Effects of Pyridine Ring Substitutions on Affinity, Efficacy, and Subtype Selectivity of Neuronal Nicotinic Receptor Agonist Epibatidine

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ABSTRACT

2-\textsuperscript{-}Pyridine ring substituted analogs of epibatidine were assessed for equilibrium binding affinity, functional potency, and efficacy at rat neuronal nicotinic receptors expressed in \textit{Xenopus} oocytes. Binding affinities were determined in membrane homogenates from oocytes expressing \(\alpha2\beta2\), \(\alpha2\beta4\), \(\alpha3\beta2\), \(\alpha3\beta4\), \(\alpha4\beta2\), or \(\alpha4\beta4\). Efficacy (relative to acetylcholine) and potency were measured electrophysiologically with oocytes expressing \(\alpha3\beta4\), \(\alpha4\beta2\), and \(\alpha4\beta4\). Hydroxy, dimethylamino, and trifluoromethanesulfonate analogs had affinities too low for accurate measurement. The bromo analog had affinities 4- to 55-fold greater at \(\beta2\) than at \(\beta4\)-containing receptors, modestly greater efficacy at \(\alpha4\beta4\) than at \(\alpha4\beta2\), and 5- to 10-fold greater potency at \(\alpha4\beta4\) than at \(\alpha3\beta4\) or \(\alpha4\beta2\). The fluoro analog displayed affinities 52- to 875-fold greater at \(\beta2\)- than at \(\beta4\)-containing receptors, efficacy at \(\alpha4\beta4\) receptors 3-fold greater than at \(\alpha4\beta2\) and \(\alpha3\beta4\), and was equipotent at all receptors tested. The norchloro analog showed affinities 114- to 3500-fold greater at \(\beta2\)- than at \(\beta4\)-containing receptors, 2-fold greater efficacy at \(\alpha4\beta2\) and \(\alpha4\beta4\) than at \(\alpha3\beta4\), and 4- to 5-fold greater potency at \(\alpha4\beta4\) and \(\alpha3\beta4\) than at \(\alpha4\beta2\). The amino analog displayed affinities 10- to 115-fold greater at \(\beta2\)- than at \(\beta4\)-containing receptors, 3-fold greater efficacy at \(\alpha3\beta4\) than at \(\alpha4\beta2\), and 2- to 4-fold greater potency at \(\alpha3\beta4\) and \(\alpha4\beta4\) than at \(\alpha4\beta2\). Although these compounds displayed a variety of differences in affinity, efficacy, and potency, with one exception (binding affinity and functional potency at \(\alpha4\beta4\) receptors) there were no significant correlations among these properties.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels assembled as pentamers of \(\alpha\) and \(\beta\) subunits, forming a variety of different receptor subtypes. There are nine known \(\alpha\) subunits (\(\alpha2\)-\(\alpha10\)) and three known \(\beta\) subunits (\(\beta2\)-\(\beta4\)) for neuronal nAChRs (Corringer et al., 2000; Elgoyhen et al., 2001). These subunits assemble in a variety of homomeric and heteromeric combinations to form receptors with different biophysical and pharmacological properties and with distinct distributions in the central and peripheral nervous systems (Corringer et al., 2000). A predominant CNS nAChR is the \(\alpha4\beta2\) subtype, whereas a predominant ganglionic nAChR is the \(\alpha3\beta4\) subtype (Lucas et al., 1999; Xu et al., 1999; Picciotto et al., 2000; Quik et al., 2000). Neuronal nAChR ligands with potential subtype selectivity are under investigation as analgesics (Qian et al., 1993; Rogers and Iwamoto, 1993; Badio and Daly, 1994), anxiolytics (Decker et al., 1995; Brioni et al., 1997), and as therapeutics for CNS disorders, including Alzheimer’s Disease, Parkinson’s Disease, and schizophrenia (Qian et al., 1993; Rogers and Iwamoto, 1993; Vidal, 1996; Brioni et al., 1997; Hellstrom-Lindahl et al., 1999).

Epibatidine is a potent agonist at neuronal nAChRs (Badio and Daly, 1994; Gerzanich et al., 1995; Akondon et al., 1998). Epibatidine is reported to exhibit high-potency analgesic activity with a longer duration of action than nicotine (Qian et al., 1993; Rogers and Iwamoto, 1993; Badio and Daly, 1994), and epibatidine analogs are currently under investigation as nonopioid analgesics. However, epibatidine is reported to produce various toxicities in rodents, including increased heart rate, motor incoordination, and seizure (Sulivan et al., 1994; Bonhaus et al., 1995; Horti et al., 1998). The toxicity of epibatidine may arise from its capacity to activate many different neuronal nAChR subtypes. Separ-
tion of the analgesic effects from the toxicities may be possible if subtype-selective analogs of epibatidine can be developed.

