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Neuronal Nicotinic Receptor β2 and β4 Subunits Confer Large Differences in Agonist Binding Affinity

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ABSTRACT

We used equilibrium binding analysis to characterize the agonist binding properties of six different rat neuronal nicotinic receptor subunit combinations expressed in *Xenopus laevis* oocytes. The α4β2 receptor bound [^{3}H]cytisine with a $K_{d\text{app}}$ of 0.74 ± 0.14 nM. The rank order of $K_{i\text{app}}$ values of additional nicotinic ligands, determined in competition assays, was cytisine < nicotine < acetylcholine < carbachol < curare. These pharmacological properties of α4β2 expressed in oocytes are comparable to published values for the high affinity cytisine binding site in rat brain (α4β2), demonstrating that rat neuronal nicotinic receptors expressed in *X. laevis* oocytes display appropriate pharmacological properties. Use of [^{3}H]epibatidine allowed detailed characterization of multiple neuronal nicotinic receptor subunit combinations. $K_{d\text{app}}$ values for [^{3}H]epibatidine binding were 10 pM for α2β2, 87 pM for α2β4, 14 pM for α3β2, 300 pM for α3β4, 30 pM for α4β2, and 85 pM for α4β4. Affinities for six additional agonists (acetylcholine, anabasine, cytisine, 1,1-dimethyl-4-phenylpiperazinium, lobeline, and nicotine) were determined in competition assays. The β2-containing receptors had consistently higher affinities for these agonists than did β4-containing receptors. Particularly striking examples are the affinities displayed by α2β2 and α2β4, which differ in 1,1-dimethyl-4-phenylpiperazinium, nicotine, lobeline, and acetylcholine affinity by 120-, 86-, 85-, and 61-fold, respectively. Although smaller differences in affinity could be ascribed to different α subunits, the major factor in determining agonist affinity was the nature of the β subunit.

Neuronal nAChRs form as pentameric assemblies of subunits, similar to muscle nAChRs (Anand *et al*., 1991; Cooper *et al*., 1991). There are 11 known neuronal nAChR subunits, α2–9 and β2–4 (Sargent, 1993; Elgoyhen *et al*., 1994). Many different combinations of these subunits can assemble to form functional nAChRs when expressed in *Xenopus laevis* oocytes or mammalian cell lines, with each functional subunit combination displaying a distinct array of biophysical and pharmacological properties (Role, 1992; Patrick *et al*., 1993; Sargent, 1993). Thus, differential subunit assembly is likely to underlie biophysical and pharmacological observations of multiple subtypes of neuronal nAChRs in the nervous system.

Nicotinic ligands are potentially useful as anxiolytics and analgesics and are potentially useful in the treatment of neurological disorders such as schizophrenia, Parkinson's disease, and Alzheimer's disease (Brioni *et al*., 1997). Neuronal nAChRs also are the sites at which nicotine exerts its psychoactive and addictive effects (Dani and Heinemann, 1996). Thus, pharmacological intervention at neuronal nAChRs holds promise for treating the effects of diseases of the central nervous system and for understanding and treating addictive processes. Critical to the realization of this potential is the development of subtype-selective nAChR ligands. Pursuit of this goal requires an understanding of the molecular structure of the ligand binding sites of neuronal nAChRs. In particular, the features of nicotinic binding sites that are responsible for nAChR subtype selectivity must be identified.

Affinity labeling and mutagenesis techniques have been used to identify a series of residues on the α, γ/ε, and δ subunits that participate in the structure of the neurotransmitter binding sites of muscle-type nAChRs (Karlin and Akabas, 1995). The identification of critical residues on the γ/ε and δ subunits, together with the repeated demonstration that the two binding sites on muscle nAChRs are pharmacologically distinct, has led to the concept that the neurotransmitter binding sites are located at the interface between α and non-α (γ/ε and δ) subunits (Blount and Merlie, 1989; Galzi and Changeux, 1995). The neurotransmitter binding sites on neuronal nAChRs seem to be formed in a similar manner, because both α and β subunits make contributions to the pharmacological properties of these receptors (Luetje

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMPP, 1,1-dimethyl-4-phenylpiperizinium.

and Patrick, 1991). Many of the residues identified as part of the binding sites of muscle type nAChRs are highly conserved among neuronal nAChR subunits. Thus, although these residues are common features of nicotinic binding sites, they cannot account for the pharmacological differences that have been observed among neuronal nAChR subtypes. Amino acid residues that differ among subunits must be responsible for this pharmacological diversity.

By constructing chimeras and mutants of pharmacologically distinct subunits and analyzing them in an electrophysiological assay, we have identified residues on both α and β subunits that determine sensitivity to the competitive antagonist toxins α-conotoxin MII and neuronal bungarotoxin (Harvey and Luetje, 1996; Harvey *et al*., 1997; Luetje *et al*., 1998). These residues are most likely involved in binding of toxin. However, when the agonist sensitivity of neuronal nAChRs is determined using electrophysiological techniques, differences in agonist sensitivity may be due to differences in affinity, efficacy, desensitization, application flow rate, or a complex combination of these processes. As an alternative, we decided to use equilibrium binding to examine the subunit dependence of agonist affinity. Although neuronal nAChRs undergo transitions among multiple states, with each state having an affinity for agonist, the desensitized state has a much higher agonist affinity and, at equilibrium, predominates. Thus, it is primarily the affinity of the desensitized state that is being measured in an equilibrium assay of neuronal nAChRs (see Discussion).

