Extracellular Domain Nicotinic Acetylcholine Receptors Formed by α4 and β2 Subunits*

Alexandra M. Person1, Kathy L. Bills1, Hong Liu1, Shaleen K. Botting4, Jon Lindstrom1, and Gregg B. Wells1†

From the 1Department of Pathology and Laboratory Medicine, College of Medicine, Texas A&M University System Health Science Center, College Station, Texas 77843-1114 and the 4Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104-6074

Models of the extracellular ligand-binding domain of nicotinic acetylcholine receptors (nAChRs), which are pentameric integral membrane proteins, are attractive for structural studies because they potentially are water-soluble and better candidates for x-ray crystallography and because their smaller size is more amenable for NMR spectroscopy. The complete N-terminal extracellular domain is a promising foundation for such models, based on previous studies of α7 and muscle-type subunits. Specific design requirements leading to high structural fidelity between extracellular domain nAChRs and full-length nAChRs, however, are not well understood. To study these requirements in heteromeric nAChRs, the extracellular domains of α4 and β2 subunits with or without the first transmembrane domain (M1) were expressed in Xenopus oocytes and compared with α4β2 nAChRs based on ligand binding and subunit assembly properties. Ligand affinities of detergent-solubilized, extracellular domain α4β2 nAChRs formed from subunits with M1 were nearly identical to affinities of α4β2 nAChRs when measured with [3H]epibatidine, cysteine, nicotine, and acetylcholine. Velocity sedimentation suggested that these extracellular domain nAChRs predominantly formed pentamers. The yield of these extracellular domain nAChRs was about half the yield of α4β2 nAChRs. In contrast, [3H]epibatidine binding was not detected from the extracellular domain α4 and β2 subunits without M1, implying no detectable expression of extracellular domain nAChRs from these subunits. These results suggest that M1 domains on both α4 and β2 play an important role for efficient expression of extracellular domain α4β2 nAChRs that are high fidelity structural models of full-length α4β2 nAChRs.

Nicotinic acetylcholine receptors (nAChRs)2 are ligand-gated ion channels expressed mainly in the nervous system and at the neuromuscular junction (1–3), although they also are expressed elsewhere (4). They are members of the superfamily of nicotinoid receptors, which includes γ-aminobutyric acid (GABA) type A and C, glycine, and serotonin 5-HT3 receptors. They contain five homologous subunits, with each subunit containing an N-terminal extracellular domain sequences around the agonist-binding site (19–23) to most neurotransmitters, including roles in addiction, neurodegeneration, epilepsy, myasthenia gravis, and congenital myasthenic syndromes (5).

Knowledge of the structure of nAChRs has come from biochemical, electrophysiological, and imaging methods (6–8). These methods, however, do not provide comprehensive structure at atomic resolution. A significant advance toward such information has come recently from the crystallographic structure of acetylcholine-binding protein (AChBP), a water-soluble pentamer that has been the basis for homology modeling of the extracellular domain of nAChRs and other nicotinoid receptors (9–18). Continuing to move to structures of native nAChRs is needed for accurate knowledge of how these receptors function as ion channels and signal transducers, for understanding differences in pharmacological profiles and kinetics of different receptors, and for designing drugs to therapeutically target specific types of receptors for specific effects in the central and peripheral nervous systems. Structures of full-length nAChRs have been elusive because they are large, integral membrane proteins.

One attractive strategy to make nAChRs more tractable for high-resolution structural analysis is to produce water-soluble models from the extracellular domain without transmembrane domains. Compared with full-length nAChRs, water-soluble extracellular domain nAChRs are expected to crystallize more readily and, because of their smaller molecular mass, are expected to be better candidates for structural studies by NMR spectroscopy. This approach has shown promise for nAChRs. Models of extracellular domains of nAChRs have ranged from short amino acid sequences around the agonist-binding site (19–23) to most or all the extracellular domain from α1 or α7 subunits expressed in bacteria or yeast (24–30). These models, however, have only partially reproduced ligand binding affinities and subunit assembly properties of full-length receptors, motivating a search for models that more fully reflect the properties of full-length receptors.

Extracellular domain nAChRs are defined here as comprising truncated subunits containing N-terminal extracellular domain sequences and having ligand binding affinities or subunit assembly properties similar to full-length nAChRs. They have been expressed with several subunit combinations. Dimeric extracellular domain nAChRs were produced by extracellular domains of α1 and β8 subunits that also contained M1 or another membrane tether, suggesting that membrane attachment of subunits might be important for forming these extracellular domain nAChRs (31, 32). Extracellular domains of α7 subunits linked to a glycosylphosphatidylinositol moiety were transported to the surface of Xenopus oocytes and bound α-bungarotoxin (αBgt) and d-tubocurarine (33), implying the formation of multimers. Water-soluble extracellular...
domain α7 nAChRs without any transmembrane domains bound α8gt, nicotine, and acetylcholine with affinities similar to α7 nAChRs and likely were pentamers (34). Their yield, however, was substantially smaller than the yield of detergent-solubilized, extracellular domain α7 nAChRs that contained M1, suggesting that M1 was important for expressing these extracellular domain nAChRs. Milligram amounts of a nAChRs that contained M1, suggesting that M1 was important for formation of extracellular domain nAChRs, leading to the inclusion of “WS” in the subunit names. Superscript NT designates subunit designs with no epitope tag. Numbering of the amino acid residues in the extracellular domain is shown in parentheses. In comparison, the total length of α4 is 627 amino acids; total length of α2 is 502 amino acids. The epitope tags for mAb 142 on α4M1 and mAb 236 on β2M1 are labeled “142” and “236”, respectively. The signal peptides are designated SP. Assignments of residues to topological and functional regions of the primary sequences were based on α4 and β2 entries in the Swiss-Prot data base.

**Experimental Procedures**

**Design of Subunit DNA Plasmids**—The cDNAs for human α4 subunit (37) and human β2 subunit (38) were cloned into pSP64 poly(A) (Promega). The DNA sequences for α4M1, β2M1, α4WS, and β2WS extracellular domain constructs (Fig. 1) were derived from the α4 or β2 sequences by PCR. The N-terminal primers for PCR were GCGC ACGCGT CTC TTT GCG GCG AAT GAT GAA GTC for α4M1, CGC TGC GGC CCC for both α4-derived extracellular domain constructs and GCGC CTGCAG ATG GCC CGG CCG TGC GGC CCC for both β2-derived extracellular domain constructs. The C-terminal primers for PCR were GCGC ACGCGT CTC GGA GGG CAG GTA GAA GAC for α4M1, GCGC ACGCGT GTC GGA TGG CAG GTA GAA GAC for β2M1, GCGC ACGCGT CAG CCG CCG GAT GAC GAA GGC for α4WS, and GCGC ACGCGT CTT GCG GCG AAT GAT GAA GTC for β2WS. PCR with Pfu DNA polymerase (Stratagene) was performed with 10 ng of template DNA and 50 pmol of each primer for 30 cycles in 4% Me2SO with annealing at 60 °C for 1 min and extension at 72 °C for 3 min. The α4M1 and α4WS products from PCR were cut with HindIII and MluI; the β2M1 and β2WS products were cut with PstI and MluI. These restricted PCR products were ligated into pSP64 poly(A) along with a double-stranded oligonucleotide cassette coding in-frame for two copies of the mAb 142 epitope QVTGEVIFQTPLIKNP (39, 40) for α4M1, one copy of the mAb 142 epitope for α4WS, and one copy of the mAb 236 epitope VSIPSRESRDLSTF (39, 40) for β2M1 and β2WS. The DNA sequences of each epitope tag contained an MluI site before and a stop codon after the sequence coding the epitope tag. To reduce the length of subunit sequence that was derived from PCR, the α4 sequence upstream from its BstYI restriction site and the β2 sequence upstream from its BsiWI were replaced by DNA sequences that were taken from the full-length subunits. The last native residues in the constructs were Glu-270 (α4 numbering from Swiss-Prot (41) entry P34681) for α4M1, Leu-242 for α4WS, Asp-261 (β2 numbering from Swiss-Prot entry P17787) for β2M1, and Lys-233 for β2WS. The DNA sequences for α4M1<sup>NT</sup> and β2M1<sup>NT</sup> were prepared by replacing the MluI site at the 5’ end of the epitope tag coding sequence with an MluI site followed by a stop codon. The DNA sequences for α4WS<sup>NT</sup> and β2WS<sup>NT</sup> were prepared by replacing the native coding sequence distal to the BglII site near the 3’ end of α4WS and distal to the BsiWI near the 3’ end of β2WS with the identical coding sequence from synthetic oligonucleotides up to and including Leu-242 (α4WS<sup>NT</sup>) or Lys-233 (β2WS<sup>NT</sup>) followed by a stop codon. The correctness of products derived from PCR or from chemically synthesized oligonucleotides was confirmed by DNA sequencing.