In this study, we examine the effects of modifying the epibatidine molecule on equilibrium binding affinity, efficacy, and functional potency at simple heteromeric neuronal nAChRs. In particular, we focus on the effect that 2′-pyridine ring substitutions have on selectivity for subunit combinations (α4β2 and α3β4) representative of neuronal nAChRs found in the central and peripheral nervous systems, respectively.

Materials and Methods

Materials. Xenopus laevis frogs were purchased from Nasco ( Ft. Atkinson, WI). Care and use of Xenopus frogs in this study have been approved by the University of Miami Animal Research Committee and meets the guidelines of the National Institutes of Health. RNA transcription kits were from Ambion (Austin, TX). [3H]Epibatidine was from PerkinElmer Life Sciences (Boston, MA). Acetylcholine, (+)-epibatidine, gentamicin, HEPES, polyethyleneimine, and 3-amino-nobenoic acid ethyl ester were from Sigma-Aldrich (St. Louis, MO). Collagenase B was from Roche Molecular Biochemicals (Indianapolis, IN). 934-AH glass microfiber filters were from Whatman (Clifton NJ).

Epibatidine Analogs. Norchloro-epibatidine (NEP), fluoro-norchloro-epibatidine (FNEP), bromo-norchloro-epibatidine (BNEP), amino-norchloro-epibatidine (ANNEP), hydroxy-norchloro-epibatidine (HOEPEP), dimethylamino-norchloro-epibatidine (DMNEP), and trifluoromethanesulfonate-norchloro-epibatidine (NTEP) were synthesized as described previously (Carroll et al., 2001). All analogs are racemic. Structures are shown in Fig. 1.

Expression of Neuronal nAChRs in Xenopus Oocytes. cDNA clones encoding rat α2, α3, α4, β2, and β4 subunits in the pGEMHE high-expression vector (Liman et al., 1992) were used for cRNA transcription. mG(5′)ppp(5′)G-capped cRNA was synthesized in vitro from linearized template cDNA using an mMessage mMACHINE kit (Ambion). Mature X. laevis frogs were anesthetized by submerging in a 10 mM solution of tricaine. Three frogs were used per condition. Oocytes were injected with 8 to 18 ng of cRNA in 23 to 50 nl of water and incubated at 19°C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 100 μg/ml gentamicin, 15 mM HEPES, pH 7.6) for 2 to 10 days. RNA transcripts encoding each subunit were injected into oocytes at a molar ratio of 1:1.

Competition Binding Assays. Crude membrane homogenates were prepared from Xenopus oocytes expressing various neuronal nAChR subunit combinations as described previously (Parker et al., 1998). Briefly, up to 15 oocytes (depending on expression levels) were homogenized per milliliter of buffer containing 140 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, and 25 mM HEPES, pH 7.5, with 0.1 mM phenylmethylsulfonyl fluoride added immediately before the experiment, using a model PT 10/35 homogenizer (Brinkman, Atlanta, GA). Homogenates were centrifuged at 4°C at 2000g for 10 min. The supernatant was removed for use in experiments, avoiding both the surface lipid layer and the pellet. Receptor expression levels averaged 480 fmoi/mg of total protein (16 fmol/oocyte).

Competition studies were performed using a reaction volume of 0.5 ml. The radioligand concentration in all reactions was 500 pM [3H]-epibatidine, and the competing ligand concentration varied from 1 pM to 3 μM. Reactions were initiated by the addition of oocyte homogenate (0.5–5 oocyte equivalents/tube) and were incubated at 25°C in a shaking water bath for 3.5 to 4.0 h. The reactions were stopped by filtration onto glass fiber filters (934-AH; Whatman) pretreated with 0.1% polyethyleneimine using a model M-24 harvester (Brandel Inc., Gaithersburg, MD) and counted on an LS 6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Nonspecific binding was determined in parallel reactions containing 100 nM epibatidine. Nonspecific binding did not exceed 45%. We have observed similar levels of nonspecific binding using 1 mM nicotine and 100 μM dimethylphenylpiperazinum (Parker et al., 1998, 2001). 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 maximum specific binding.