We used [^{3}H]epibatidine in most of our analyses because epibatidine has been shown to bind with high affinity to multiple nAChR subtypes in the central and peripheral nervous systems (Marks *et al*., 1986; Houghtling *et al*., 1995; Flores *et al*., 1996). We adapted equilibrium binding assays originally developed for use with brain homogenates (Pabreza *et al*., 1991; Houghtling *et al*., 1994; Marks *et al*., 1998) for analysis of cloned neuronal nAChRs expressed in *X. laevis* oocytes. We demonstrate that neuronal nAChRs expressed in *X. laevis* oocytes display appropriate pharmacological properties when compared with nAChRs expressed in the brain. We then use saturation and competition assays to determine the affinities of six different neuronal nAChR subunit combinations for ACh, anabasine, cytisine, DMPP, epibatidine, lobeline, and nicotine. We find large differences in agonist affinities among different receptor subunit combinations to be due primarily to the identity of the β subunit present in the receptor.

Experimental Procedures

Materials. *X. laevis* frogs were purchased from Nasco (Ft. Atkinson, WI). The care and use of *X. laevis* frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. RNA transcription kits were from Ambion (Austin, TX). [^{3}H]Cytisine and [^{3}H]epibatidine were from New England Nuclear (Boston, MA). Acetylcholine, anabasine, carbachol, curare, cytisine, DMPP, lobeline, mecamylamine, nicotine, and 3-aminobenzoic acid ethyl ester were from Sigma Chemical (St. Louis, MO). Collagenase B was from Boehringer-Mannheim (Indianapolis, IN).

Expression of neuronal nAChRs in *X. laevis* oocytes. cDNA clones encoding α2, α3, α4, β2, and β4 subunits of rat neuronal nicotinic receptors were engineered into the pGEMHE high expression vector (Liman *et al*., 1992). In preliminary experiments, we found that injection of cRNA transcribed from pGEMHE constructs resulted in receptor expression levels that were 10–100-fold higher than expression levels achieved by injection of cRNA transcribed from pSP64/65 constructs (data not shown). m^{7}G(5′)ppp(5′)G capped cRNA was synthesized *in vitro* from linearized template cDNA using an Ambion mMessage mMachine kit. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and oocytes were surgically removed. Follicle cells were removed by treatment with collagenase B for 2 hr at room temperature. Oocytes were injected with 20 ng of cRNA encoding various subunit combinations in 23 nl of water and incubated at 19° in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 μg/ml gentamicin, 15 mM HEPES, pH 7.6) for 2–7 days.

Preparation of oocyte homogenates. Membrane preparation from *X. laevis* oocytes can be problematic due to the abundance of yolk and pigment granules. We found that a radioligand binding assay could be successfully performed using a crude oocyte homogenate after the removal of lipids and pigment granules. From 0.25 to 15 oocytes (depending on expression levels) were homogenized per milliliter of buffer containing freshly added 0.1 mM phenylmethylsulfonyl fluoride (see below), using a Brinkmann Instruments (Westbury, NY) model PT 10/35 homogenizer. Homogenates were centrifuged at 4° at 2000 × g for 10 min. The supernatant was removed for use in experiments, avoiding both the surface lipid layer and the pellet. Approximately 30 μg of protein/oocyte was recovered in the crude homogenate. Receptor expression levels ranged from 16 to 968 fmol/mg of protein, averaging 480 fmol/mg of protein (16 fmol/oocyte). We also examined a more purified membrane preparation. A crude homogenate of $\alpha4\beta2$-expressing oocytes, prepared as described above, was centrifuged at 4° at 45,000 × *g* for 20 min. The supernatant was discarded, and the pellet was resuspended in buffer (see below). We found no difference in affinity for cytisine between the crude and more purified membrane preparations (data not shown). However, approximately half the specific binding was lost in the more purified preparation; therefore, the crude membrane preparation was better suited for our needs.

[^{3}H]Cytisine binding. The oocyte homogenate was prepared in 50 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$, pH 7.0, using a modification of the assay of Pabreza *et al*. (1991). Assay volume was 0.5 ml. Assays were initiated by the addition of membrane homogenate and were incubated on ice for 90 min with gentle shaking. For saturation analysis, the concentrations of [^{3}H]cytisine ranged from 30 pM to 7.0 nM. Nonspecific binding was determined using 1 μM cytisine. For competition studies, 1.5 nM [^{3}H]cytisine was used. For reactions involving ACh, the homogenate was preincubated for 30 min with 200 nM diisopropylfluorophosphate, a cholinesterase inhibitor, before the addition of ligands. The reactions were stopped by filtration onto glass-fiber filters (934-AH; Whatman, Clifton, NJ), and the filters were counted with a Beckman Instruments (Fullerton, CA) LS 1801 scintillation counter. Nonspecific binding was 10–20% of the total binding at [^{3}H]cytisine concentrations near the $K_{d\mathrm{app}}$ and did not exceed 41% at the highest radioligand concentrations.

[^{3}H]Epibatidine binding. The oocyte homogenate was prepared in buffer containing 140 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM HEPES, pH 7.5. Our protocol is a modification of the methods of Houghtling *et al*. (1994) and Marks *et al*. (1998). To avoid problems with ligand depletion during saturation experiments, the reaction volumes varied at different epibatidine concentrations. Final reaction volumes of 0.5 ml were used for epibatidine concentrations between 2 nM and 5 nM, 1-ml volumes were used for concentrations between 500 pM and 1 nM, 2-ml volumes were used for epibatidine concentrations between 15 pM and 250 pM, and 5-ml volumes were used for concentrations below 15 pM epibatidine. In competition studies, 100 pM [^{3}H]epibatidine was used for all $\beta2$-containing receptors, whereas 500 pM [^{3}H]epibatidine was used for all $\beta4$-containing receptors. Reaction volumes of 0.5 ml were suffi-

cient to avoid ligand depletion in the competition studies for the concentrations of [^{3}H]epibatidine and competitors used. Both competition and saturation experiments contained ≤25 fmol of receptor/reaction tube. Reactions involving ACh were treated as described above. Reactions were initiated by the addition of oocyte homogenate and were incubated at 25° in a shaking water bath.

