an EK-specific protease site between the extracellular domain and M1. In contrast to the two membrane-tethered proteins, α7WS does not contain M1; the mAb 142 epitope tag of the α7WS protein follows directly after the ECD.
AChR, measurements of binding affinity with 125I-
structural properties of the three
appropriate starting points for analyzing the functional and
pare favorably with the published
assays in which the Triton-solubilized proteins were bound in
containing AChR from chick brain (37). Binding of 125I-
glycosylation than was present on
of bands before deglycosylation suggested more heterogeneous
lated mass of 26 kDa (Fig. 2). The detection of a diffuse pattern
inclusion dissociation constant,
ECD constructs. The equi-
7 AChR and for
Bgt was 1.6 nM for
7EK constructs. The equi-
7. The presence of 2% Triton
during the binding assay of
7WS for 125I-
aBgt to a meas-
5
n2).

(a) Glycosidase

(b) Molecular Mass (kDa)

FIG. 2. Protein expression and glycosylation of α7M1, α7EK,
and α7WS demonstrated by immunoblotting with 125I-mAb 142.
Deglycosylated protein is compared with control protein that was pro-
cessed in parallel except without glycosidase. The presence (+) or
absence (−) of glycosidase is noted above each lane. Each lane of α7M1
and α7EK contains the protein from about two oocytes; each lane of
α7WS contains the protein secreted from about 24 oocytes. Although
α7WS has the lowest molecular weight as calculated from amino acid
sequences, it is the most heavily glycosylated and migrates at the
largest apparent molecular weight. Triton extracts and incubation me-
dium from un.injected oocytes were used as negative controls and
showed no binding of 125I-mAb142. The positions of molecular mass
markers are indicated on the left.

calculated from amino acid compositions. The detection of pre-
dominately a single band before deglycosylation suggested that
post-translational modifications were comparatively uniform
on all molecules. Deglycosylation shifted each band to approx-
imately the molecular mass of the protein calculated without
modifications.

Without M1, α7WS was secreted in soluble form into the
incubation medium by oocytes that had been injected with
α7WS cRNA. The secreted protein displayed an apparent mass
of about 41 kDa by immunoblotting, compared with a cal-
culated mass of 26 kDa (Fig. 2). The detection of a diffuse pattern
of bands before deglycosylation suggested more heterogeneous
glycosylation than was present on α7M1 and α7EK. Deglyco-
sylation shifted the apparent mass down to about 26 kDa,
confirming that α7WS had the most extensive glycosylation of
the three proteins. The larger shift in apparent mass compared
with α7M1 and α7EK also suggested that the pattern of glyco-
sylation differed between membrane-tethered and soluble
forms of the ECD. An additional observation from the immu-
noblot is that the yield of secreted α7WS was about 10-fold less
than the yields of α7M1 and α7EK from Triton-solubilized
membrane fractions, based on a comparison of the numbers of
injected oocytes needed to produce approximately equal signals
on the immunoblot.

High Affinity Binding of 125I-aBgt to α7 ECD AChRs—Be-
cause high affinity binding of aBgt is a hallmark of the α7
AChR, measurements of binding affinity with 125I-aBgt were
appropriate starting points for analyzing the functional and
structural properties of the three α7 ECD constructs. The equi-
librium dissociation constant, Kd, of 125I-aBgt was 1.6 nM for
α7M1 and 1.9 nM for α7EK when measured in solid phase
assays in which the Triton-solubilized proteins were bound in
mAb 142-coated wells (Fig. 3 and Table I). These values com-
pare favorably with the published Kd of 1.6 nM for
125I-aBgt binding to Triton-solubilized full-length α7 AChR and for α7-
containing AChR from chick brain (37). Binding of 125I-aBgt
was measured for α7EK by also using the mAb 236 epitope tag
to bind the protein to mAb 236-coated wells. The Kd was 2.3 nM,
demonstrating that the mAb 236 epitope tag at the C-terminal
end of the ECD did not interfere significantly with the function
of the aBgt binding site.

