Alkaloids Indolizidine 235B’, Quinolizidine 1-epi-207I, and the Tricyclic 205B are Potent and Selective Noncompetitive Inhibitors of Nicotinic Acetylcholine Receptors

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ABSTRACT
Nicotinic acetylcholine receptors are key molecules in cholinergic transmission in the nervous system. Because of their structural complexity, only a limited number of subtype-specific agonists and antagonists are available to study nicotinic receptor functions. To overcome this limitation, we used voltage-clamp recordings to examine the effects of several frog skin alkaloids on acetylcholine-elicited currents in Xenopus laevis oocytes expressing major types of neuronal nicotinic receptors (α4β2, α7, α3β2, α3β4, and α4β4). We found that the 5,8-disubstituted indolizidine (−)-235B’ acted as a potent noncompetitive blocker of α4β2 nicotinic receptors (IC50 = 74 nM). This effect was highly selective for α4β2 receptors compared with α3β2, α3β4, and α4β4 receptors. The inhibition of α4β2 currents by (−)-235B’ was more pronounced as the acetylcholine concentration increased (from 10 nM to 100 μM). Moreover, the blockade of α4β2 currents by (−)-235B’ was voltage-dependent (more pronounced at hyperpolarized potentials) and use-dependent, indicating that (−)-235B’ behaves as an open-channel blocker of this receptor. Several other 5,8-disubstituted indolizidines (5-n-propyl-8-n-butylindolizidines), two 5,6,8-trisubstituted indolizidines (−)-223A and (+)-6-epi-223A), and a 1,4-disubstituted quinolizidine ((−)-207I) were less potent than (−)-235B’, and none showed selectivity for α4β2 receptors. The quinolizidine (−)-1-epi-207I and the tricyclic (+)-205B had 8.7- and 5.4-fold higher sensitivity, respectively, for inhibition of the α7 nicotinic receptor than for inhibition of the α4β2 receptor. These results show that frog alkaloids alter the function of nicotinic receptors in a subtype-selective manner, suggesting that an analysis of these alkaloids may aid in the development of selective drugs to alter nicotinic cholinergic functions.

Nicotinic acetylcholine receptors (nAChRs) are widely expressed in the mammalian brain, and act as key molecules in the physiological processes of reward, cognition, learning, and memory (Changeux et al., 1998; Dani, 2001; Lindstrom, 2003). Nicotinic receptors are ligand-gated ion channels composed of five subunits. To date, 12 nAChR subunits (α2-α10 and β2-β4 subunits) have been identified (Hogg et al., 2003). Because different combinations of these subunits produce different subtypes of receptors, the potential for nAChR diversity is vast. Based on their pharmacology, function, and location, different categories of nAChR subtypes have been created. Three predominant subtypes in the mammalian central nervous system are those containing the α7 subunit (α7*), the β2 subunit (β2*), or the β4 subunit (β4*) (Alkondon and Albuquerque, 1993; Zoli et al., 1998).

Evidence indicates that nAChRs are implicated in several neurological disorders. In particular, significant loss of α4β2 nAChRs has been observed in cortical autopsies from patients with Alzheimer’s disease, and schizophrenia and bipolar disorder exhibit some linkage to the α7-subunit gene (Weiland et al., 2000). Mutations in the α4 or the β2 subunit also underlie autosomal-dominant nocturnal frontal lobe epilepsy in some families (Raggenbass and Bertrand, 2002). Nicotinic agonists and antagonists would therefore be very
valuable and are being investigated for possible use in these diseases (Lloyd and Williams, 2000; Dani et al., 2004). However, selective ligands for the multiple subtypes of neuronal nicotinic receptors are still scarce.

Extracts from the skin of certain poison frogs provide a variety of pharmacologically active alkaloids, and more than 500 alkaloids have been isolated to date (Daly et al., 1999). Some of them are known to target nAChRs: a nicotinic agonist, epibatidine, and nicotinic antagonists, histriotoxins, pumiliotoxins, and indolizidines (Spivak et al., 1982; Wannick et al., 1982; Aronstam et al., 1986; Daly et al., 1991, 1999). However, the selectivity of most amphibian alkaloids for each subtype of nAChR remains to be characterized.