Preliminary time course experiments were performed before saturation and competition analyses to determine the time required for each receptor subunit combination to reach equilibrium with [^{3}H]epibatidine. $K_{d\text{app}}$ values were estimated for each subunit combination in preliminary saturation experiments. One fifth to one half of this concentration then was used in the time course experiments. The reactions were stopped by filtration at 15-min intervals over 4 hr. The half-times to equilibrium from these data ranged from 5 to 36 min. To be confident of reaching equilibrium, we used an incubation time that exceeds five times the longest half-time; thus, all reactions were incubated for 3.5–4.0 hr.

Reactions were stopped by filtration and counted as described above. Nonspecific binding was determined in parallel reactions containing 1 mM nicotine. Nonspecific binding was 10–15% of total binding at [^{3}H]epibatidine concentrations near the $K_{d\text{app}}$ and did not exceed 45% at the highest radioligand concentration.

Calculations. Due to the complexities of agonist interactions with receptors (reflected in Hill coefficients that deviate from 1.0; see Discussion), K_d and K_i values should be considered empirical descriptions of the data and not true equilibrium dissociation constants. For this reason, we refer to these values as $K_{d\text{app}}$ (apparent K_d) and $K_{i\text{app}}$ (apparent K_i).

Data from saturation experiments were analyzed using the equation $B = (B_{\max} * L^n)/(K_{d\text{app}}{}^n + L^n)$, where B is the binding at free ligand concentration, L; $B_{\max}$ is the maximal specific binding; $K_{d\text{app}}$ is the apparent equilibrium dissociation constant; and n is the Hill coefficient. Values for $B_{\max}$, $K_{d\text{app}}$, and n were calculated by nonlinear regression with Prism 2.0 (GraphPAD, San Diego, CA). Scatchard plots were generated using the Rosenthal method for linearizing binding data outlined in the Prism 2.0 manual. IC_{50} values were derived using the equation $B = B_o/[1 + (I/IC_{50})^n]$, where B is ligand bound at competitor concentration, I; B_o is binding in the absence of competitor; IC_{50} is the concentration of ligand that reduces the specific binding by one half; and n is the Hill coefficient. $K_{i\text{app}}$ values were calculated using the equation $K_{i\text{app}} = IC_{50}/[1 + ([L]/K_{d\text{app}})]$. Because of the variation in receptor expression level from day to day after injection of the oocytes and among oocyte batches, all results were normalized as the percentage of maximal specific binding.

Results

Rat α4β2 nAChRs expressed in *X. laevis* oocytes display pharmacological properties similar to those of the high affinity cytisine binding site in rat brain. Our intention to characterize the agonist binding affinities of various neuronal nAChR subunit combinations on expression in *X. laevis* oocytes raises a critical question. Are the pharmacological properties of neuronal nAChRs expressed in *X. laevis* oocytes an accurate reflection of the pharmacological properties of neuronal nAChRs expressed by neurons? We previously addressed this issue for muscle-type nAChR and found a close correspondence between the pharmacological properties of the mouse muscle α1β1γδ nAChRs expressed in oocytes (Luetje and Patrick, 1991) and those of the nAChR expressed by the mouse muscle cell line, BC3H-1 (Sine and Steinbach, 1986, 1987). An opportunity to compare directly the properties of a neuronal nAChR (α4β2) expressed in both oocytes and brain is presented by the detailed pharmacological analysis of the high affinity cytisine binding site in rat brain (Pabreza *et al.*, 1991) and the subsequent identification of this site as α4β2 (Flores *et al.*, 1992). To make this comparison, we expressed rat α4β2 neuronal nAChRs in *X. laevis* oocytes and performed saturation analysis using a modification of the [^{3}H]cytisine binding assay of Pabreza *et al.* (1991) (see Experimental Procedures). The α4β2 receptor bound [^{3}H]cytisine with a $K_{d\text{app}}$ value of 0.74 ± 0.14 nM (Fig. 1). This is very similar to the value of 0.9 ± 0.1 nM obtained by Pabreza *et al.* (1991) for α4β2 in rat brain. To extend our characterization of the rat α4β2 nAChR expressed in oocytes, we performed competition studies with cytisine and four additional ligands that compete for [^{3}H]cytisine binding (Fig. 2). The ligands tested included three agonists (nicotine, ACh, and carbachol) and one competitive antagonist (curare). The rank order of IC_{50} values obtained (cytisine < nicotine < ACh < carbachol < curare) was identical to the rank order reported by Pabreza *et al.* (1991). In Table 1, we compare $K_{d\text{app}}$ and $K_{i\text{app}}$ values calculated from data presented in Figs. 1 and 2 (as described in Experimental Procedures), with values calculated from data presented in Pabreza *et al.* (1991). Only minor differences were observed between the $K_{i\text{app}}$ values obtained for α4β2 expressed in oocytes and α4β2 expressed in brain. For cytisine, nicotine, and carbachol, the differences were ~2-fold. The curare and ACh values differed by ~4- and ~6-fold, respectively.

The identical rank orders and similar $K_{i\text{app}}$ values led us to conclude that rat neuronal α4β2 nAChRs expressed in *X. laevis* oocytes display pharmacological properties similar to what α4β2 nAChRs display in neurons. It seems likely that other neuronal nAChRs expressed in oocytes also will display appropriate pharmacological properties.