The affinity of secreted α7WS for 125I-aBgt was measured
first without Triton (Fig. 4 and Table I). The Kd value of 0.4 nM
was about 4-fold smaller than the values for Triton-solubilized
α7M1, α7EK, and full-length α7. The presence of 2% Triton
during the binding assay of α7WS for 125I-aBgt increased the
value of Kd to 1.7 ± 0.1 nM (Fig. 4), which is in the range
measured for the Triton-solubilized AChRs. Triton, however,
did not significantly affect the total number of 125I-aBgt bind-
ingsites. The ratio of the number of binding sites calculated
from Equation 1 in the absence of Triton compared with the
number of binding sites in the presence of Triton from other-
wise identical pools of secreted α7WS was 0.99 ± 0.13 (n = 2).

Only the secreted fraction of α7WS bound 125I-aBgt to a meas-
urable extent, despite the significant amount of α7WS protein
that was retained intracellularly. The total amount of α7WS
protein per oocyte that was detected in the cytoplasmic and the
Triton-solubilized membrane fractions by immunoblotting was approximately equal to the amount of Triton-solubilized α7M1 or α7EK protein in similarly-injected oocytes. Hence all three constructs apparently were synthesized and accumulated to an approximately equal extent. The intracellular pool of α7WS, however, showed no significant binding of $^{125}$I-aBgt.

The yield of $^{125}$I-aBgt binding sites from each of the three ECD proteins was measured by the binding of $^{125}$I-aBgt to AChR on mAb 142-coated wells (Fig. 5). These measurements assumed that the mAb 142 epitope in each protein was equally accessible to the antibody. The yield of $8 \pm 1$ fmol of $^{125}$I-aBgt binding sites per oocyte with α7M1 AChR was the largest among the three proteins. This value is near the value of 15 fmol per oocyte that was reported for Triton-solubilized AChR. As with α7M1, the addition of the peptide spacer in α7EK decreased the yield of AChR by about three-quarters to 1.9 ± 0.6 fmol of $^{125}$I-aBgt binding sites per oocyte. The yield of secreted α7WS AChR was the smallest of the three at 0.21 ± 0.02 fmol of $^{125}$I-aBgt binding sites per oocyte. These comparisons indicate that insertion of residues before M1 or the absence of M1 decreases the yield of ECD AChRs.

High affinity for $^{125}$I-aBgt was the first step in demonstrating the structural similarity of α7M1, α7EK, and α7WS AChRs to the full-length α7 AChR. It was only an initial test of the properties that we hoped to find in a model AChR. Specifically, we were seeking a model that also was a pentamer and that had native-like affinity for agonists like nicotine and ACh. High affinity for aBgt does not necessarily imply either of these properties. Muscle-type AChR illustrates this point. The human α1 subunit in the absence of other muscle-type subunits binds aBgt relatively tightly, with $K_d$ of 0.6 nM compared with $K_d$ of the pentameric muscle-type AChR of 0.1 nM (35). Instead of a pentamer, however, it exists as a monomer according to its velocity sedimentation profile relative to the profiles of fully assembled muscle-type AChR and αα and αγ dimers (38, 39). Moreover, it does not bind agonists with native-like affinity. In light of these characteristics for α1, other experiments besides binding of $^{125}$I-aBgt were required for assessing the oligomerization of the α7 ECD constructs.

Velocity Sedimentation Imply α7M1, α7EK, and α7WS Form Multimeric AChRs, Probably Pentamers—Oligomerization of α7M1, α7EK, and α7WS proteins was examined first with velocity sedimentation profiles in sucrose gradients. The proteins were detected by binding of $^{125}$I-aBgt in solid-phase assays. These profiles were compared with full-length AChRs and monomeric α1 subunits as sedimentation standards. Fully assembled, full-length α7 AChR (calculated molecular mass of 272 kDa from amino acid sequence) were pentameric standards (37). Muscle-type AChR from T. californica were pentameric (i.e. a monomeric AChR with calculated molecular mass of 268 kDa) and decameric standards (i.e. a dimeric AChR with calculated molecular mass 536 kDa) (40). The monomeric α1 subunit (calculated molecular mass of 52 kDa) from the human rhabdomyosarcoma cell line TE671 was the monomeric standard (35). As expected because of their much larger molecular masses, the pentameric AChR standards sedimented faster than monomeric α1 subunit (Fig. 6).