**Protein Expression in Xenopus Oocytes**—*Xenopus laevis* were purchased from NASCO (Fort Atkinson, WI) and maintained in 12-h light/12-h dark cycle at 18 °C. The Institutional Animal Care and Use Committee of Texas A & M University approved all animal procedures. Oocytes were prepared following standard procedures (42, 43), using collagenase type 1 (Invitrogen) for enzymatic stripping of the follicular layer. cRNA was synthesized from linearized plasmid DNA using an SP6 mMessage mMachine kit (Ambion, Austin, TX). A 40-nl aliquot containing 20 ng of cRNA for each subunit being studied was injected into the cytoplasm of each oocyte. The oocytes then were incubated at 18 °C for 3–4 days in 50% Leibovitz’s L-15 medium (Invitrogen) in 10 mM HEPES, pH 7.5, containing 10 units/ml penicillin and 10 μg/ml streptomycin.

The membrane-bound fraction of subunit protein was extracted from oocyte membranes by homogenizing 25–50 oocytes by hand in ice-cold buffer A (in mM: 50 sodium phosphate, 50 NaCl, 5 EDTA, 5 EGTA, 5 benzamidine, 15 iodoacetamide, pH 7.5), centrifuging this
mixture for 30 min at 14,500 × g and 4 °C, solubilizing the pellet in buffer B (buffer A with 2% w/v Triton X-100) during gentle agitation for 2 h at 4 °C, and again centrifuging for 30 min at 14,500 × g and 4 °C (44). The membrane fraction was defined as the detergent extract. The membrane fraction was used for immunoblotting and ligand binding assays of subunits with transmembrane domains. For α4WS, α4WSNT, β2WS, and β2WSNT, the secreted fraction from injected oocytes was defined as the L-15 medium incubating injected oocytes; the cytoplasmic fraction from injected oocytes was defined as the supernatant above the pellet after homogenizing oocytes in buffer A and centrifuging.

**Immunoblotting**—Membrane fractions of α4M1, β2M1, α4WS, and β2WS were deglycosylated with peptide:N-glycosidase F (PNGase F, New England Biolabs), a glycosylasparaginase, according to the manufacturer’s instructions. Membrane fractions were processed in parallel without PNGase F. After deglycosylation, the samples were denatured by heating at 95 °C for 90 s in 1% SDS and 15 mM dithiothreitol. The samples were run by SDS-PAGE on a 12.5% acrylamide gel and transferred to Immune-Blot™ polyvinylidene difluoride membrane (Bioread) using a GENIE™ electrophoretic transfer device (Idea Scientific). The membrane was blocked overnight at 4 °C with 5% powdered milk in PBS buffer and 0.05% Tween. Primary antibody (mAb 142 for α4M1 and α4WS; mAb 236 for β2M1 and β2WS) at 3 nM in blocking solution was incubated with the membrane overnight at 4 °C, followed by washing for several hours with PBS and 0.05% Tween. Goat anti-rat secondary antibody conjugated with peroxidase (Pierce) was diluted 1:5000 in PBS containing 0.05% Tween and 1% bovine serum albumin. The membrane was incubated with the membrane overnight at 4 °C, followed by washing for 30 min at 14,500 × g.

**Ligand Binding Assays**—Immulon 4 HBX plastic microwells (Thermo Labsystems) for solid phase binding assays were coated with mAb 142 or mAb 236 for subunits with epitope tags (39, 40); or mAb 299 (Sigma), which binds the extracellular domain of α4 in its native or denatured conformation (45, 47). The wells were blocked with 3% bovine serum albumin, fraction V (EM Science). The membrane fraction was used for immunoblotting and ligand binding assays, except these fractions. Processing of these microwells was similar to processing of microwells measuring membrane-bound fractions, except buffer A replaced buffer B. Nonspecific binding was measured from un.injected oocytes and was less than 5% of specific binding at 30 nM total [3H]epibatidine and was less than 1% of specific binding at concentrations below 1 nM total [3H]epibatidine.

**Models of [3H]Epibatidine Binding**—Disaccharide constants in the picomolar range have been reported for [3H]epibatidine binding to α4β2 nACHRs (48–51). This high affinity raised the possibility of ligand depletion during binding assays. Ligand depletion means the free concentration of [3H]epibatidine differs significantly from the total concentration (52). To estimate dissociation constants for [3H]epibatidine, binding data were described with a one-site model and with cooperative and independent two-site models (Fig. 2). To allow for ligand depletion, the equations that described binding in the models were functions of the total concentration of [3H]epibatidine and did not assume free and total concentrations of [3H]epibatidine to be equal.

Binding of [3H]epibatidine for the one-site model was based on the mass action Equation 1 derived from the one-site binding mechanism in Fig. 2A,

$$K_d = \frac{R \cdot E}{RE} \quad \text{(Eq. 1)}$$

where $R$ is the concentration of free ligand-binding sites (unbound receptor); $E$ is the concentration of free [3H]epibatidine; $R_E$ is the concentration of bound [3H]epibatidine; and $K_d$ is the equilibrium dissociation constant. Equation 2 describes the concentration of [3H]epibatidine-binding species $RE$ for this model allowing for [3H]epibatidine depletion (52).

$$RE = \frac{R_t + E_t + K_d}{2} - \sqrt{\left(\frac{R_t + E_t + K_d}{2}\right)^2 - 4RE} \quad \text{(Eq. 2)}$$

where $R_t$ and $E_t$ are the total concentrations of receptor-binding sites and of [3H]epibatidine, respectively. Equation 2 is derived from Equa-
Estimation of Dissociation Constants—$K_d$ for the one-site binding model was estimated with two methods. First, $K_{d,0.5}$ was calculated for each data set from the half-maximum concentration, $K_{d,0.5}$ and total concentration of binding sites, $R_t$, as determined from least squares fitting of Equation 10 to the data set and from Equation 11.

In Equation 10, $n$ is the Hill coefficient. The best estimate of $K_d$ with this method, which is based on the relationship between $K_d$ and $K_{d,0.5}$ in the one-site model (52, 56), was the average of the $K_d$ values from all data sets. Second, $K_d$ was estimated with least squares fitting of Equation 2 simultaneously to all data sets of $[3H]$epibatidine binding. The independent variable was $E_t$. The value of $K_d$ was constrained to be identical for all data sets. Because the total concentration of receptor varied from data set to data set, a unique $R_t$ parameter value for each data set also was included in the simultaneous fitting to all data sets.

Values for $K_{d,0.5}$ and $K_d$ were estimated by least squares fitting of the equation $RE + 2\times RE_2$ (total concentration of bound $[3H]$epibatidine) simultaneously to all data sets of $[3H]$epibatidine binding, where $RE$ and $RE_2$ are defined by Equations 3 and 4. The values of $K_{d,0.5}$ and $K_d$ were constrained to be identical for all data sets and were defined as microscopic or intrinsic dissociation constants and not statistical dissociation constants based on two equivalent binding sites per receptor (57). A unique parameter $R_t$ for each data set also was included in the simultaneous fitting, where $R_t = RE + RE_2$.

Values for $K_{d,0.5}$ and $K_d$ were estimated by least squares fitting of the equation $R^{(1)}E + R^{(2)}E$ (total concentration of bound $[3H]$epibatidine) from Equations 6 and 7 simultaneously to all relevant data sets. The values of $K_{d,0.5}$ and $K_d$, and the ratio $R_t^{(1)}/R_t^{(2)}$ were constrained to be identical for all data sets. A unique $R_t$ parameter for each data set was included in the simultaneous fitting where $R_t = R_t^{(1)}/R_t^{(2)}$.

$K_h$ the dissociation constant for an inhibitor, was estimated with two methods based on the one-site competitive inhibition model (Fig. 2D). First, $K_h$ was calculated from the half-maximum concentration $IC_{50}$ as determined from fitting to Equation 12 and from the Cheng-Prusoff equation (58), Equation 13.