[^{3}H]Epibatidine allows radioligand binding analysis of six different neuronal nAChR subunit combinations. We used [^{3}H]epibatidine to examine the pharmacology of additional neuronal nAChR subunit combinations. Epibatidine has been reported to have exceptionally high affinity for multiple neuronal nAChRs in the nervous system (Qian *et al.*, 1993; Badio and Daly, 1994; Houghtling *et al.*, 1995; Flores *et al.*, 1996; Khan *et al.*, 1997). We adapted the

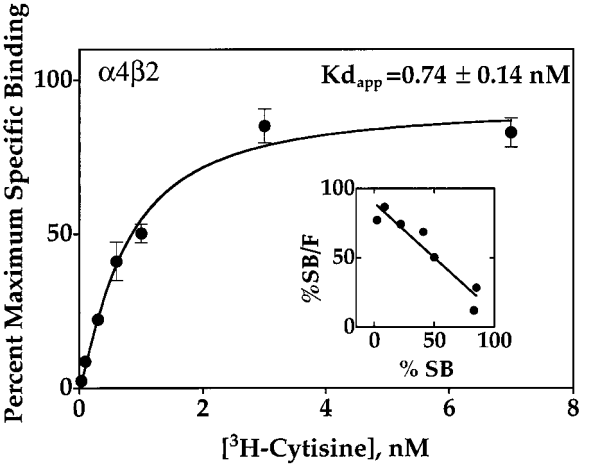

Fig. 1. Saturation of specific [^{3}H]cytisine binding to homogenates of *X. laevis* oocytes expressing α4β2. *Inset*, Scatchard analysis of specific binding of [^{3}H]cytisine. Homogenates were incubated with [^{3}H]cytisine (30 pM to 7.0 nM) for 90 min on ice. Nonspecific binding was determined in the presence of 1.0 μM cytisine. Data are the mean ± standard error of six different experiments, each performed in triplicate. Data were fit as described in Experimental Procedures.

[^{3}H]epibatidine binding assay originally developed for brain membrane preparations (Houghtling *et al.*, 1995; Marks *et al.*, 1998) for use with oocyte homogenates. This allowed determination of the epibatidine binding affinity of the six possible receptors formed by α/β combinations of $\alpha2$, $\alpha3$, $\alpha4$, $\beta2$, and $\beta4$. Two characteristics of epibatidine binding complicate the experiments. First, the exceptionally high affinity of epibatidine for some receptors requires that precautions be taken to avoid ligand depletion in the assay (see Experimental Procedures). Second, epibatidine binding displays relatively slow kinetics compared with other ligands such as cytisine and nicotine (Houghtling *et al.*, 1995). Thus, longer incubation periods are needed to reach equilibrium. To decrease the time to equilibrium, we conducted all experiments at 25°. Incubation times (3.5–4 hr) were chosen to exceed five times the half-time to equilibrium for each receptor (see Experimental Procedures).

Once the parameters for the binding assay were established, we performed saturation analysis on the six different neuronal nAChR subunit combinations (Fig. 3). The $K_{d\mathrm{app}}$ values for [^{3}H]epibatidine binding ranged from 10 pM for $\alpha2\beta2$ to 303 pM for $\alpha3\beta4$. The $\beta2$-containing receptors had consistently higher affinities for epibatidine than did $\beta4$-containing receptors. For $\alpha2$, $\alpha3$, and $\alpha4$, the difference in affinity between the $\beta2$ and $\beta4$ context was 8-, 22-, and 3-fold, respectively. Differences were also observed for the binding affinities among the α subunits, but these differences were not consistent between the different β subunit contexts. For example, the $\alpha3\beta2$ receptor had a higher affinity than the $\alpha4\beta2$ receptor (14 versus 30 pM), whereas the $\alpha4\beta4$ receptor had a higher affinity than the $\alpha3\beta4$ receptor (85 versus 303 pM).

The β subunits confer large differences in agonist binding affinity. We conducted a series of competition binding experiments for each receptor subtype using the agonists ACh, anabasine, cytisine, DMPP, lobeline, and nicotine. The results of the competition analyses are shown in Fig. 4, and the calculated $K_{i\mathrm{app}}$ values derived from these results are shown in Table 2. We found that the trend in $K_{i\mathrm{app}}$ values for these agonists was similar to what we observed in saturation analysis with epibatidine; that is, for each α subunit, the $\beta2$-containing receptors had consistently higher affinities for all agonists than did $\beta4$-containing receptors. In fact, only in the case of the cytisine affinity for $\alpha3\beta2$ was the affinity of any agonist for any $\beta2$-containing receptor lower than the affinity for any $\beta4$-containing receptor (compare $\alpha3\beta2$ with $\alpha2\beta4$ and $\alpha4\beta4$).