The single $^{125}$I-aBgt binding peak from α7M1 and from α7EK also sedimented much faster than the monomeric α1 subunit and slightly slower than the full-length AChRs (Fig. 6). Two conclusions can be drawn from these velocity sedimentation data in the absence of measurements of diffusion and partial specific volume. First, the position of the $^{125}$I-aBgt binding peak was distant from the α1 monomer standard, strongly suggesting that a multimeric species was responsible for the $^{125}$I-aBgt binding. Second, this multimeric species probably was a pentamer. This conclusion about subunit stoichiometry was deduced from both the single peak and its relatively narrow distribution. The single peak implied that a multimer with a single distinct stoichiometry was the dominant species that bound $^{125}$I-aBgt with nanomolar affinity. In contrast, if multiple stoichiometries such as dimers and trimers and tetramers had significant representation in the high affinity population, then one would have expected either several discrete peaks or a low, broad profile. Because of the pentameric structure of the full-length α7 AChR (41), the most likely stoichiometry for α7M1 and α7EK AChRs also was pentameric, although minor fractions of smaller oligomers may also have been present. Moreover, it has been reported that only pentameric α1 subunits bind $^{125}$I-aBgt with nanomolar affinity, whereas monomeric full-length subunits and aggregated or incompletely assembled forms do not (37).

Velocity sedimentation of the secreted α7WS in sucrose gradients also revealed a single $^{125}$I-aBgt binding peak (Fig. 6). It sedimented slightly slower than the peaks for α7M1 and α7EK. As with α7M1 and α7EK, the position of the single, relatively narrow peak that was distant from the peak of the α1 subunit did not suggest a monomeric form for α7WS AChR and was consistent with the conclusion that the dominant form of α7WS AChR was a pentamer.

High Affinity Binding of Nicotine and ACh to α7 ECD AChRs—High affinity binding for agonists was the second test of oligomerization and the last test of structural equivalence between the α7 ECD AChRs and the full-length α7 AChR. Interpretation of this test was based on an analogy with characteristics of the α1 subunit of the muscle-type AChR. High affinity for agonists develops for α1 only after it assembles with
or g (39, 42), because the agonist binding site consists of structural components from both subunits at ad and ag interfaces (3). Similarly, if a7M1, a7EK, and a7WS AChRs indeed were pentamers and structural models of the full-length a7 AChR, then we expected native-like affinities for nicotine and ACh.

The affinities of a7M1 and a7EK for the small ligands nicotine and ACh were measured by competitive inhibition of the binding of 125I-αBgt (Fig. 7 and Table I). The equilibrium dissociation constants, K_d, of a7M1 and a7EK were 1.0 μM for nicotine and 50 μM for ACh. In the case of a7EK, tethering in the solid phase assays via the mAb236 epitope tag slightly increased the values of K_d for nicotine and ACh to 1.4 μM and 60 μM, respectively. For both proteins, the K_d values are similar to those for Triton-solubilized, full-length a7 and for a7-containing AChRs from chick brain (Table I). Competitive binding with each ligand eliminated almost all binding of 125I-αBgt, implying that the majority of the 125I-αBgt binding sites also bound nicotine and ACh.

In inhibition assays with 125I-αBgt and a7WS, the values of K_d for nicotine binding in the absence of Triton were 10-fold smaller than the values for nicotine binding to a7M1 and a7EK in the presence of Triton (Fig. 8 and Table I). The values for ACh binding to a7WS without Triton were 40-fold smaller than the values for ACh binding to a7M1 and a7EK with Triton. The water-soluble a7WS AChR without Triton, therefore, bound to small ligands more tightly than did the full-length a7 AChR in Triton. The presence of 2% Triton in the inhibition assays increased the values of K_d by about 3-fold (Fig. 8). As with a7M1 and a7EK, the majority of the 125I-αBgt binding sites of a7WS AChR also bound nicotine and ACh.