Inhibition of $[3H]$epibatidine binding by cytisine, ($\sim$)-nicotine, and acetylcholine was described with one site competition inhibition model (Fig. 2D). Total concentrations of $[3H]$epibatidine for inhibition experiments were 1 and 3 nM. These concentrations were large enough that depletion of $[3H]$epibatidine and inhibitors could be neglected. Equation 9 describes the concentration of the $[3H]$epibatidine-binding species for the one-site competitive inhibition model, 

$$RE = K_d E_t R_t/(K_d R_d + K_d E_t + K_d t)$$ (Eq. 9)

Based on the theory of ligand binding with ligand depletion (54, 55), Equations 3–9 were derived with the program Maple (Maplesoft, Waterloo, Ontario, Canada) from the mass action equations for the two-site models and one-site competitive inhibition model, combined with the conservation equations for $R_t$, $R_t^{(1)}$, $R_t^{(2)}$, $E_t$ and $I_t$.

Estimation of Dissociation Constants—$K_d$ for the one-site binding model was estimated with two methods. First, $K_{d,0.5}$ was calculated for each data set from the half-maximum concentration, $K_{d,0.5}$ and total concentration of binding sites, $R_t$, as determined from least squares fitting of Equation 10 to the data set and from Equation 11,
the add-in function set Xnumbers (Foxes Team, digilander libero.it/foxes). To compute the concentration of bound \[^{3}H\]epibatidine in Equations 3 and 4 and in Equations 6 and 7, the positive root of Equation 5 and of Equation 8 was selected at each iteration during the least squares fitting with Solver. Confidence intervals for parameter values were determined with a model comparison method based on F ratios (60). Quality of fit from different models was compared with an F test (61). Uncertainties are presented as standard error of the mean.

Sucrose Gradient Sedimentation—Detergent extracts of membranes from 3 to 5 oocytes injected with \(\alpha 4\) and \(\beta 2\) subunits or with \(\alpha 4M1\) and \(\beta 2M1\) subunits were processed as described for ligand binding assays. Membrane vesicles from TE671, a human rhabdomyosarcoma cell line (62) that contains muscle nAChRs, were solubilized in buffer B. A 200-μl aliquot of each detergent extract was layered on a 5-ml sucrose gradient (5–20% w/v) in a 0.5% Triton solution (in mM: 100 NaCl, 10 sodium phosphate, 5 EGTA, 5 EDTA, 1 NaN3, pH 7.5). The gradients were centrifuged for 75 min at 70,000 rpm (340,000 g) and 4 °C in a Beckman NVT90 rotor. Each gradient was collected in aliquots of 11 drops (130 μl) into 40 microwells coated with mAb 236 for \(\alpha 4M1/\beta 2M1\) nAChRs, mAb 295 for \(\alpha 4\beta 2\) nAChRs, or mAb 210 (63) for nAChRs from TE671. After incubation at 4 °C for 24 h that allowed for binding of protein to antibodies, the microwells were washed with buffer B and filled with 100 μl of 2 nm \[^{3}H\]epibatidine for \(\alpha 4M1/\beta 2M1\) and \(\alpha 4\beta 2\) nAChRs or 4 nm \[^{125}I\]-Bgt for muscle nAChRs in buffer B for 24 h at 4 °C. Microwells were washed with buffer B. Bound \[^{3}H\]epibatidine was stripped from the microwells with a solution 5% SDS and 25 mM dithiothreitol in buffer B and measured in a liquid scintillation counter. Bound \[^{125}I\]-Bgt was measured in a \(\gamma\)-counter.

Chemical Cross-linking—Detergent extracts of \(\alpha 4M1/\beta 2M1\) nAChRs were cross-linked at room temperature with 2 mM dimethylsuberimidilidate (64). Products were visualized by immunoblotting with mAb 142.

Comparison of nAChR Yields—The yield of \[^{1}H\]epibatidine-binding sites for each subunit combination was defined as the amount of bound \[^{1}H\]epibatidine with a total \[^{1}H\]epibatidine concentration of 1–3 nM. The absence of ligand depletion and absence of saturation of mAb-binding sites were confirmed by determining that the amount of bound \[^{1}H\]epibatidine was directly proportional to the amount of membrane fraction. Yield was calculated in two ways as follows: 1) the amount of \[^{1}H\]epibatidine-binding sites per oocyte and 2) the amount of \[^{1}H\]epibatidine-binding sites per oocyte relative to the yield of a standard subunit combination that was expressed and measured in parallel. The standards were \(\alpha 4\beta 2\) or \(\alpha 4M1/\beta 2M1\) nAChRs. Relative yield helped control for variations in protein expression that depended on time of year or the particular frog from which oocytes were harvested.

RESULTS

Protein Expression in Xenopus Oocytes—The \(\alpha 4M1, \beta 2M1, \alpha 4WS,\) and \(\beta 2WS\) subunits, when the corresponding cRNAs were injected as \(\alpha 4M1\) and \(\beta 2M1\) or as \(\alpha 4WS\) and \(\beta 2WS\) pairs, were glycosylated in oocytes (Fig. 3). The relatively narrow band of each glycosylated subunit suggested homogeneity in the attached carbohydrate structure. The molecular mass of each protein after deglycosylation with PNGase F, which cleaves N-linked glycoproteins, corresponded closely to the calculated value based on amino acid composition. The extracellular domain of \(\alpha 4\) has three potential N-linked glycosylation sites (Asn-57, Asn-108, and Asn-174), based on an NX(S/T) motif, \(\beta 2\) has two potential sites (Asn-51 and Asn-168).

Binding Properties of \[^{1}H\]Epibatidine with \(\alpha 4M1/\beta 2M1\) nAChRs—To begin analyzing the binding properties with \[^{1}H\]epibatidine, the affinities of \(\alpha 4\beta 2\) nAChRs and \(\alpha 4M1/\beta 2M1\) nAChRs were estimated according to the one-site model (Fig. 2A). \(K_d\) was estimated in two ways as follows: 1) from \(K_{d0,5}\) and \(R_i\) values of the individual binding data sets according to Equation 11, and 2) by fitting Equation 2 simultaneously to all data sets (TABLE ONE). \(K_{d0,5}\) values, determined with Equation 10, ranged from 23 to 200 pm for \(\alpha 4\beta 2\) nAChRs and from 20 to 100 pm for \(\alpha 4M1/\beta 2M1\) nAChRs. The total concentration of \[^{1}H\]epibatidine-binding sites ranged from 23 to 400 pm for \(\alpha 4\beta 2\) nAChRs and 34 to 160 pm for \(\alpha 4M1/\beta 2M1\) nAChRs. The average \(K_d\) values calculated from \(K_{d0,5}\) were 13 ± 3 pm (n = 8) for \(\alpha 4\beta 2\) nAChRs and 8 ± 2 pm (n = 6) for \(\alpha 4M1/\beta 2M1\) nAChRs. One data set for \(\alpha 4M1/\beta 2M1\) nAChRs was not included in the estimate because its calculated \(K_d\) value based on \(K_{d0,5}\) and \(R_i\) for that data set was negative. The \(K_d\) values from simultaneous fitting of all data sets to the one-site model were similar to the estimates based on \(K_{d0,5}\) values: \(K_d\) was 16 pm (95% CI, 7–29 pm, n = 8) for \(\alpha 4\beta 2\) nAChRs and \(K_{d0,5}\) was 10 pm (95% CI, 5–17 pm, n = 7) for \(\alpha 4M1/\beta 2M1\) nAChRs. To determine whether binding of \[^{1}H\]epibatidine reached equilibrium after 24 h of incubation, \(K_d\) values estimated with the one-site model from paired incubations at 24 and 72 h were compared. The average ratio of \(K_{d0,5}/K_{d24}\) was 0.95 ± 0.07 (n = 2) for \(\alpha 4\beta 2\) and 1.18 ± 0.02 (n = 2) for \(\alpha 4M1/\beta 2M1\) nAChRs. These values were close to 1, which was the expected ratio if equilibrium was reached by 24 h of incubation, and imply that 24 h was a sufficient incubation time to reach binding equilibrium for \[^{1}H\]epibatidine.