The competition binding experiments revealed much larger differences in agonist affinity than did the epibatidine saturation experiments. The largest differences were observed between receptors containing either the $\beta2$ or $\beta4$ subunit coexpressed with the same α subunit. Particularly striking are the differences in affinity of $\alpha2\beta2$ and $\alpha2\beta4$ for nicotine (86-fold), lobeline (85-fold), and DMPP (120-fold). Interestingly, the magnitude of the difference observed between $\beta2$- and $\beta4$-containing receptors was dependent on the α subunit. Differences were generally largest for $\alpha2$-containing receptors and smallest for $\alpha4$-containing receptors. For example, the $\alpha2\beta2$ and $\alpha2\beta4$ receptors differ in ACh affinity by 61-fold, whereas $\alpha3\beta2$ and $\alpha3\beta4$ differ by 19-fold, and $\alpha4\beta2$ and $\alpha4\beta4$ differ by only 2-fold. Affinity differences due to the identity of the α subunit also were observed, although these differences were smaller than those ascribed to β subunits. The largest differences were seen with cytisine affinity. The $\alpha3\beta2$ receptor had a 37-fold lower affinity for cytisine than $\alpha2\beta2$ and a 14-fold lower affinity for cytisine than $\alpha4\beta2$. The $\alpha3\beta4$ receptor had a 47-fold lower affinity for cytisine than $\alpha4\beta4$ and an 11-fold lower affinity for cytisine than $\alpha2\beta4$. The $\alpha2\beta2$ receptor had a higher affinity than the $\alpha3\beta2$ receptor for nicotine (20-fold) and ACh (16-fold). The affinity of $\alpha2\beta2$ for ACh was also 19-fold higher than that of $\alpha4\beta2$. All other differences in agonist affinity due to α subunits were $<$10-fold. Also dependent on subunit combination was the range of affinities for the agonists (excluding epibatidine). At the extremes were $\alpha3\beta2$, for which the six agonist affinities were within 4-fold of each other, and $\alpha4\beta4$, for which the affinities were spread across a 833-fold range (Fig. 4, Table 2).

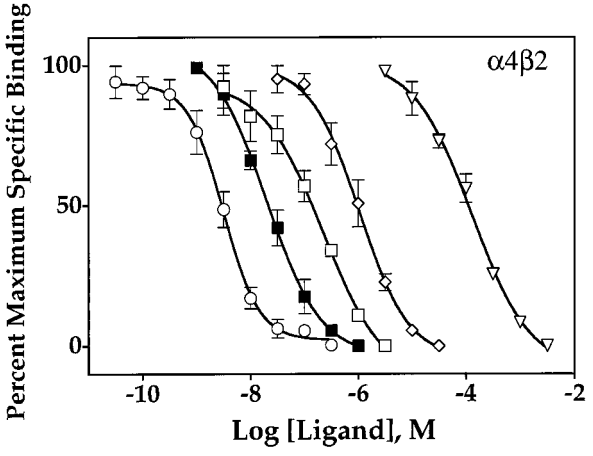

Fig. 2. Competition for [^{3}H]cytisine binding sites by the nicotinic agonists ACh (□), cytisine (○), carbachol (◇), and nicotine (■) and the competitive antagonist curare (▽). Homogenates of oocytes expressing $\alpha4\beta2$ receptors were incubated with 1.5 nM [^{3}H]cytisine for 90 min on ice in the presence of various concentrations of competitor. Data are the mean ± standard error of three experiments, each performed in triplicate. Data were fit as described in Experimental Procedures.

TABLE 1

Binding affinities of $\alpha4\beta2$ receptors expressed in oocytes are similar to the binding affinities of $\alpha4\beta2$ expressed in rat brain

The $K_{i\mathrm{app}}$ value for [^{3}H]cytisine binding to $\alpha4\beta2$ expressed in oocytes was taken from the fit data in Fig. 1, whereas the value for rat brain is from Pabreza *et al.* (1991). $K_{i\mathrm{app}}$ values for $\alpha4\beta2$ expressed in oocytes were calculated from the IC_{50} values taken from the fit data in Fig. 2, whereas the values for rat brain were calculated from IC_{50} values presented in Pabreza *et al.* (1991) (see Experimental Procedures).

	Ligand	$\alpha4\beta2$ in oocytes	Rat brain[a]
$K_{d\mathrm{app}}$ (n_H)	[^{3}H]cytisine	0.74 ± 0.14nM (0.96 ± 0.09)	0.9 ± 0.1 nM (0.96 ± 0.1)
$K_{i\mathrm{app}}$ (n_H)	Cytisine	1.03 ± 0.06nM (1.3 ± 0.09)	0.45 ± 0.15nM (0.9 ± 0.1)
	Nicotine	6.52 ± 0.54nM (0.86 ± 0.07)	3.3 ± 0.96nM (0.9 ± 0.1)
	Acetylcholine	73.5 ± 15.4nM (0.77 ± 0.13)	12.3 ± 4.0 nM (0.9 ± 0.1)
	Carbachol	351 ± 49 nM (0.95 ± 0.14)	225.0 ± 33 nM (0.9 ± 0.1)
	Curare	43.0 ± 7.9 μM (0.81 ± 0.14)	10.5 ± 0.8 μM (NA)[b]

[a] Pabreza *et al.* (1991).
[b] NA, not available.

Discussion

Our characterization of six different neuronal nicotinic subunit combinations ($\alpha2\beta2$, $\alpha2\beta4$, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, $\alpha4\beta4$) using radioligand binding analysis demonstrates that binding affinities for a variety of agonists are dependent on both the α and β subunit present in the receptor. The largest differences occurred as a consequence of changing the β subunit, but differences also were seen when the α subunit was changed. These results emphasize the potential for the formation of multiple, pharmacologically distinct nAChR subtypes in the nervous system. In fact, recent work using epibatidine, cytisine, and nicotine as radioligands and competitors has demonstrated the presence of multiple nAChR subtypes (Flores *et al.*, 1996; Marks *et al.*, 1998; Zoli *et al.*, 1998).