In light of the velocity sedimentation data, these results confirm that the a7M1, a7EK, and a7WS AChRs are oligomers and probably are pentamers. These results demonstrate that the a7 ECD with or without M1 is sufficient for the expression of a7 ECD AChR with native-like affinities for αBgt and nicotinic agonists.

DISCUSSION

Structural Implications of the Ligand Binding Properties and Oligomerization of ECD AChRs—We have shown that the N-terminal ECD of the a7 AChR subunit with or without M1 forms an AChR whose ligand-binding and sedimentation prop-
properties imply that it is a structural model of the full-length AChR. In particular, the $\alpha_7$WS AChR is water-soluble. The ECD, therefore, constitutes an autonomous unit capable of folding and assembling in the absence of the transmembrane, cytoplasmic, and other extracellular portions of the $\alpha_7$ subunit sequence. We examined three characteristics that implied retention of the native-like structure in the three ECD AChRs: 1) high affinity binding of $\alpha$Bgt, which is a hallmark of $\alpha_7$ AChRs; 2) velocity sedimentation, which is most consistent with assembly of each of the three $\alpha_7$ ECD subunits into pentamers; and 3) native-like affinities for nicotine and ACh.

First, the affinity for $^{125}$I-$\alpha$Bgt of each of the three $\alpha_7$ ECD AChRs was equal to or higher than that of the fully assembled, full-length $\alpha_7$ AChR. Because the $\alpha$Bgt binding site incorporates noncontiguous regions of primary sequence, these affinities suggest that the global structure of each $\alpha_7$ ECD AChR models the structure of the ECD in the full-length $\alpha_7$ AChR. The moderately higher affinity of the $\alpha_7$WS AChR for $^{125}$I-$\alpha$Bgt reverted back into the range of $K_d$ values observed with $\alpha_7$M1 and $\alpha_7$EK AChRs when Triton was included in the assay. Triton could have exerted its effects by modifying the interaction of $\alpha$Bgt with either the AChR or with the solvent environment, since both interactions can affect $K_d$. In other words, Triton may have caused structural perturbations of the AChR that affected the binding site, or $^{125}$I-$\alpha$Bgt may have partitioned differently in Triton-containing solvent compared with detergent-free solvent.

Second, velocity sedimentation data suggested that the $\alpha_7$ ECD subunits had assembled into multimers that probably were pentamers. Other AChRs or AChR subunits were used as oligomerization standards for estimating the extent of oligomerization of the $\alpha_7$ ECD subunits. The single $^{125}$I-$\alpha$Bgt binding peak for each of the ECD AChRs migrated on the sucrose gradient slightly slower than full-length, pentameric $\alpha_7$ AChR and Torpedo AChR and much faster than the peak of monomeric $\alpha_1$ subunit. Although differences in the amount of
bound detergent (40) and in molecular shape (43, 44) will affect the sedimentation of AChRs, we do not think that these effects alone can account for the differences between the position of the monomeric subunit standard and the positions of α7 ECD AChRs on the gradients. A reasonable interpretation of the position of the 125I–Bgt binding peaks relative to the monomeric α1 subunit peak was that these α7 ECD subunits were not monomeric. Multimeric stoichiometry also is consistent with their native-like affinity for agonists. Given that full-length α7 AChR are pentamers (41), the single, relatively narrow 125I–Bgt binding peak from each α7 ECD AChR probably arose from pentamers.