To determine whether a two-sites model was better than the one-site model, models of two cooperative sites (Fig. 2B) and two independent sites (Fig. 2C) were fit to the data. For \(\alpha 4\beta 2\) nAChRs, the best values for the dissociation constants for the two-cooperative sites model were \(K_{d0,5}\) = 23 pm and \(K_{d24}\) = 14 pm. This cooperative model, however, did not fit the data better than the one-site model (p = 0.54). For \(\alpha 4M1/\beta 2M1\) nAChRs, the best values were \(K_{d0,5}\) = 4.8 pm and \(K_{d24}\) = 15 pm; however, this cooperative model did not fit the data significantly better than the one-site model (p = 0.13). These results showed the two-cooperative sites model was not better than the one-site model for either \(\alpha 4\beta 2\) or \(\alpha 4M1/\beta 2M1\) nAChRs.

The two-independent sites model for \(\alpha 4\beta 2\) nAChRs contained a large fraction of a high affinity site (picomolar for \(K_{d0,5}\)) and a small fraction of a low affinity site (nanomolar for \(K_{d24}\)) (TABLE ONE). The best values for the dissociation constants were \(K_{d0,5}\) = 13 pm (population...
TABLE ONE

| Dissociation constants for $[^3H]$epibatidine binding to α4β2, α4M1/β2M1, and α4M1NT/β2M1NT nAChRs |
|--------------------------------------------------|--------------------------------------------------|
| Units for dissociation constants and CI are picomolar. mAbs for immunosolation are as follows: α4β2, mAb 295; α4M1/β2M1, mAb 142; and α4M1NT/β2M1NT, mAb 295. |
| $K_d$ | $K_d$ | $K_d$ |
| α4β2 | α4M1/β2M1 | α4M1NT/β2M1NT |
| 13 ± 3 | 8.4 ± 2.2 | 9.0 ± 4.3 |

From mechanisms in Fig. 2a

| $K_d$ | $K_d$ | $K_d$ |
|-------|-------|-------|
| 16    | 10    | 8.0   |

| 95% CI |
|--------|
| Lower  | 7.2   | 5.0   | 2.3   |
| Upper  | 29    | 17    | 17    |

| Two independent sites* |
|------------------------|
| $K_d$ | $K_d$ | $K_d$ |
| 13    | 7.4   | 3.7   |
| 12,000| 350   | 1700  |

| $K_d$ |
|-------|
| 17    |

| $K_d$ | $K_d$ | $K_d$ |
|-------|-------|-------|
| 23    | 5.6   | 0.39  |
| 14    | 15    |       |
|       |       |       |

| Two cooperative sites’ |
|------------------------|
| $K_d$ | $K_d$ | $K_d$ |
| 4     | 0.5   | 0.84  |
| 14    | 15    | 0.91  |
| 0.84  | 0.91  | 1.0   |

| $K_d$ |
|-------|
| 14    |

| Two independent sites* |
|------------------------|
| $K_d$ | $K_d$ | $K_d$ |
| 13    | 7.4   | 3.7   |
| 12,000| 350   | 1700  |

| $K_d$ |
|-------|
| 17    |

* Data are from Equations 10 and 11. Number of data sets are as follows: 8 for α4β2, 6 for α4M1/β2M1, and 2 for α4M1NT/β2M1NT.

** Number of data sets are as follows: 8 for α4β2, 7 for α4M1/β2M1, and 3 for α4M1NT/β2M1NT.

† Data are from Equation 2.

‡ Data are from Equations 3 and 4.

| Dissociation constants for $[^3H]$epibatidine binding to α4β2, α4M1/β2M1, and α4M1NT/β2M1NT nAChRs |
|--------------------------------------------------|--------------------------------------------------|
| Units for dissociation constants and CI are picomolar. mAbs for immunosolation are as follows: α4β2, mAb 295; α4M1/β2M1, mAb 142; and α4M1NT/β2M1NT, mAb 295. |
| $K_d$ | $K_d$ | $K_d$ |
| α4β2 | α4M1/β2M1 | α4M1NT/β2M1NT |
| 13 ± 3 | 8.4 ± 2.2 | 9.0 ± 4.3 |

From mechanisms in Fig. 2a

| $K_d$ | $K_d$ | $K_d$ |
|-------|-------|-------|
| 16    | 10    | 8.0   |

| 95% CI |
|--------|
| Lower  | 7.2   | 5.0   | 2.3   |
| Upper  | 29    | 17    | 17    |

| Two cooperative sites’ |
|------------------------|
| $K_d$ | $K_d$ | $K_d$ |
| 4     | 0.5   | 0.84  |
| 14    | 15    | 0.91  |
| 0.84  | 0.91  | 1.0   |

| $K_d$ |
|-------|
| 14    |

| Two independent sites* |
|------------------------|
| $K_d$ | $K_d$ | $K_d$ |
| 13    | 7.4   | 3.7   |
| 12,000| 350   | 1700  |

| $K_d$ |
|-------|
| 17    |

| $K_d$ | $K_d$ | $K_d$ |
|-------|-------|-------|
| 23    | 5.6   | 0.39  |
| 14    | 15    |       |

| Two independent sites* |
|------------------------|
| $K_d$ | $K_d$ | $K_d$ |
| 13    | 7.4   | 3.7   |
| 12,000| 350   | 1700  |

| $K_d$ |
|-------|
| 17    |

| $K_d$ | $K_d$ | $K_d$ |
|-------|-------|-------|
| 14    | 15    |       |

| $K_d$ |
|-------|
| 14    |

fraction, 0.84; 95% CI = 3.4–23 pm at fixed population ratios) and $K_{d2}$ was 12 nM (population fraction, 0.95; 95% CI = 11 pm, no upper limit at fixed population ratios). The value of $K_{d2}$ was similar to the value of $K_d$ in the one-site model (16 pm). The two-independent sites model fit the data better than the one-site model (p < 0.002). When the analysis was restricted to the three data sets with 30 nM total $[^3H]$epibatidine, $K_{d2} = 4.9$ pm (population fraction, 0.76) and $K_{d2} = 0.59$ nM (population fraction, 0.24). The two-independent sites model fit these three data sets better than the one-site model (p = 0.0001). A three-independent sites model did not fit the data better than the two-independent sites model (p = 0.9997).

The two-independent sites model for α4M1/β2M1 nAChRs contained one site with a large population fraction and picomolar affinity and a second site with a small population fraction with subnanomolar affinity: $K_{d1} = 7.4$ pm (population fraction, 0.91) and $K_{d2} = 0.35$ nM (population fraction, 0.09) (TABLE ONE). The value of $K_{d2}$ was similar to the value of $K_d$ in the one-site model (10 pm). The two-independent sites model, however, did not fit the data significantly better than the one-site model (p = 0.09). When the analysis was restricted to the three data sets with a maximum of 30 nM total $[^3H]$epibatidine, then $K_{d1} = 8.0$ pm (population fraction, 0.95) and $K_{d2} = 3.0$ nM (population fraction, 0.05). The two-independent sites model, however, did not fit these three data sets significantly better than the one-site model (p = 0.09).

In summary, the two-independent sites model provided the best fit for α4β2 nAChRs, and the one-site model provided the best fit for α4M1/β2M1 nAChRs (Fig. 4). The high affinity site contributed a large majority of the $[^3H]$epibatidine-binding sites for α4β2 nAChRs. The value of $K_{d1}$ (13 pm) for the high affinity site on α4β2 nAChRs was similar to the value of $K_d$ (10 pm) for the single site on α4M1/β2M1 nAChRs.

To determine whether the presence of the epitope tag at the C terminus of the α4M1 and β2M1 affected the binding properties of α4M1/β2M1 nAChRs with $[^3H]$epibatidine, binding was measured with α4M1NT and β2M1NT, which did not have epitope tags. $K_d$ values for α4M1NT/β2M1NT nAChRs estimated from either $K_{d1}$ and $R_d$ or from the single site model were similar to $K_d$ values for α4M1/β2M1 nAChRs and for α4β2 nAChRs (TABLE ONE). The two-cooperative sites model did not fit the data significantly better than the one-site model (p = 0.07). In contrast, the two-independent sites model fit significantly better than the one-site model (p = 0.0005). $K_{d1}$ for α4M1NT/β2M1NT nAChRs was comparable with $K_{d1}$ for α4M1/β2M1 nAChRs and characterized 85% of the binding sites. $K_{d2}$ characterized 15% of the binding sites and showed nanomolar affinity. These results showed the epitope tag was not necessary for forming the major high affinity site, although the tag might modify the heterogeneity of the binding sites. The major binding sites from α4β2 nAChRs, α4M1/β2M1 nAChRs, and α4M1NT/β2M1NT nAChRs showed similar affinities for $[^3H]$epibatidine (TABLE ONE).