In Table 1 and Fig. 2, Kd values for (+)-epibatidine, obtained by saturation analysis in Parker et al. (1998), are compared with Kd values derived from competition analysis (as described above). To ensure the validity of this comparison, we performed competition analysis for (+)-epibatidine and α4β2 receptors. We obtained a Kd of 42 ± 2 pM (mean ± S.E.M. of three experiments, each in triplicate), similar to the Kd of 30 ± 4 pM obtained in saturation analysis. IC50 values were derived using the equation $B = B_max/(1 + I/IC_{50})$, where B is ligand bound at competitor concentration (I), B_max is the concentration of ligand that reduces the specific binding by one-half, and nH is the Hill coefficient. Kd values were calculated using the Cheng and Prusoff equation $K_d = IC_{50}/(1 + ([I]/K_p))$ (Cheng and Prusoff, 1973). Kd values shown in Table 1 were used in the calculation of Kd.

Electrophysiological Methods. Agonist-induced current responses were measured under two-electrode voltage clamp, using a TEV-200A voltage-clamp unit (Dagan, Minneapolis, MN). Micropipettes were filled with 3 M KCl and were used at resistances of 0.3 to 2.0 MΩ. Current responses were recorded at a holding potential of −40 mV to minimize the contribution of calcium-activated chloride channels. Recordings were sampled at 100 Hz and filtered at 20 Hz (−3 db). Data was acquired, stored, and analyzed on a Macintosh G3 computer using AxDGraph 4.6 software (Axon Instruments, Union City, CA).

Oocytes were continuously perfused in perfusion solution (115 mM NaCl, 1.8 mM CaCl2, 2.5 mM KCl, 0.1 μM atropine, and 10 mM HEPES, pH 7.2) at a rate of 20 μl/min. Agonists were dissolved in 10 mM NaOH and applied for 10 s, with 5-min washes between applications. Dose-response curves were determined for epibatidine and each analog as follows. Acetylcholine was applied before each test perfusion to determine the EC20 for acetylcholine for each receptor subunit combination (α4β2, 20 μM; α3β4, 110 μM; and α4β4, 5 μM). Current responses to epibatidine and epibatidine analogs were normalized to the preceding acetylcholine-induced response, allowing determination of each test drug response relative to the maximum response to acetylcholine at each receptor. Initial experiments with epibatidine determined with and without this normalization resulted in different efficacies (Fig. 3A). All subsequent experiments were normalized as described above.

Dose-response data were fit to the equation $I = I_{max}/(1 + (EC_{50}/X)^n)$, where I is the current response at agonist concentration X, I_{max} is the maximum current, EC_{50} is the agonist concentration
TABLE 1
Equilibrium binding affinities for epibatidine analogs on neuronal nAChRs