Third, affinities of the three α7 ECD AChRs for nicotine and ACh also matched or were slightly higher than those of the full-length α7 AChR. In the case of α7WS AChR, Triton reduced the affinity of nicotine and ACh compared with that observed without Triton. The differences in affinity for agonists of α7WS AChR compared with α7M1 and α7EK AChRs were not caused solely by Triton and may have been contributed in part by M1. Overall, these results imply that the local structure of the ACh binding site of each of the α7 ECD AChRs closely matches that of the full-length α7 AChR. By analogy with the behavior of the muscle-type AChR (39, 42), the binding site for small ligands is thought to form at interfaces between subunits. Therefore, native-like affinities for agonists by the α7 ECD AChRs, combined with the results from velocity sedimentation, confirmed that the α7 ECD subunits had assembled into multimers.

The α7EK subunit demonstrated another structural property of the ECD AChRs. Splicing a peptide spacer of 27 amino acid residues between these two domains did not significantly alter ligand affinity. Therefore, strict continuity of the native primary amino acid sequence between the ECD and M1 is not required for formation of the agonist binding site or for oligomerization. In other words, an ECD that is displaced away from M1 by a peptide spacer retains its assembly and binding properties. This flexibility in design raises the possibility of first expressing a membrane-bound ECD AChR, followed by release of a water-soluble ECD AChR from M1 by enzymatic proteolysis in vitro.

Elimination of transmembrane domains either by recombinant techniques or by proteolysis of native protein has been the starting point for structural studies of other integral membrane proteins. In the family of ionotropic glutamate receptors, the soluble agonist binding domain of GluR-B and GluR-D was successfully designed by fusion of discontinuous sections of the primary sequence that apparently are separated by two transmembrane domains (45, 46). This strategy also has been successful for the x-ray crystallography of many integral membrane proteins including growth hormone receptor (47), prolactin receptor (48), tissue factor (49), interferon-γ receptor (50), human class II histocompatibility antigen (51), insulin receptor protein-tyrosine kinase domain (52), α and β chains of the T cell receptor (53, 54), neuraminidase (55), hemaggutin (56), and bacterial aspartic proteinase (57).

Yield of ECD AChRs—The protein sequence between the beginning of M1 and the start of M2 (residues Leu430 to Ile450) was needed for production of AChRs from the ECD at a level comparable to that of the full-length AChR. M1 probably was the key component of this region. Compared with the yield of AChRs from α7M1 AChR, the yield was smaller from α7EK and even smaller from α7WS. The differences were unlikely to be caused by reduced translation efficiencies of α7EK and α7WS, because of the extent of sequence identity among all three designs and because the amounts α7EK and intracellular α7WS proteins per oocyte that were detected by immunoblotting were approximately equal to the amount of α7M1. Instead, the difference in the case of α7EK AChR suggests some adverse effects on folding and assembly caused by the alteration of the primary sequence at the ECD/membrane interface.

The low yield in the case of α7WS AChR highlighted an important role for M1. M1 has been shown to function as a tether to the ER membrane during the dimerization of truncated α1 and δ subunits in the ER (23). It was successfully replaced in mouse α1 by unrelated transmembrane domains with unrelated primary sequences or with a glycosylphosphatidylinositol (GPI) moiety (24). A similar role for M1 probably applies to the production of α7 ECD AChRs. M1 constrains the subunits to a membrane surface, which may favor orientations...
between subunits that are required for assembly and may help retain them in the endoplasmic reticulum (ER) and Golgi apparatus for folding, subunit assembly, and post-translational modifications. In contrast, failure to retain the subunits on the ER and Golgi membranes removes such constraints and decreases the efficiency of these processes. In particular, M1 may enhance folding and assembly by increasing the local concentration of subunits relative to folding cofactors, such as calnexin, that are found in the ER membrane (58, 59).

Attaching the ECD to a membrane may not be the sole function of M1 for α7M1 and α7EK, however. Instead, the yield of AChRs may depend on particular properties of M1. Chimeras of α7α3 (26) and point mutations in M1 of α7 (60) demonstrate that the specific sequence used in the first transmembrane domain affects the yield of surface AChRs. Similarly, the total amount of bound 125I-Bgt per oocyte varied when unrelated transmembrane domains replaced any one of the regions M1–M4 of α1 in recombinantly expressed Torpedo AChRs (61).