To determine whether binding properties for α4M1/β2M1 nAChRs depended on the presence of mAb 142 in the assay, α4M1/β2M1 nAChRs were immunosolated on microwells with three other antibodies as follows: mAb 295, mAb 236, and mAb 299 (Fig. 5). Values of $K_d$ estimated from either $K_{d1}$ and $R_d$ or from fitting the one-site model to binding data with a maximum of 30 nM total $[^3H]$epibatidine ranged from 7 to 11 pm (TABLE TWO) and were similar to the value of $K_d$ for mAb 142 (10 pm). The two-independent sites model did not fit these data better than the one-site model (p > 0.28 for all mAbs). No site, therefore, with nanomolar affinity was identified with any of the three
mAbs. These results with different mAbs showed that binding affinity of α4β1/β2M1 nAChRs for [3H]epibatidine did not depend on the antibody used for immunoisolation.

**Binding Properties of Cytisine, Nicotine, and ACh with α4M1/β2M1 nAChRs**—Binding properties of α4M1/β2M1 nAChRs and α4β2 nAChRs were further compared with cytisine, nicotine, and ACh. The dissociation constants for these ligands were estimated with the following two methods based on their competitive inhibition of binding by [3H]epibatidine: 1) from IC_{50} values and the Cheng-Prusoff equation (Equation 12) and (Equation 2) to a specific data set. For these calculations, K_d and total receptor concentrations for each data set (R_t) were determined by fitting the model to all data sets simultaneously for a given mAb. Each line was calculated with the total receptor concentration that best fit the corresponding data set. The dissociation constant did not depend significantly on which mAb immunoisolated α4M1/β2M1 nAChRs (TABLE TWO).

The values of dissociation constants of cytisine, nicotine, and ACh with α4M1/β2M1 nAChRs were similar to the values with α4β2 nAChRs (TABLE THREE), whether the estimates were based on IC_{50} values and the Cheng-Prusoff equation (Fig. 6) or the one-site competitive inhibition model (Fig. 7). A two-independent sites model of inhibition (Equation 14) did not fit the data significantly better for either α4β2 nAChRs (p > 0.15) or α4M1/β2M1 nAChRs (p > 0.36) for any of these ligands. Estimates of dissociation constants did not depend significantly on whether the total [3H]epibatidine concentration was 1 or 3 nM.

For α4β2 nAChRs, about 20% of the [3H]epibatidine bound to α4β2 nAChRs was not displaced at the highest concentrations of inhibitor (Fig. 6). The base line from Equation 12 as a fraction of binding of 1 nM [3H]epibatidine in the absence of inhibitor was 0.18 (95% CI, 0.068–0.26) for cytisine, 0.18 (95% CI, 0.074–0.26) for nicotine, and 0.23 (95% CI, 0.10–0.34) for ACh. With 3 nM [3H]epibatidine, the average base line was 0.22 (95% CI, 0.12–0.29) for cytisine, 0.28 (95% CI, 0.088–0.40) for nicotine, and 0.23 (95% CI, 0.034–0.37) for ACh. To test whether a larger concentration of nicotine could more completely displace 1 nM [3H]epibatidine bound to α4β2 nAChRs, the largest concentration of nicotine was extended from 10 μM to 1 mM. The base line was reduced to 0.08 ± 0.03 (n = 2). A possible interpretation of these features is that inhibitors displaced [3H]epibatidine with high affinity from an initially fully bound, high affinity site and displaced [3H]epibatidine with much lower affinity from a different site or set of sites, leading to non-zero base lines.

To determine whether the antibody used to immunoisolate α4β2 nAChRs affected the base line of inhibition assays, nicotine inhibition of [3H]epibatidine binding was measured with α4β2 nAChRs immunoisolated with mAb 299 (Fig. 8). The IC_{50} values with mAb 299 and mAb 295 were similar. In contrast to incomplete inhibition with mAb 295, nicotine completely displaced [3H]epibatidine bound to α4β2 nAChRs immunoisolated with mAb 299, as evidenced by a zero base line (95% CI, 0–0.14).

All three inhibitors displaced nearly all [3H]epibatidine bound to α4M1/β2M1 nAChRs at the highest concentrations of inhibitor (Fig. 6). The base line from Equation 12 as a fraction of binding without inhibitor and with 1 nM [3H]epibatidine was 0.0038 (CI, 0–0.055) for cytisine, 0.017 (CI,
Inhibition of [3H]epibatidine binding to α4β2 and α4M1/β2M1 nAChRs by cytisine, nicotine, and ACh

|   | Cytine | Nicotine | ACh |
|---|---|---|---|
| α4β2* |   |   |   |
| From IC₅₀ and Cheng-Prusoff equation | 8.1 | 46 | 300 |
| Kᵢ | 0.13 | 0.74 | 4.9 |
| From one-site inhibition model |   |   |   |
| Kᵢ | 0.12 | 0.84 | 4.9 |
| 95% CI |   |   |   |
| Lower | 0.030 | 0.18 | 0.72 |
| Upper | 0.43 | 3.2 | 27 |
| α4M1/β2M1* |   |   |   |
| From IC₅₀ and Cheng-Prusoff equation | 19 | 160 | 910 |
| Kᵢ | 0.19 | 1.6 | 9.2 |
| From one-site inhibition model |   |   |   |
| Kᵢ | 0.22 | 1.5 | 8.3 |
| 95% CI |   |   |   |
| Lower | 0.091 | 0.52 | 2.8 |
| Upper | 0.45 | 3.7 | 20 |

* Number of data sets are as follows: 2 for cytine, 3 for nicotine, and 3 for ACh.

Data are from Equation 9. For α4β2, Kᵢ for [3H]epibatidine was set to 16 ps for estimating Kᵢ. Lower and upper limits of Kᵢ for confidence intervals of Kᵢ were 7 and 29 ps for α4M1/β2M1. Kᵢ for [3H]epibatidine was set to 10 ps for estimating Kᵢ. Lower and upper limits of Kᵢ for determining confidence intervals of Kᵢ were 5 and 17 ps.

The average base line as fractions of the binding without inhibition were 0.34 (95% CI, 0.15–0.46) for mAb 295, 0.00 (95% CI, 0–0.014) for nicotine, and 0.00018 (95% CI, 0–0.20) for ACh.

To determine whether the antibody used to immunoisolate α4M1/β2M1 nAChRs affected the inhibition assays, the Kᵢ value for nicotine was estimated with mAb 295, mAb 236, and mAb 299. The values of Kᵢ from the one-site inhibition model did not significantly depend on the mAbs (TABLE TWO). The base line, however, did depend on the mAb, with the base line for mAb 295 being significantly different from zero (Fig. 9). The average base lines as fractions of the binding without inhibitor were 0.34 (95% CI, 0.15–0.46) for mAb 295, 0.00 (95% CI, 0–0.15) for mAb 236, and 0.00 (95% CI, 0.00–0.14) for mAb 299. For both α4β2 nAChRs and α4M1/β2M1 nAChRs, immunoisolation by mAb 295 was associated with complete inhibition of [3H]epibatidine binding. In contrast, immunoisolation by mAb 142, mAb 236, and mAb 299 was associated with complete inhibition of [3H]epibatidine binding.

Oligomerization of α4M1 and β2M1 Subunits—[3H]Epibatidine-binding sites arose from a heteromeric combination of α4M1 and β2M1 subunits because neither α4M1 nor β2M1 subunits alone produced detectable [3H]epibatidine binding. To estimate the number of subunits in α4M1/β2M1 nAChRs, the molecular mass of α4M1/β2M1 nAChRs was estimated from sedimentation on sucrose gradients (Fig. 10A). The size standards were pentameric α4β2 nAChRs and pentameric (α1)₄βγy nAChRs and α1 subunit monomers from TE671, a human rhabdomyosarcoma cell line. The peak from α4β2 nAChRs ran slightly faster than the peak from TE671 nAChRs, as was predicted from their molecular masses of 297 kDa for α4β2 nAChRs and 268 kDa for human muscle nAChRs. The peak of the monomeric α1 subunit, although broad, was near the top of the gradient, as was predicted from its molecular mass of 52 kDa. The distribution of [3H]epibatidine binding by α4M1/β2M1 nAChRs was narrow and formed a single peak, suggesting that one multimeric species contributed a significant fraction of the binding sites. In contrast, binding by a mixture of oligomeric states with significant fractions of dimers, trimers, tetramers, pentamers, and higher order aggregates would be expected to form a broad distribution or several different peaks. The molecular mass of the α4M1/β2M1 nAChRs as estimated from the size standards was 170 kDa (Fig. 10A, insets). The calculated molecular mass of a pentamer with a stoichiometry of (α4M1)₄β2M1₃ was 154 kDa without glycosylation. This estimate for molecular mass, therefore, is consistent with a pentamer predominantly but not necessarily exclusively forming the high affinity [3H]epibatidine-binding sites.