Expression of mammalian nAChRs in the *X. laevis* oocytes raises concern as to whether the pharmacological properties that we identify and characterize are an accurate reflection of the properties that these receptors would display in their native context. In earlier work, the agonist pharmacology of mouse muscle $\alpha1\beta1\gamma\delta$ expressed in oocytes and assayed electrophysiologically (Luetje and Patrick, 1991) was found to be quite similar to the agonist pharmacology of the same receptor natively expressed by BC3H-1 cells (Sine and Steinbach,

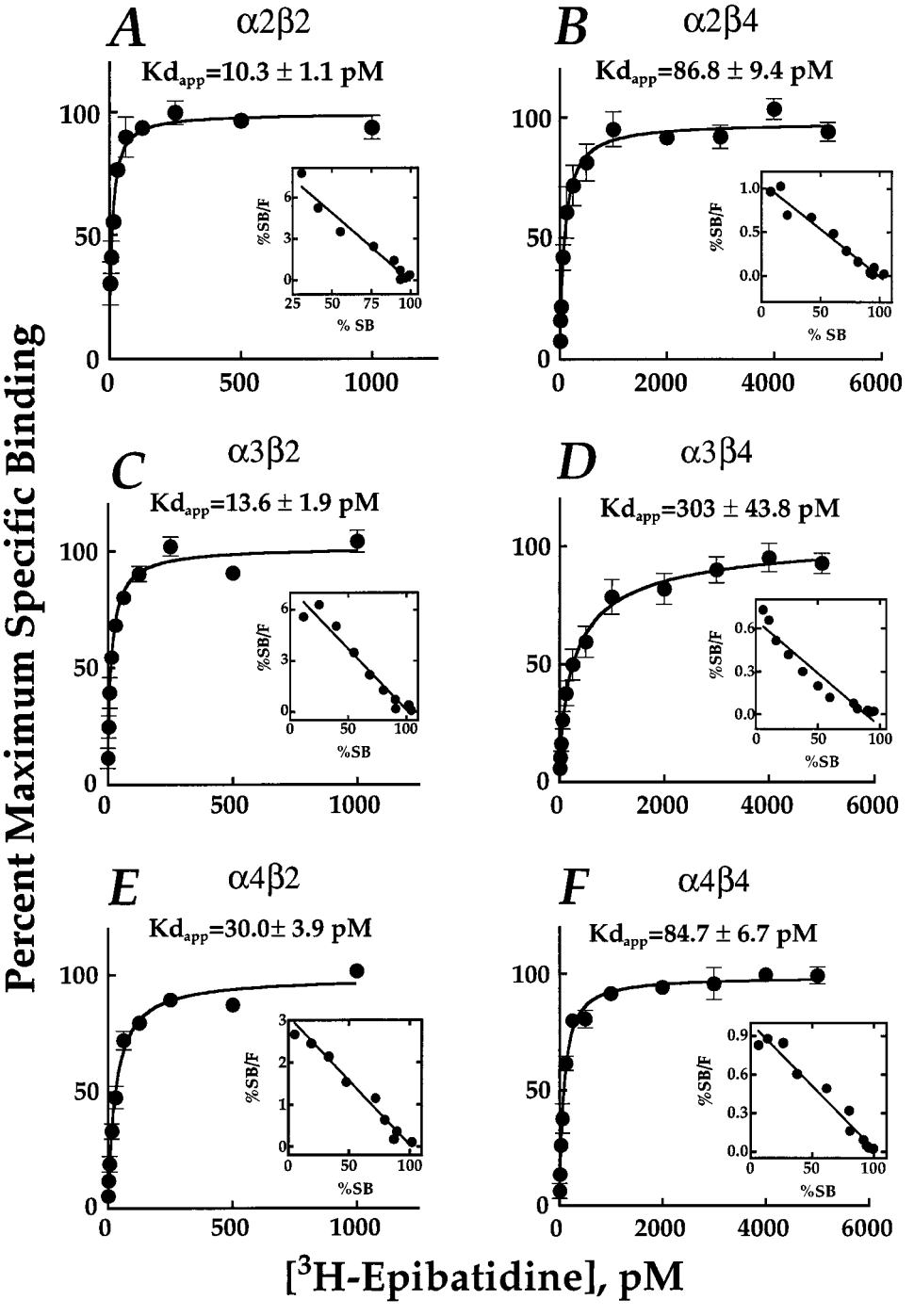

Fig. 3. Saturation of specific [^{3}H]epibatidine binding to homogenates of *X. laevis* oocytes expressing six different neuronal nAChRs. *Insets*, Scatchard analyses of specific binding of [^{3}H]epibatidine. Homogenates of oocytes expressing nAChRs were incubated with [^{3}H]epibatidine (1.95 pM to 5 nM) for ≥3.5 hr at 25°. Nonspecific binding was determined in the presence of 1 mM nicotine. Data are the mean ± standard error of three to six experiments each performed in triplicate. Data were fit as described in Experimental Procedures.

1986, 1987). We now demonstrate that rat neuronal nicotinic $\alpha4\beta2$ receptors expressed in oocytes display agonist and antagonist binding affinities similar to the native $\alpha4\beta2$ receptor in rat brain (Table 1). We also find a close correspondence between the agonist binding affinities of the rat $\alpha3\beta4$ receptor expressed in oocytes (Table 2) and the same receptor expressed in human embryonic kidney 293 cells (Xiao *et al*., 1998). More importantly, the [^{3}H]epibatidine affinity of $\alpha3\beta4$ expressed in oocytes is quite similar to the affinity of $\alpha3\beta4$ expressed in rat trigeminal ganglion (Flores *et al*., 1996). We conclude that the pharmacological properties of mammalian neuronal nAChRs expressed in *X. laevis* oocytes are an accurate reflection of the pharmacological properties of nAChRs natively expressed in the nervous system.