Strategy for Structural Studies of α7 ECD AChRs—What is the optimum design of an α7 ECD AChR for structural studies? The complete ECD of the α7 subunit incorporating all of the amino acid sequence up to the start of M1 probably is necessary to satisfactorily reproduce the ligand-binding and assembly properties of the full-length α7 AChR. Although we did not attempt to truncate the α7 ECD N-terminal to Thr208 results from the mouse α1 subunit suggest that there is little flexibility for moving the truncation point closer to the N terminus and into residues known to contribute to the Bgt binding site (23). Truncation of mouse α1 after Pro211, which is homologous with our truncation point in α7WS, did not disrupt the formation of a high affinity binding site for Bgt. In contrast, truncation of the mouse α1 subunit after Met207 (mouse α1 numbering) caused a loss of affinity for Bgt.

More extreme truncations of the ECD of α1 have been explored in attempts to bypass the difficulties presented by AChRs for structural studies. None, however, appears as successful in duplicating properties of full-length AChRs as the α7 ECD AChRs described here. For example, peptide sequences from the region around residues 170–200 of the α1 subunit have been used as potential mimics of the Bgt binding site for NMR and ligand-binding studies (62, 63). The affinity for the binding of Bgt, however, typically is three orders of magnitude less than with full-length AChRs (64). In addition, peptide models from the α1 subunit are incapable of oligomerization and do not show significant affinity for small ligands.

Three conclusions from our investigation are relevant to a strategy for obtaining α7 ECD AChRs for structural studies. First, the extracellular domain constitutes a stable, fully water-soluble AChR that quantitatively retains essential ligand-binding properties of the full-length AChR and eliminates confounding factors of detergent solubilization that hamper the crystallography of membrane proteins. Second, M1 in the α7M1 and α7EK constructs significantly enhances the yield of ECD AChRs compared with the yield from α7WS. Although membrane tethering of the AChR subunit proteins was essential for association of the ECD of the mouse muscle-type α1 subunit and the full-length δ subunit when expressed in COS cells (24), it was advantageous but not essential for assembly of α7WS AChR in oocytes. Third, the α7 ECD still can form an AChR even when a peptide spacer separates it from M1 and the adjacent membrane surface.

These findings suggest that a water-soluble α7 ECD AChR more likely will be produced in large amounts in two stages via a membrane-bound intermediate than directly from a water-soluble design of the ECD. The first stage is in vitro synthesis of a membrane-bound AChR that subsequently will be removed from its membrane tether by in vitro processing. The second stage is enzymatic removal of the membrane tether in vitro. α7EK demonstrates that an AChR substrate can be designed for specific proteolysis within a peptide spacer between the ECD and M1. Our attempts to separate an ECD AChR from the M1 domain of α7EK using EK, however, resulted in substantial nonspecific proteolysis. An alternative method that allows truncated α1 subunits to dimerize with full-length δ is substitution of M1 with a GPI moiety (24, 65). A GPI tether also leads to the expression of membrane-bound extracellular domains of chick α7 on the surface of oocytes (66). A GPI tether may be preferred at the stage of enzymatic processing, because the carbohydrate portion of the anchor at the C terminus of the protein can be separated from the more distal, membrane-embedded fatty acid portion with phosphatidylinositol-specific phospholipase C (67, 68). This enzyme is expected to eliminate the risk of nonspecific enzymatic proteolysis.

In addition to α7, we anticipate that ECD receptors of other members of the family of AChR subunits and other subunits of this entire superfamily of neurotransmitter-gated ion channels will mimic the structure and function of their respective full-length receptors. Demonstrations that ECD sequences are important in the assembly of both glycine receptors (69) and γ-aminobutyric acidA receptors (70) suggest that the ECD of other members of the superfamily also will fold and assemble autonomously. The ultimately successful design of an ECD AChR for high level expression may require other design modifications. Moreover, structural aspects of the gating and ion permeation functions of transmembrane domains will have to be explored with different strategies. A water-soluble, recombinantly generated ECD AChR, however, appears to be a promising foundation for structural studies of this superfamily of ion channels.

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