\[ K_i \] values for \([^3]H\)epibatidine were taken from Parker et al. (1998). \([K_i]\) values for the epibatidine analogs were calculated from the IC\(_{50}\) values taken from the fit data (see Materials and Methods). Hill coefficients are in parentheses. All values are presented as the mean ± S.E.M. of two to three experiments, each performed in sextuplicate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>(\alpha_2\beta_2)</th>
<th>(\alpha_2\beta_4)</th>
<th>(\alpha_3\beta_2)</th>
<th>(\alpha_3\beta_4)</th>
<th>(\alpha_4\beta_2)</th>
<th>(\alpha_4\beta_4)</th>
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</thead>
<tbody>
<tr>
<td>Epibatidine</td>
<td>10 ± 1</td>
<td>87 ± 9</td>
<td>14 ± 2</td>
<td>300 ± 44</td>
<td>30 ± 4</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>(K_i) pM</td>
<td>(1.1 ± 0.2)</td>
<td>(1.2 ± 0.1)</td>
<td>(1.2 ± 0.1)</td>
<td>(1.2 ± 0.04)</td>
<td>(1.1 ± 0.1)</td>
<td>(0.9 ± 0.1)</td>
</tr>
<tr>
<td>NEP pM</td>
<td>13 ± 1</td>
<td>55 ± 8</td>
<td>4.0 ± 2</td>
<td>220 ± 18</td>
<td>10 ± 1</td>
<td>44 ± 11</td>
</tr>
<tr>
<td>(K_i) pM</td>
<td>(1.0 ± 0.1)</td>
<td>(0.8 ± 0.1)</td>
<td>(1.0 ± 0.03)</td>
<td>(1.0 ± 0.1)</td>
<td>(1.1 ± 0.1)</td>
<td>(1.1 ± 0.3)</td>
</tr>
<tr>
<td>NNEP</td>
<td>2.4 ± 0.6</td>
<td>1200 ± 190</td>
<td>3.8 ± 0.5</td>
<td>2100 ± 210</td>
<td>9.2 ± 1.5</td>
<td>480 ± 160</td>
</tr>
<tr>
<td>(K_i) pM</td>
<td>(0.7 ± 0.1)</td>
<td>(0.9 ± 0.1)</td>
<td>(1.0 ± 0.1)</td>
<td>(0.9 ± 0.1)</td>
<td>(0.9 ± 0.1)</td>
<td>(0.7 ± 0.2)</td>
</tr>
<tr>
<td>NFEP</td>
<td>28 ± 2</td>
<td>3300 ± 550</td>
<td>3.1 ± 0.1</td>
<td>11,000 ± 750</td>
<td>8.5 ± 0.7</td>
<td>3200 ± 600</td>
</tr>
<tr>
<td>(K_i) pM</td>
<td>(1.0 ± 0.1)</td>
<td>(1.0 ± 0.2)</td>
<td>(1.0 ± 0.1)</td>
<td>(1.2 ± 0.1)</td>
<td>(1.1 ± 0.1)</td>
<td>(0.9 ± 0.2)</td>
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<tr>
<td>NTEP</td>
<td>62 ± 14</td>
<td>3500 ± 370</td>
<td>77 ± 12</td>
<td>7200 ± 900</td>
<td>350 ± 24</td>
<td>4300 ± 330</td>
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<tr>
<td>(K_i) pM</td>
<td>(0.8 ± 0.2)</td>
<td>(1.0 ± 0.1)</td>
<td>(0.8 ± 0.1)</td>
<td>(1.1 ± 0.1)</td>
<td>(1.0 ± 0.1)</td>
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<td>NDMNNEP</td>
<td>&gt;100,000</td>
<td>N.D.</td>
<td>&gt;100,000</td>
<td>N.D.</td>
<td>&gt;100,000</td>
<td>N.D.</td>
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<tr>
<td>(K_i) pM</td>
<td>&gt;100,000</td>
<td>N.D.</td>
<td>&gt;100,000</td>
<td>N.D.</td>
<td>&gt;100,000</td>
<td>N.D.</td>
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</table>

N.D., not determined.

Results

Epibatidine Analogs. Seven racemic analogs of epibatidine, with substitutions at the 2′ position of the pyridine ring, were synthesized as described previously (Carroll et al., 2001). The structures of epibatidine and the analogs are shown in Fig. 1.

Competition for \([^3]H\)Epibatidine Binding by Novel Epibatidine Analogs. We determined the equilibrium binding affinities of the epibatidine analogs for several neuronal nAChR subunit combinations (\(\alpha_2\beta_2\), \(\alpha_3\beta_2\), \(\alpha_4\beta_2\), \(\alpha_2\beta_4\), \(\alpha_3\beta_4\), and \(\alpha_4\beta_4\)) by competition for \([^3]H\)epibatidine binding to \(Xenopus\) oocyte homogenates. The calculated \(K_i\) values and Hill coefficients derived from the competition analyses are shown in Table 1. Several analogs (NOHEP, NDMNNEP, and NTEP) had affinities that were too low to accurately measure. Rough estimates of the affinities of these analogs for \(\beta_2\)-containing receptors are provided in Table 1. No estimates of affinity were obtained for \(\beta_4\)-containing receptors. These three epibatidine analogs were not pursued further. All subsequent work in this study is focused on the four analogs with measurable affinities (NEP, NFEP, NBEP, and NNEP).

Similar to what we reported previously for epibatidine and other ligands (Parker et al., 1998), the \(\beta_2\)-containing receptors displayed higher affinities for the epibatidine analogs than did the \(\beta_4\)-containing receptors. However, some of the analogs displayed much greater selectivity for \(\beta_2\)-containing receptors over \(\beta_4\)-containing receptors than we have observed with other compounds. Differences in affinity for different receptors based on the \(\alpha\) subunit were also observed, but the differences were modest. The binding affinities of epibatidine and the four analogs for the various neuronal nAChR subunit combinations are compared in Fig. 2.

The bromo-substitution in NBEP resulted in an analog with affinities for the various subunit combinations that were very similar to those of epibatidine itself. The fluorosubstitution (NFEP) resulted in an analog with increased affinity for \(\beta_2\)-containing receptors and decreased affinity for \(\beta_4\)-containing receptors. The largest difference was seen for \(\alpha_3\)-containing receptors, with the \(\alpha_3\beta_2\) receptor having a 550-fold higher affinity than the \(\alpha_3\beta_4\) receptor. The norchloro analog (NEP) was also highly selective for \(\beta_2\)-containing receptors. Again, the greatest difference in affinity was seen with \(\alpha_3\beta_2\) and \(\alpha_3\beta_4\) (a 3500-fold difference). The amino substitution (NNEP) caused a loss of affinity for both \(\beta_2\)- and \(\beta_4\)-containing receptors; however, NNEP remained selective for \(\beta_2\) receptors over \(\beta_4\) receptors.