In our assay conditions, both surface and intracellular nAChRs may be detected. Although surface receptors are

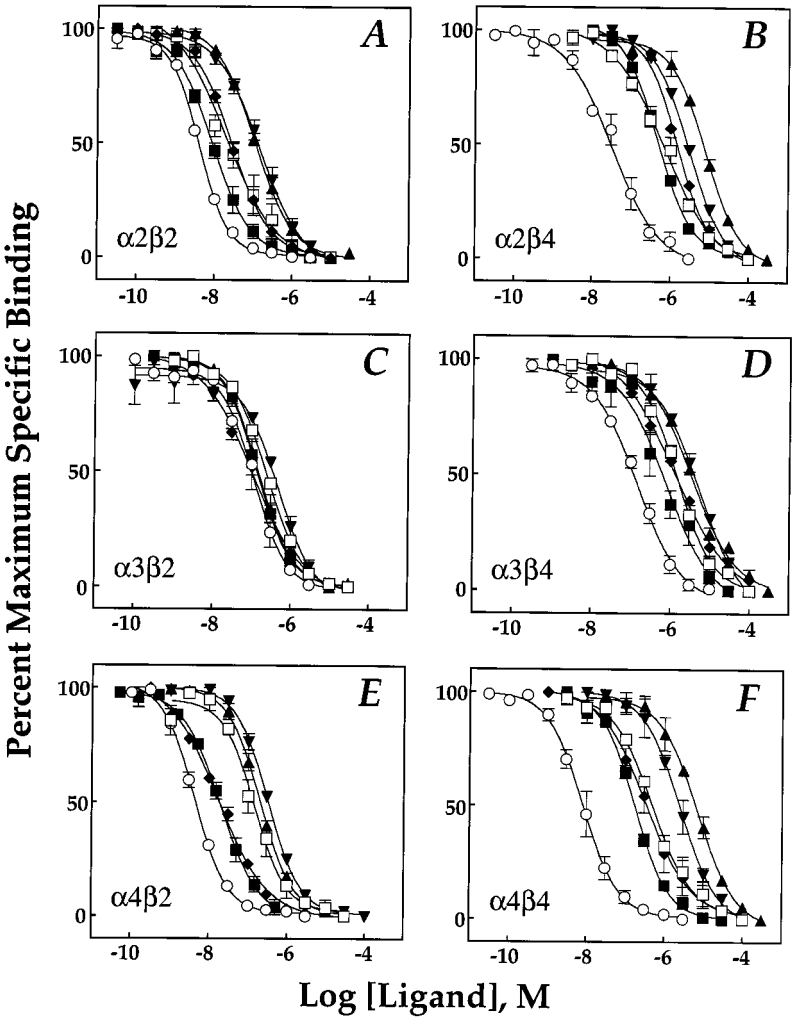

Fig. 4. Competition for [^{3}H]epibatidine binding by the nicotinic agonists ACh (□), anabasine (▼), cytisine (○), DMPP (▲), lobeline (♦), and nicotine (■). Homogenates of oocytes expressing nAChRs were incubated with 100 pM [^{3}H]epibatidine (for $\beta2$-containing receptors) or 500 pM [^{3}H]epibatidine (for $\beta4$-containing receptors) in the presence of various concentrations of competitor for ≥3.5 hr at 25°. Data are the mean ± standard error of two or three experiments, each performed in sextuplicate. Data were fit as described in Experimental Procedures.

TABLE 2

Agonist binding affinities of neuronal nAChRs

K_{dapp} values for [^{3}H]epibatidine were taken from the fit data in Fig. 3. K_{iapp} values for acetylcholine, anabasine, cytisine, DMPP, lobeline, and nicotine were calculated from the IC_{50} values taken from the fit data in Fig. 4 (see Experimental Procedures).

	Ligand	α2β2	α2β4	α3β2	α3β4	α4β2	α4β4
K_{dapp} (pM) (n_H)	[^{3}H]Epibatidine	10.3 ± 1.1 (1.13 ± 0.20)	86.8 ± 9.4 (1.17 ± 0.08)	13.6 ± 1.9 (1.21 ± 0.10)	303.0 ± 44 (1.18 ± 0.04)	30.0 ± 3.9 (1.07 ± 0.06)	84.7 ± 6.7 (0.91 ± 0.06)
K_{iapp} (nM) (n_H)	Acetylcholine	1.8 ± 0.51 (0.67 ± 0.13)	110 ± 20 (0.64 ± 0.08)	29.0 ± 1.9 (0.92 ± 0.05)	560 ± 72 (0.96 ± 0.11)	34 ± 6.6 (1.1 ± 0.21)	72 ± 9.2 (0.85 ± 0.09)
	Anabasine	14 ± 1.1 (0.79 ± 0.05)	450 ± 32 (0.96 ± 0.06)	57 ± 14 (0.92 ± 0.17)	2100 ± 480 (0.79 ± 0.12)	76 ± 3.6 (1.04 ± 0.05)	370 ± 22 (0.90 ± 0.05)
	Cytisine	0.38 ± .02 (1.2 ± 0.08)	4.9 ± 1.1 (0.71 ± 0.11)	14 ± 1.6 (1.04 ± 0.11)	56 ± 7.3 (0.77 ± 0.07)	0.99 ± 0.07 (1.1 ± 0.07)	1.2 ± 0.08 (.96 ± 0.06)
	DMPP	10 ± 0.58 (0.87 ± 0.04)	1200 ± 130 (0.99 ± 0.09)	18 ± 1.0 (0.98 ± 0.05)	1300 ± 210 (0.78 ± 0.10)	48 ± 1.8 (1.03 ± 0.04)	1000 ± 75 (0.90 ± 0.05)
	Lobeline	2.6 ± 0.24 (0.88 ± 0.07)	220 ± 33 (1.1 ± 0.17)	13 ± 1.3 (0.68 ± 0.05)	480 ± 56 (0.60 ± 0.05)	4.0 ± 0.63 (0.68 ± 0.06)	49 ± 5.3 (0.75 ± 0.06)
	Nicotine	0.81 ± .08 (0.88 ± 0.07)	70 ± 4.2 (0.98 ± 0.06)	16 ± 0.52 (1.0 ± 0.03)	300 ± 110 (0.74 ± 0.18)	4.6 ± 0.33 (0.87 ± 0.05)	26 ± 2.4 (1.0 ± 0.09)