Time-dependent chemical cross-linking of α4M1 and β2M1 subunits expressed together provided additional evidence for oligomerization into pentamers (Fig. 10B). The products of cross-linking were denatured and reduced and analyzed by immunoblotting with mAb 142. A ladder of α4M1-containing protein bands of increasing apparent molecular masses formed as the cross-linking reaction proceeded. This time-dependent ladder was consistent with formation initially of dimers and trimers, then tetramers, and ultimately pentamers by cross-linking between subunits of α4M1/β2M1 pentamers. The disappearance of lower molecular mass
bands with increasing time suggested that most α4M1 subunits in oligomers were present in pentamers in the cross-linking reaction.

**Yield of α4M1/β2M1 nAChRs**—To evaluate the efficiency of expressing extracellular domain nAChRs, yields of nAChRs from derivatives of α4 and β2 subunits were measured in absolute amounts of [3H]epibatidine-binding sites and in amounts relative to binding sites from α4β2 nAChRs or α4M1/β2M1 nAChRs assayed in parallel (Fig. 11). Evaluating relative amounts helped control for seasonal variation and frog-to-frog variation of oocyte expression. The averages of absolute amounts varied 10-fold from 46 (α4 and β2M1) to 440 fmol/oocyte (α4M1NT and β2M1NT). The relative amounts, however, varied about 2-fold across the different subunit combinations, ranging from 0.32 (α4M1 and β2M1NT) to 0.63 (α4M1 and β2) relative to α4β2 nAChRs. α4M1 produced [3H]epibatidine-binding sites with β2 or β2M1NT. Similarly, β2M1 produced [3H]epibatidine-binding sites with α4 or α4M1NT. These results showed the cytoplasmic loop or the C-terminal epitope tag on only one of the subunit types did not prevent forming of [3H]epibatidine-binding sites.

**Binding Properties of [3H]Epibatidine with α4WS/β2WS**—The α4WS and β2WS subunits did not contain a transmembrane domain and potentially could form water-soluble extracellular domain nAChRs. Both α4WS and β2WS proteins were expressed on oocytes and were glycosylated (Fig. 3). In contrast to α4M1 and β2M1 subunits, however, α4WS and β2WS did not produce a detectable amount of [3H]epibatidine.
295-coated microwells, no \(^{3}H\)epibatidine-binding sites were detected in the secreted, cytoplasmic, or membrane fractions. These results showed that factors other than the epitope tags were responsible for the failure to detect \(a4WS/b2WS\) nAChRs.

To determine whether the presence of transmembrane domains on either only \(a4\)-type or only \(b2\)-type subunits was sufficient for forming \(^{3}H\)epibatidine-binding sites, the following pairs of subunits were expressed in oocytes: \(a4WS/b2M1, a4WS/b2, a4WSNT/b2M1, a4WSNT/b2, a4M1/b2WS, a4M1/b2WS, a4M1/b2WSNT, and a4/b2WSNT\). With microwells containing membrane fractions from five oocytes, only the \(a4WSNT/b2\) subunit combination immunosolated with mAb 295 produced a detectable amount of \(^{3}H\)epibatidine binding from Triton-extracted oocyte membranes. The yield was 0.12 ± 0.07 fmol/oocyte (\(n = 2\)); the ratio of \(^{3}H\)epibatidine-binding sites from \(a4WSNT/b2\) compared with \(a4b2\) nAChRs was 0.0013 ± 0.0004. These results showed that M1 in both \(a4\) and \(b2\) subunits greatly increased the efficiency of forming the extracellular domain \(a4b2\) nAChRs.

**DISCUSSION**

The nearly identical ligand affinities of \(a4M1/b2M1\) nAChRs, the predominant species for \(a4b2\) nAChRs, and the assembly of \(a4M1\) and \(b2M1\) subunits into multimers that likely are pentamers suggest that extracellular domains with the M1 domains of \(a4\) and \(b2\) subunits form high fidelity structural models of \(a4b2\) nAChRs. The values of dissociation constants for binding between \(a4b2\) nAChRs and \(^{3}H\)epibatidine, cysteine, nicotine, and acetylcholine are similar to values reported from other laboratories (48–51, 65–67). A two-site model of binding of \(^{3}H\)epibatidine that previously was applied to \(a4b2\) nAChRs expressed in *Xenopus* oocytes and immunosolated with mAb 299 found dissociation constants of 14 nm (population fraction, 0.73) and 1.2 nm (population fraction, 0.27) (49). Based on the relatively low precision of the estimated value of \(K_{d}^{2}\) for \(a4b2\) nAChRs, the difference between 1.2 and 12 nm for these two estimates of the dissociation constant of the low affinity \(^{3}H\)epibatidine site was not significant.

With respect to design requirements for extracellular domain \(a4b2\) nAChRs, the extracellular domains with the first transmembrane domains of \(a4\) and \(b2\) subunits were sufficient for assembly into extracellular domain \(a4M1/b2M1\) nAChRs that were stable after solubilization in detergent. The cytoplasmic loop was not required for assembly or for stability of these extracellular domain nAChRs after solubilization. M1, however, played an important role for expressing these extracellular domain nAChRs. These results, with the properties of extracellular domain \(a7\) nAChRs (33, 34) and evidence of oligomerization of extracellular domains from \(a1\) and \(\delta\) subunits (31, 32), from extracellular domains of muscle-type subunits (35), from chimeric \(a7/\alpha1\)CBP subunits (26), from glycine receptor subunits (68, 69), and from \(\gamma2\) receptor subunits (70), suggest that producing extracellular domain receptors might be possible for many subunits throughout the nicotinoid family. Expression of extracellular domain nicotinoid receptors with high structural fidelity, however, might be more feasible with M1 included in the subunit design than without M1.

Conclusions about the extent of structural similarity between \(a4M1/b2M1\) and \(a4b2\) nAChRs have limitations. First, the data for \(^{3}H\)epibatidine binding to \(a4b2\) nAChRs were fit better by the two-independent sites model than by the one-site model, implying a heterogeneous population of binding sites. In contrast, the data for \(^{3}H\)epibatidine binding to \(a4M1/b2M1\) nAChRs were fit better by the one-site model, suggesting that \(a4M1/b2M1\) nAChRs model only a subset of different \(a4b2\) nAChR structures. One possible cause of a heterogeneous popu-
lation of binding sites in \(\alpha_4\beta_2\) nAChRs could be the presence of two or more relative subunit stoichiometries between \(\alpha_4\) and \(\beta_2\) subunits. The \(\alpha_4M1:\beta_2M1\) ratio and \(\alpha_4:\beta_2\) ratio are likely 2:3, based on evidence from chicken \(\alpha_4\beta_2\) nAChRs expressed in oocytes with a 1:1 ratio of injected \(\alpha_4\) and \(\beta_2\) cRNAs (71) or with a 1:1 ratio of nuclear-injected cDNAs (72). In addition to the 2:3 ratio, however, an \(\alpha_4:\beta_2\) ratio of 3:2 in \(\alpha_4\beta_2\) nAChRs has been found in human embryonic kidney tsA201 cells (73). Other \(\alpha_4:\beta_2\) ratios besides 2:3 also have been implied by pharmacologic properties of \(\alpha_4\beta_2\) nAChR expressed from different ratios of injected \(\alpha_4\) and \(\beta_2\) subunits (74, 75).