Electrophysiological Analysis. We examined the functional potency and efficacy of (+)-epibatidine and the four analogs that displayed measurable equilibrium binding affinities (NEP, NFEP, NEP, and NNEP) for activation of three neuronal nAChR subunit combinations. \(\alpha_2\beta_2\) is a major subtype in the CNS, whereas \(\alpha_3\beta_4\) represents a subtype (\(\alpha_3\beta_4^{*}\)) found in the periphery (for explanation of nomenclature, see Lucas et al., 1999). The \(\alpha_4\beta_4\) subunit combination was also examined as a point of comparison between \(\alpha_2\beta_2\) and \(\alpha_3\beta_4\).

To investigate the wide variation in the efficacy of epibatidine at the \(\alpha_4\beta_2\) receptor reported in the literature (see Discussion), we initially constructed concentration-response curves for epibatidine at \(\alpha_4\beta_2\) receptors using two different protocols (Fig. 3A). In both protocols, three applications of acetylcholine (20 \(\mu\)M, the EC\(_{20}\) for \(\alpha_4\beta_2\) receptors) were given before epibatidine applications to ensure stability of responses. In the first protocol, the epibatidine dose-response curve was determined by measuring epibatidine responses in succession and normalizing to the ACh response immediately preceding the first epibatidine response. In the second protocol, a normalizing ACh application (20 \(\mu\)M) was added before each epibatidine application to correct for possible receptor desensitization or inactivation after repeated agonist application. When dose-response curves for epibatidine were determined by the first method, an efficacy of 25 ± 3% (relative to the maximum ACh response) was obtained. When dose-response curves were determined by the second method, an efficacy of 58 ± 6% was obtained. Although the efficacy values determined by the two methods were quite different...
Affinity and Efficacy of Epibatidine Analogs

Affinity of Epibatidine Analogs

Affinity (K_i) values derived from competition analysis for binding of NBEP, NFEP, NEP, and NNEP to αβ2 and αβ4 (A), αβ3 and αβ4 (B), and αβ2 and αβ4 (C) are shown. Hatched columns indicate β-containing receptors, white columns indicate β-containing receptors. Affinity (K_i) values for binding of epibatidine (Parker et al., 1998) are shown for reference. All values are mean ± S.E.M. of two to three experiments, each performed in sextuplicate.

(p < 0.01), the EC_{50} values were similar (11 ± 5 nM by the first method, 14 ± 5 nM by the second method).

Our observation that the measured efficacy of epibatidine varies depending on the normalization method (Fig. 3A) suggested that epibatidine may be causing a long-lasting inactivation of the receptors. To test this idea, we examined the effect of high concentrations of ACh or epibatidine on subsequent responses to ACh (Fig. 3B). Agonists were applied to αβ2-expressing oocytes for 10 s at 5-min intervals. Three applications of 20 μM ACh were followed by an application of 75 μM ACh, or a concentration of epibatidine (45 nM), yielding a similar degree of receptor activation. Three more applications of 20 μM ACh followed. These last three applications of 20 μM ACh are plotted in Fig. 3B as a percentage of the 20 μM ACh application immediately preceding application of the high concentration of ACh or epibatidine. Although application of the high concentration of ACh had no effect on subsequent ACh responses, application of the high concentration of epibatidine resulted in a decrease in subsequent ACh responses. This effect of epibatidine did not reverse after 15 min of washing. In light of this apparent inactivation of a portion of the receptors upon epibatidine exposure, we decided to use the second method (renormalizing after each epibatidine application) in all subsequent work.

In addition to αβ2, we also examined epibatidine activation of the αβ4 and αβ4 receptors. Figure 3C compares the epibatidine dose-response curves for αβ2, αβ3, and αβ4 receptor subtypes. At αβ4 receptors, epibatidine exhibited an efficacy of 71 ± 7%, similar to the efficacy at αβ2 receptors. At the αβ4 receptor, epibatidine exhibited an efficacy of 39 ± 3%, significantly lower than at either the αβ2 or αβ4 receptor.