likely to consist solely of fully assembled pentamers, the intracellular receptor population consists of fully assembled pentamers, as well as various assembly intermediates. Intracellular pentamers might be expected to have the same properties as surface pentamers. However, the pharmacological properties of assembly intermediates could differ from those of fully assembled pentamers and might affect our results. Although little is known about the assembly of neuronal nAChRs, the assembly of muscle nAChRs has been more extensively studied (Blount and Merlie, 1989). If neuronal nAChR assembly is analogous to muscle nAChR assembly, then we could expect pairs of α and β subunits to form functional binding sites before pentamer assembly. Pairs of muscle nAChR subunits ($\alpha\gamma$, $\alpha\epsilon$, $\alpha\delta$) form binding sites capable of binding agonists, but they seem to be unable to undergo transition to the high affinity desensitized state (Prince and Sine, 1996). The agonist affinity of these binding site pairs seems to resemble that of closed activatable receptors (Prince and Sine, 1998). If this is also true for neuronal nAChR $\alpha\beta$ pairs, then given the ~3 orders of magnitude difference in affinity between the closed activatable and desensitized states (see below), binding to pairs of subunits is unlikely to be a factor in our assays. This is consistent with our observation that the Hill coefficient derived from fitting [^{3}H]epibatidine saturation binding data is near 1.0 for each receptor tested (Fig. 3 and Table 2).

Neuronal nAChRs, like muscle nAChRs, undergo transitions between closed activatable, open, and desensitized states. Each of these states can have a different affinity for ligand. The desensitized state, in particular, has an exceptionally high affinity for agonists. However, the binding affinity that we measure under equilibrium conditions can not be considered a pure measure of the affinity of any single state. This is because the receptors are in equilibrium among these various states and the apparent binding affinity we measure depends on the affinities of the individual states, as well as the equilibrium constants for transitions among the states. The EC_{50} value for activation in a functional assay can be taken as a crude estimate of the agonist affinity of the closed activatable state. Rat neuronal nAChRs expressed in oocytes display EC_{50} values for ACh activation ranging from 55 to 210 μM (Harvey *et al.*, 1996), suggesting that the closed activatable state of each rat neuronal nAChR has an affinity for ACh 2–4 orders of magnitude lower than the affinity of the desensitized state. It is also interesting to note that EC_{50} values for ACh activation of the various neuronal nAChR subunit combinations differ by <4-fold, whereas the equilibrium ACh binding affinities of these receptors differ by >300-fold (Table 2). The rank order of ACh affinities of the closed activatable states ($\alpha4\beta4 > \alpha3\beta2 > \alpha2\beta2 > \alpha2\beta4 > \alpha4\beta2 > \alpha3\beta4$) and desensitized states ($\alpha2\beta2 > \alpha3\beta2 > \alpha4\beta2 > \alpha4\beta4 > \alpha2\beta4 > \alpha3\beta4$) also are markedly different. The affinity of epibatidine for the closed activatable state (as crudely estimated from the EC_{50} value for activation) and the desensitized state (as estimated from the $K_{d\mathrm{app}}$ value for equilibrium binding) of several neuronal nAChR subunit combinations also has been observed to differ by ~3 orders of magnitude (Gerzanich *et al.*, 1995; Gopalakrishnan *et al.*, 1996). Thus, in our assay, the concentration of agonist generally is too low for a significant amount of the binding to be to the closed activatable state. This fact, combined with the transience of the open state, suggests that the binding affinity we derive from our equilibrium binding experiments is dominated by the affinity of the desensitized receptor. Consistent with this conclusion is our observation of Hill coefficients at or near 1.0 for binding of epibatidine and most agonists (suggesting binding to a single class of sites). It should be noted, though, that in several cases (e.g., ACh binding to α2-containing receptors and lobeline binding to α3- and α4-containing receptors), the Hill coefficients are substantially <1.0, suggesting negatively cooperative interactions between binding sites or heterogeneity among binding sites. However, this is unlikely to explain our observations of differences in affinity among receptors because there is no correlation between deviation of the Hill coefficient from 1.0 and the observed affinity.

Different rat neuronal nAChR subunit combinations expressed in oocytes have been shown to differ in their susceptibility to desensitization (Vibat *et al.*, 1995; Fenster *et al.*, 1997). Could differences in desensitization rates rather than differences in affinities underlie our results? Arguing against this possibility is the observation that neither the rank order of decay time constants for nicotine-induced desensitization of $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$ nor the rank order of extent of desensitization after repeated exposure to nicotine (Vibat *et al.*, 1995) correlates with the rank order of nicotine affinities we observe for these subunit combinations. In addition, Fenster *et al.* (1997) found that the desensitization rate of various subunit combinations is not a good predictor of the affinity of the desensitized state.

Our use of a [^{3}H]epibatidine binding assay to characterize nAChRs expressed in *X. laevis* oocytes has allowed detailed analysis of six different neuronal nAChR subunit combinations. The results of these analyses reveal that the large differences in agonist affinity among different neuronal nAChR subunit combinations are primarily determined by the nature of the β subunit. The pharmacological characteristics defined in this study will be useful in classifying different neuronal nAChR subtypes in the nervous system. The radioligand binding assay developed here also will be useful, in conjunction with chimeric and mutant receptor subunit constructs, to identify structural features of neuronal nAChRs responsible for differences in agonist affinity.

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