Second, additional evidence suggesting structural heterogeneity in \(\alpha_4\beta_2\) nAChRs as well as in \(\alpha_4M1:\beta_2M1\) nAChRs was incomplete inhibition of \(\left[^{3}H\right]\)epibatidine binding by cytisine, nicotine, and acetylcholine that was associated with immunoisolation by mAb 295. In contrast, complete inhibition of \(\left[^{3}H\right]\)epibatidine binding was associated with immunoisolation by mAb 142, mAb 236, and mAb 299. These differences in inhibition behavior might identify minor populations of nAChRs captured by mAb 295 and possessing binding properties distinguishable from the dominant population. These binding properties might arise from unusual subunit stoichiometries or post-translational modifications or might be associated with immature subunit assemblies. A protein population with structural homogeneity facilitates structural analysis. Therefore, determining the range of relative subunit stoichiometries of extracellular domain \(\alpha_4\beta_2\) nAChRs and developing methods for purifying populations with homogeneous subunit composition and structure will be important steps in the future for developing these nAChRs as structural models.

Third, similarity of ligand dissociation constants does not require global structural fidelity of the \(\alpha_4M1:\beta_2M1\) nAChRs with the extracellular domain of \(\alpha_4\beta_2\) nAChRs. In other words, structural fidelity at the ligand-binding site might coexist with structural differences outside that site. Similar ligand binding properties likely reflect similar global structures in nAChRs, however, because the ligand-binding site likely is formed by widely dispersed, noncontiguous sections of the primary sequence, based on the structure of AChBP (9, 76). Similar to the effect of M1 on expression of extracellular \(\alpha_7\) nAChRs (34), the presence of M1 in both \(\alpha_4M1\) and \(\beta_2M1\) appears to greatly increase the efficiency of forming extracellular \(\alpha_4\beta_2\) nAChRs in oocytes. This conclusion is based on detecting \(\left[^{3}H\right]\)epibatidine-binding sites formed with \(\alpha_4M1\) and \(\beta_2M1\) but not with \(\alpha_4\) and \(\beta_2\). What hindered the expression of \(\alpha_4\) and \(\beta_2\) nAChRs? The failure of \(\alpha_4\) and \(\beta_2\) to form extracellular domain nAChRs was not caused by failure to synthesize these proteins or by excessive instability of these proteins. They were synthesized and were stable enough to be detected by immunoblotting at levels in detergent extract similar to the levels \(\alpha_4M1\) and \(\beta_2M1\). The C-terminal epitope tags were not the sole block to forming extracellular domain nAChRs because removing these epitope tags from \(\alpha_4\) and \(\beta_2\) did not lead to expression of...
[3H]epibatidine-binding sites. A possible explanation is that the truncation points in the primary sequences of α4 and β2, chosen because those points juxtaposed with M1, might be unsuitable for forming extracellular domain α4β2 nAChRs. In that case, shorter designs of α4WS and β2WS might be better suited for expressing water-soluble extracellular domain α4β2 nAChRs. Increasing the hydrophilic character of the Cys loop in the extracellular domain is another potentially favorable change in the designs of α4WS and β2WS, based on the increased water solubility of a chimeric α7 extracellular domain with a more hydrophilic Cys loop sequence taken from AChBP (26).

Why might M1 be necessary for efficiently forming extracellular domain nAChRs? Previous speculation has centered on a possible role of M1 as a tether to membrane surfaces, which might reduce the degrees of spatial freedom within and between subunits and favor correct folding and assembly (31, 32). Such tethering might also favor interaction with membrane-bound host components of the endoplasmic reticulum or Golgi that contribute to formation of nAChRs. The presence of a sequence motif in the M1 of α7 that governs surface trafficking (77) supports an alternative possibility that M1 is not acting simply as a membrane tether. Instead, properties specific to M1 and not found in other transmembrane domains might be important for formation of extracellular domain nAChRs.

With evidence for the importance of M1 to extracellular domain nAChRs, how can water-soluble extracellular domain nAChRs be made efficiently? First, they might be produced by in vitro proteolysis that removes transmembrane domains from nAChRs. This strategy would require engineering of specific proteolysis sites into subunit sequences or Golgi host factors that contribute to subunit folding and assembly also might have begun to explore the feasibility of this approach. Fourth, increasing the hydrophilic character of the Cys points juxtaposed with M1, might be unsuitable for forming extracellular nAChRs. Previous speculation has centered on a possible role of M1 that governs surface trafficking (77) and AChBP.

References

1. Lindstrom, J. M. (2003) Annu. N. Y. Acad. Sci. 998, 41–52
2. Gotti, C., and Clementi, F. (2004) Prog. Neurobiol. 74, 363–396
3. Hogg, R. C., Raggenbass, M., and Bertrand, D. (2003) Rev. Physiol. Biochem. Pharmacol. 147, 1–46
4. Sharma, G., and Vijayaraghavan, S. (2002) J. Neurobiol. 53, 524–534
5. Lindstrom, J. (1997) Mol. Neurobiol. 15, 193–222
6. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
7. Unwin, N. (2003) FEBS Lett. 555, 91–95
8. Unwin, N. (2005) J. Mol. Biol. 346, 967–989
9. Brejc, K., van Dijk, W. J., Klassens, R. V., Schuurmans, M., van Der Oost, J., Smit, A. B., and Sancia, T. K. (2001) Nature 411, 269–276
10. Karlin, A. (2002) Nat. Rev. Neurosci. 3, 102–114
11. Reeves, D. C., Sayed, M. F., Chau, P. L., Price, K. L., and Lummis, S. C. (2003) Biophys. J. 84, 2338–2344
12. Trudell, J. R., and Bertaccini, F. (2004) J. Mol. Graph. Model. 23, 39–49
13. Nevin, S. T., Cromer, B. A., Haddrell, J. L., Morton, C. J., Parker, M. W., and Lynch, J. W. (2003) J. Biol. Chem. 278, 28985–28992
14. Ernst, M., Brauchart, D., Boresch, S., and Sieghart, W. (2003) Neuroscience 119, 933–943
15. Cromer, B. A., Morton, C. J., and Parker, M. W. (2002) Trends Biochem. Sci. 27, 180–187
16. Le Novère, N., Gutter, T., and Changeux, J. P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3210–3215
17. Maksg, B., Bikadi, Z., and Simonyi, M. (2003) J. Recept. Signal Transduct. Res. 23, 255–270
18. Grudzinska, J., Scherm, R., Haeger, S., Nicke, A., Schmalzing, G., Betz, H., and Laube, B. (2005) Neurosci. 45, 727–739
19. Basus, V. J., Song, C., and Hawrot, E. (1993) Biochemistry 32, 12290–12298
20. Zeng, H., Moise, L., Grant, M. A., and Hawrot, E. (2001) J. Biol. Chem. 276, 22930–22940
21. Moise, L., Pisccherio, A., Basus, V. J., and Hawrot, E. (2002) J. Biol. Chem. 277, 12406–12417
22. Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., and Maelicke, A. (1990) Biochemistry 29, 6221–6230
23. Harel, M., Kasher, R., Nicolas, A., Guss, J. M., Balas, M., Fridkin, M., Smit, A. B., Brejc, K., Sancia, T. K., Tschaki-Katzi, E., Sussman, J. L., and Fuchs, S. (2001) Neuron 32, 265–275
24. Yao, Y., Wang, J., Viroonchatapan, N., Samson, A., Chill, J., Rothe, E., Anglister, J., and Wang, Z. Z. (2002) J. Biol. Chem. 277, 12613–12621
25. Psaridi-Linardaki, L., Mamalaki, A., Remoundos, M., and Tzartos, S. J. (2002) J. Biol. Chem. 277, 26980–26986
26. Avramopoulou, V., Mamalaki, A., and Tzartos, S. J. (2004) J. Biol. Chem. 279, 38287–38293
27. Fischer, M., Corringer, P. J., Schott, K., Bachner, A., and Changeux, J. P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3567–3570
28. Tsetsin, V. I., Dergousova, N. I., Azeve, E. A., Kryukova, E. V., Kudelina, I. A., Shibanovo, E. D., Kasheverov, I. E., and Methfessel, C. (2002) Eur. J. Biochem. 269, 2801–2809
29. Alexeev, T., Kirovsoin, A., Shevalier, A., Kudelina, L., Telyakov, O., Vincent, A., Ultik, Y., Hucro, F., and Tsettin, V. (1999) Euro. J. Biochem. 259, 310–319
30. Schrattenholz, A., Pfeiffer, S., Pejovic, V., Rudolph, R., Godovac-Zimmermann, J., and Maelicke, A. (1998) J. Biol. Chem. 273, 32393–32399
31. Wang, Z. Z., Hardy, S. F., and Hall, Z. W. (1996) J. Biol. Chem. 271, 27575–27584
32. Wang, Z. Z., Hardy, S. F., and Hall, Z. W. (1996) J. Cell. Biol. 135, 809–817
33. West, A. P., Björkman, P. J., Dougherty, D. A., and Lester, H. A. (1997) J. Biol. Chem. 272, 25468–25473
34. Wells, G. B., Anand, R., Wang, F., and Lindstrom, J. (1998) J. Biol. Chem. 273, 964–974
35. Tierney, M. L., and Unwin, N. (2000) J. Mol. Biol. 303, 185–196
36. Wang, D. X., and Abdoog, L. G. (1996) J. Neurosci. Res. 44, 350–354
37. Kuryatov, A., Gerzanich, V., Nelson, M., Olale, F., and Lindstrom, J. (1997) J. Neurosci. 17, 9035–9047
38. Anand, R., and Lindstrom, J. (1990) Nucleic Acids Res. 18, 4272
39. Das, M. K., and Lindstrom, J. (1991) Biochemistry 30, 2470–2477
40. Anand, R., Baron, L., Saedi, M. S., Gerzanich, V., Peng, X., and Lindstrom, J. (1993) Biochemistry 32, 9975–9984
41. Bairoeh, A., Apweiler, R., Wu, C. H., Barker, W. C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M. J., Natale, D. A., O’Donovan, C., Redaschi, N., and Yeh, L. S. (2005) Nucleic Acids Res. 33, D154–D159
42. Matthews, G. M. (1999) in Protein Expression: A Practical Approach (Higgins, S. J., ed) pp. 29–59, Oxford University Press, Oxford, UK
43. Colman, A. (1984) in Transcription and Translation: A Practical Approach (Hames, B. D., and Higgins, S. J., eds) pp. 271–302, IRL Press, Oxford, UK
44. Gerzanich, V., Anand, R., and Lindstrom, J. (1994) Mol. Pharm. 45, 212–220
Extracellular Domain α4β2 Nicotinic Acetylcholine Receptors