The fluoro-substituted analog (NFEP) displayed the greatest improvement in subtype selectivity (in terms of efficacy). Dose-response curves for NFEP at each receptor subtype are shown in Fig. 3D. Although NFEP exhibited high efficacy at αβ4 receptors (131 ± 9%), efficacies were significantly lower at αβ2 and αβ4 receptors (41 ± 10 and 40 ± 3%, respectively). Although NFEP produced differences in maximum response, the EC_{50} values were similar for the three receptor subtypes (Table 2). Dose-response curves were also constructed for NBEP, NEP, and NNEP activation of αβ2, αβ3, and αβ4 receptors. Efficacies relative to ACh for epibatidine and the four ANALOGS are compared in Fig. 4. Efficacy, EC_{50}, and n_{H} values are provided in Table 2.

NEP exhibited efficacies similar to ACh at αβ2, αβ3, and αβ4 receptors (65 ± 8, 82 ± 20, and 102 ± 10%, respectively). The efficacy at αβ2 receptors was significantly less than at αβ4 receptors. NEP was significantly less potent at αβ2 (EC_{50} = 94 ± 6 nM) and αβ4 (EC_{50} = 189 ± 51 nM) receptors relative to ACh (EC_{50} = 20 ± 8 nM).

The amino-substituted analog (NNEP) was the only analog to display selectivity (in terms of efficacy) for the αβ4 receptor over the αβ2 receptor. NNEP displayed an efficacy at the αβ4 receptor (82 ± 20%) that was similar to ACh, whereas the efficacy of NNEP at the αβ2 receptor was significantly lower (29 ± 4%). NNEP was less potent at αβ2 (EC_{50} = 7600 ± 627 nM) than at αβ4 (EC_{50} = 3240 ± 449 nM) and at αβ4 (EC_{50} = 1840 ± 622 nM).

NEP exhibited high efficacy at both αβ2 and αβ4 receptors (112 ± 19 and 147 ± 34%, respectively). This analog exhibited a significantly lower efficacy of 61 ± 10% at αβ4 receptors. NEP was less potent at αβ2 receptors (EC_{50} = 3630 ± 646 nM) than at αβ4 (EC_{50} = 702 ± 57 nM) and αβ4 (EC_{50} = 982 ± 54 nM).

**Discussion**

In this study, we have examined the equilibrium binding affinities and functional activities of several epibatidine an-
Table 2

Dose-response curve parameters for epibatidine and epibatidine analogs

<table>
<thead>
<tr>
<th></th>
<th>α4β2</th>
<th></th>
<th>α3β4</th>
<th></th>
<th>α4β4</th>
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<tr>
<td>max %</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>n&lt;sub&gt;H&lt;/sub&gt;</td>
<td>max %</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>n&lt;sub&gt;H&lt;/sub&gt;</td>
<td>max %</td>
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<tr>
<td>EP</td>
<td>58 ± 6&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>14 ± 7</td>
<td>39 ± 3&lt;sup&gt;‡‡‡&lt;/sup&gt;</td>
<td>128 ± 76</td>
<td>1.0 ± 0.2</td>
<td>71 ± 7&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>NBEp</td>
<td>65 ± 8&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>94 ± 6&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>82 ± 20</td>
<td>189 ± 51&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>1.3 ± 1.0</td>
<td>102 ± 10</td>
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<tr>
<td>NFEP</td>
<td>41 ± 10&lt;sup&gt;‡‡‡,*&lt;/sup&gt;</td>
<td>254 ± 31</td>
<td>40 ± 3&lt;sup&gt;‡‡‡&lt;/sup&gt;</td>
<td>319 ± 83</td>
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<tr>
<td>NEP</td>
<td>112 ± 19&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>3630 ± 646&lt;sup&gt;‡‡‡&lt;/sup&gt;</td>
<td>61 ± 10&lt;sup&gt;‡‡‡&lt;/sup&gt;</td>
<td>702 ± 57</td>
<td>1.1 ± 0.7</td>
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<td>NNEP</td>
<td>29 ± 4&lt;sup&gt;‡‡&lt;/sup&gt;</td>
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<td>82 ± 20</td>
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<td>1.2 ± 0.9</td>
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<td>NACH</td>
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Fig. 3. Dose-response curves for epibatidine and NFEP. A, epibatidine dose-response curves for activation of the α4β2 receptor with a single normalizing ACh response preceding epibatidine responses (open squares) or with normalizing ACh responses interleaved between the epibatidine responses (filled squares). B, epibatidine causes long-lasting inhibition of α4β2 receptors. Responses of α4β2-expressing oocytes to 20 μM ACh after 10-s exposure to 75 μM ACh (squares) or 45 nM epibatidine (circles), as a percentage of the response to 20 μM ACh before application of the high concentrations of ACh or epibatidine. Statistically significant differences from post-ACh values are indicated by asterisk (*, p < 0.05). C, epibatidine dose-response curves for α4β4 (circles), α4β2 (squares), and α3β4 (triangles). D, NFEP dose-response curves for α4β4 (circles), α4β2 (squares), and α3β4 (triangles). All values are mean ± S.E.M. (n = 3 to 5).