45. Lindstrom, J. (2000) in Neuronal Nicotinic Receptors (Clementi, F., Fornasari, D., and Gotti, C., eds) Vol. 144, pp. 101–162, Springer-Verlag, Berlin
46. Whiting, P. J., and Lindstrom, J. M. (1988) J. Neurosci. 8, 3395–3404
47. Peng, X., Gerzanich, V., Anand, R., Whiting, P. J., and Lindstrom, J. (1994) Mol. Pharmacol. 46, 523–530
48. Eaton, J. B., Peng, J. H., Schroeder, K. M., George, A. A., Fryer, J. D., Krishnan, C., Buhrman, L., Kuo, Y. P., Steinlein, O., and Lukas, R. J. (2003) Mol. Pharmacol. 64, 1283–1294
49. Sabay, N., Houn, M., Triant, A., Viseshakul, N., Figl, A., Sandhu, S., Forsayeth, J. R., Dowsok, L. P., Crooks, P. A., and Cohen, B. N. (1999) Br. J. Pharmacol. 128, 1291–1299
50. Houghtling, R. A., Davila-Garcia, M. I., and Kellar, K. J. (1995) Mol. Pharmacol. 48, 280–287
51. Houghtling, R. A., Davila-Garcia, M. I., Hurt, S. D., and Kellar, K. J. (1994) Mol. Chem. Res. 4, 538–546
52. Hulme, E. C., and Birdsall, N. J. M. (1992) in Receptor-Ligand Interactions: A Practical Approach (Hulme, E. C., ed), pp. 63–176, Oxford University Press, New York
53. Swillens, S. (1995) Mol. Pharmacol. 47, 1197–1203
54. Wells, J. W. (1992) in Receptor-Ligand Interactions: A Practical Approach (Hulme, E. C., ed) pp. 289–395, Oxford University Press, New York
55. Wang, Z. X., and Jiang, R. F. (1996) FEBS Lett. 392, 245–249
56. Cuatrecasas, P., and Hollenberg, M. D. (1976) Biochem. Pharm. 23, 3099–3108
57. Weber, G. (1992) Protein Interactions, Chapman and Hall, New York
58. Cheng, Y., and Prusoff, W. H. (1973) Biochem. Pharmac. 22, 3099–3108
59. Brown, A. M. (2001) Comput. Methods Programs Biomed. 65, 191–200
60. Motulsky, H. J., and Christophofoiu, A. (2004) Fitting Models to Biological Data Using Linear and Nonlinear Regression. A Practical Guide to Curve Fitting, pp. 109–117, Oxford University Press, New York
61. Motulsky, H. J., and Ransnas, L. A. (1987) FASEB J. 1, 365–374
62. Conroy, W. G., Saedi, M. S., and Lindstrom, J. (1990) J. Biol. Chem. 265, 21642–21651
63. Tzartos, S., Hochschwender, S., Vasquez, P., and Lindstrom, J. (1987) J. Neuroimmun. 15, 185–194
64. Brew, K., Shaper, J. H., Olsen, K. W., Trayer, I. P., and Hill, R. L. (1974) J. Biol. Chem. 250, 1434–1444
65. Sabay, K., Paradiso, K., Zhang, J., and Steinbach, J. H. (1999) Mol. Pharmacol. 55, 58–66
66. Truong, A., Xing, X., Forsayeth, J. R., Dowsok, L. P., Crooks, P. A., and Cohen, B. N. (2001) Brain Res. Mol. Brain Res. 96, 68–76
67. Whiteaker, P., Sharples, C. G., and Wonnacott, S. (1998) Mol. Pharmacol. 53, 950–962
68. Xue, H., Shi, H., Tsang, S. Y., Zheng, H., Savva, C. G., Sun, J., and Holzenburg, A. (2001) J. Mol. Biol. 312, 915–920
69. Breitinger, U., Breitinger, H. G., Bauer, F., Fahmy, K., Glockenhammer, D., and Becker, C. M. (2000) J. Biol. Chem. 275, 1627–1636
70. Xue, H., Zheng, H., Li, H. M., Kitimoto, A., Zhu, H., Lee, P., and Holzenburg, A. (2000) J. Biol. Chem. 275, 739–742
71. Anand, R., Conroy, W. G., Schoepfer, R., Whiting, P., and Lindstrom, J. (1991) J. Biol. Chem. 266, 11192–11198
72. Cooper, E., Couturier, S., and Ballivet, M. (1991) Nature 350, 235–238
73. Nelson, M. E., Kuryatov, A., Choi, C. H., Zhou, Y., and Lindstrom, J. (2003) Mol. Pharmacol. 63, 332–341
74. Zwart, R., and Vijverberg, H. P. (1998) Mol. Pharmacol. 54, 1124–1131
75. Lopez-Hernandez, G. Y., Sanchez-Padilla, J., Ortiz-Acevedo, A., Lizardi-Ortiz, J., Salas-Vincenty, J., Rojas, L. V., and Lasalde-Dominicci, J. A. (2004) J. Biol. Chem. 279, 38007–38015
76. Celic, P. H., van Rossum-Fikkert, S. E., van Dijk, W. J., Brejc, K., Smit, A. B., and Sixma, T. K. (2004) Neuron 41, 907–914
77. Wang, J. M., Zhang, L., Yao, Y., Virosch, N., Rothe, E., and Wang, Z. Z. (2002) Neuron 35, 963–970
78. Zhou, Y., Nelson, M. E., Kuryatov, A., Choi, C., Cooper, J., and Lindstrom, J. (2003) J. Neurosci. 23, 9004–9015
79. Bourrat, C., Gurnar, F., Spitzmaul, G., Wang, H. L., Rayes, D., Hansen, S. B., Taylor, P., and Sine, S. M. (2004) Nature 430, 896–900
80. Tugarinov, V., Choy, W. Y., Orekhov, V. Y., and Kay, L. E. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 622–627
81. Tugarinov, V., Hwang, P. M., and Kuryatov, A. (2004) Annu. Rev. Biochem. 73, 107–146
82. Fiaux, J., Bertelsen, E. B., Horwich, A. L., and Wuthrich, K. (2002) Nature 418, 207–211
83. Williamson, P. T., Meier, B. H., and Watts, A. (2004) Eur. J. Biochem. 312, 247–254
84. Opella, S. J., and Marassi, F. M. (2004) Chem. Rev. 104, 3587–3606