Fig. 4. Efficacies of four epibatidine analogs. The fit maximum efficacies (as a percentage of the maximal ACh response) of epibatidine (EP), NFEP, NBEp, NEP, and NNEP are shown for α4β2 (white columns), α3β4 (black columns), and α4β4 (hatched columns). All values are mean ± S.E.M. (n = 3 to 5).

We found that the epibatidine analogs with measurable binding affinities were selective for β2-containing receptors over β4-containing receptors. This is similar to what we have previously reported using epibatidine and several common agonists (Parker et al., 1998). However, the degree of selectivity was much greater than in our previous work. In particular, the fluoro-substituted (NFEB) and norchloro (NEP) analogs of epibatidine showed several hundred- to several thousand-fold selectivity for β2-containing receptors. In general, substitution of the 2′-chlorine of epibatidine with electron-withdrawing groups, such as the fluoro and bromo groups, or the neutral hydrogen, resulted in analogs with high affinity for β2-containing receptors and low, but measurable affinities for β4-containing receptors. Substitution with electron-donating groups (hydroxy and dimethylamino) resulted in affinities for all subunit combinations that were too low to accurately measure. However, effects on affinity could not be attributed solely to electron-donating or -withdrawing properties of the substituents. Substitution with the electron-
withdrawing trifluoromethanesulfonate group produced an analog with a very low affinity for all subunit combinations, perhaps due to the large increase in steric bulk. Also, substitution with the electron donating amino group resulted in an analog with moderate affinities for some subunit combinations.

Although the great increase in selectivity seen in the equilibrium binding assays was promising, the affinity values for the analogs derived from the binding assay were not generally predictive of the relative potencies determined in the functional assay (Tables 1 and 2). For example, the equilibrium binding of NFEP for the α4β2 receptor was 228-fold greater than the affinity for the α3β4 receptor. However, NFEP displayed equal functional potency at the α4β2 and α3β4 receptors. NEP provides an even more striking example. Although the binding affinity of NEP was 1300-fold greater for α4β2 than for α3β4, the functional potency of NEP was 5-fold greater at the α3β4 receptor than at the α4β2 receptor. For both α4β2 and α3β4, the functional potency of epibatidine and the four analogs failed to correlate with the equilibrium binding affinity (Table 3). However, for α4β4 there was a significant correlation between functional potency and equilibrium binding affinity.

The affinity measured in our binding assay reflects a distribution of receptor states. Because the receptors are in equilibrium between closed, open, and desensitized states, the binding affinity measured is dependent on agonist affinities for the individual states as well as the equilibrium constants for transitions among the different states. The desensitized state of the receptor has an exceptionally high affinity for agonists. Furthermore, the open activated state of the receptor is transient, and in our binding assay, the agonist concentration is too low for significant binding to the closed state of the receptor. This suggests that the equilibrium binding affinity predominantly reflects the affinity of agonists for the desensitized state. Because of the higher affinity of agonists for this state, equilibrium binding affinities are 2 to 4 orders of magnitude higher than that for the closed activeable state (estimated from EC50 values for activation) measured in functional assays (Harvey and Ludetje, 1996; Parker et al., 1998). The affinity of epibatidine for the closed activeable state and the desensitized state has been reported to differ by −3 orders of magnitude, depending on receptor subunit combination (Gerzanich et al., 1995; Gopalakrishnan et al., 1996). Thus, independent variation in ligand affinity for different states of the receptors may underlie the differences in selectivity measured in equilibrium binding assays and functional potency assays of the epibatidine analogs studied herein. The large effect of 2′-pyridine ring substitutions on β subunit selectivity in equilibrium binding assays that we have observed is interesting in light of the recently reported computational docking of epibatidine into the binding site of a homology model of the extracellular domain of an α7 homomeric neuronal nAChR (Le Novere et al., 2002). In this model, which is suggested to represent the desensitized form of the receptor, the 2′-pyridine chlorine of epibatidine interacts with residues of the “complementary component” of the binding pocket. In heteromeric neuronal nAChRs, such as α4β2 and α3β4, the complementary component is supplied by the β subunit. Although the selectivity in equilibrium binding assays will be of use in experimental identification of receptor subunit composition, identification of analogs of potential therapeutic usefulness requires assessment of functional potency and efficacy.

The reported efficacy of epibatidine for activation of α4β2 receptors varies from ~20 to ~100% of the maximum ACh response (Gerzanich et al., 1995; Buisson et al., 2000; Spang et al., 2000). We have found that the measured efficacy of epibatidine is dependent on the method used to normalize the data (Fig. 3A). If the responses to epibatidine are recorded in succession and normalized to an ACh response that precedes the entire series of epibatidine responses, the measured efficacy is quite low (25 ± 3%). If normalizing ACh responses are interleaved between the epibatidine responses then the measured efficacy is higher (58 ± 6%). This difference seems to be due to exposure to epibatidine causing an inactivation of a portion of the receptors. This inactivated state seems to be relatively long-lived, because the receptors do not reappear even after extensive washing (Fig. 3B). Buisson et al. (2000) also observed this effect of epibatidine, proposing that epibatidine remains tightly bound, maintaining the receptor in a nonactivatable state. The remaining functional receptors did not seem to be affected, because EC50 values, Hill coefficients, and desensitization rates before and after epibatidine exposure were the same (Buisson et al., 2000). This effect of epibatidine supports the necessity of repeated ACh normalization during epibatidine and analog efficacy measurements. We also observed decreases in the amplitude of the normalizing ACh applications after application of the various epibatidine analogs (data not shown).

Similar to what we observed for functional potencies, equilibrium binding affinity values for the epibatidine analogs were not generally predictive of the relative efficacies determined in the functional assay (Tables 1 and 2). For example, although NEP was selective for α4β2 over α3β4 (3-fold) in the binding assay (1300-fold) and the efficacy assay (2-fold), NFEP was selective for α4β2 over α3β4 (228-fold) and α4β4 (52-fold) in the binding assay, but was selective for α4β4 over α4β2 and α3β4 (3-fold) in the efficacy assay. Another striking example is provided by NNEP, which had a 20-fold greater affinity for α4β2 receptors than for α3β4 receptors, but was 3-fold more efficacious at α3β4 receptors than at α4β2 receptors. There were no significant correlations between equilibrium binding affinity and efficacy for α4β2, α3β4, or α4β4 (Table 3).

The large increases in neuronal nAChR subtype selectivity that we observed in equilibrium binding assays with several epibatidine analogs suggested that these analogs might have useful functional selectivity for CNS neuronal nAChRs (typified by α4β2) over peripheral neuronal nAChRs (typified by α3β4). However, differences in functional potency and efficacy were modest, suggesting that these analogs are unlikely to be selective enough for CNS neuronal nAChRs over peripheral nAChRs to avoid peripheral toxicity. This has been

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TABLE 3
Correlation analysis of affinity, efficacy, and functional potency

<table>
<thead>
<tr>
<th>Comparison</th>
<th>α4β2</th>
<th>α3β4</th>
<th>α4β4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affinity and efficacy</td>
<td>0.34</td>
<td>0.07</td>
<td>0.05</td>
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<tr>
<td>EC50 and efficacy</td>
<td>0.02</td>
<td>0.32</td>
<td>0.14</td>
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<tr>
<td>Affinity and EC50</td>
<td>0.77</td>
<td>0.26</td>
<td>0.96**</td>
</tr>
</tbody>
</table>

r2 values were obtained from correlation analysis of affinity, efficacy, and functional potency values for epibatidine, NFEP, NNEP, NEP, and NNEP. Statistical significant correlation is denoted by **, p < 0.01.
a problem with other epibatidine analogs. Halogen-substituted and methylated analogs of epibatidine have been reported to produce fewer toxicities in rodents (Badio et al., 1997; Horti et al., 1999); however, the reduction in toxic effects seen in these studies was not sufficient to ensure safe therapeutic/toxicity ratios. Epibatidine, a methylisoxazole analog of epibatidine has also been reported to produce fewer toxicities than epibatidine in mice (Badio et al., 1997). However, epibatidine was less potent than epibatidine as an antinociceptive agent and, in sodium flux assays, was not significantly less potent than epibatidine at ganglionic nAChRs. Thus, the development of CNS-selective epibatidine analogs will require continued effort. The general failure of efficacy and functional potency to correlate with binding affinity (Table 3) indicates that a combined physiological and pharmacological approach for determination of binding affinity, efficacy, and functional potency will be required for the development of epibatidine analogs with improved functional subtype selectivity.

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