In the present study, we investigated the selectivity of bicyclic alkaloids, indolizidines, and quinolizidines, and a tricyclic alkaloid, 8-azaacenaphthylene, across several major types of nicotinic receptors (α4β2, α7, α3β2, α3β4, and α4β4) expressed in Xenopus laevis oocytes.

### Materials and Methods

#### Materials

All alkaloids and their analogs used in this study were synthesized and are shown in Fig. 1. The synthesis of indolizidine (−)-235B was performed as reported previously (Momose and Toyooka, 1984; Toyooka et al., 1997; Toyooka and Nemoto, 2002). The structure of (−)-235B is identical to that of the natural product. The synthesis of indolizidine (5-α-propyl-8-n-butylindolizidine) I, II, and III were synthesized, and indicated that 223I was not a 5,8-disubstituted indolizidine. The synthesis of indolizidine I has been shown previously (Toyooka et al., 1997; Toyooka and Nemoto, 2002). The synthesis of 5,6,8-trisubstituted indolizidine (−)-223A along with revision of its structure has recently been reported (Toyooka and Nemoto, 2002; Toyooka et al., 2002) as well as the synthesis of a 6-epimer (223A proposed). This unnatural compound has been designated (+)-6-epi-223A in the present study. The 1,4-disubstituted quinolizidine (+)-207I, which is the opposite enantiomer of the natural product, was synthesized in recent studies (Toyooka and Nemoto, 2002, 2003). An unnatural 1-epimer of (+)-207I, named (−)-1-epi-207I in this study, was synthesized as described previously (Toyooka et al., 1997; Toyooka and Nemoto, 2002). Synthetic (+)-205B is the opposite enantiomer of natural (−)-205B, as reported by Toyooka et al. (2003).

The other reagents were purchased from Sigma (St. Louis, MO), unless indicated otherwise. BAPTA-AM was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA).

The cDNAs of the rat α3, β2, and β4 subunits in pcDNA1/Neo vectors were subcloned into HindIII and XhoI sites of pcDNA1.1 vectors (Invitrogen, Carlsbad, CA), and the cDNA of the rat α7 subunit in pcDNA1/Neo vectors was subcloned into HindIII and XbaI sites of pcDNA3.1 vectors (Invitrogen) to enhance the expression of αβ2, αβ4, and αβ4 nicotinic receptors in oocytes. To express αβ2 and α7 nAChRs, mouse cDNAs were used.

#### Expression in X. laevis Oocytes

All experiments were carried out in accordance with guidelines approved by the Toyama Medical and Pharmaceutical University Animal Research Committee. Oocytes were surgically removed from X. laevis frogs under anesthesia using Tricaine solution (2.4 g/l). The oocytes were rinsed in calcium-free OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.6), then defolliculated in this buffer supplemented with 1.5 mg/ml collagenase A (Roche Diagnostics, Mannheim, Germany) for approximately 2 h at room temperature. Stage V-VI oocytes were selected and microinjected with 20 ng of cDNAs in the nucleus. The mixture of two subunit cDNAs, α3 or α4 in combination with β2 or β4, was injected in a ratio of 1:1, whereas α7 subunit was injected alone. Oocytes were incubated at 19°C in standard oocyte saline solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 2 mM MgCl2, and 5 mM HEPES, 2.5 mM pyruvic acid, 1% BSA, and 25 μg/ml gentamycin, pH 7.5) for 3 to 8 days before recordings were made.

#### Electrophysiological Recording

An oocyte was placed in a 300-μl tube-like chamber in which Ringer’s solution (82.5 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.4) containing 1 μM atropine to block endogenous muscarinic receptors was perfused by gravity (15 ml/min). Current responses were recorded under two-electrode voltage-clamp at a holding potential of −60 mV using a GeneClamp 500 amplifier and pClamp7 software (Axon Instruments, Union City, CA). The sampling rate was 20 Hz. Electrodes contained 3 M KCl and had resistances of <1 MΩ.

To apply a pulse of acetylcholine (ACh) to the oocyte, the perfusion fluid was switched to one containing ACh for 5 s, using a three-way Teflon solenoid valve (Parker Hannifin Corp., General Valve Division, Fairfield, NJ) controlled by a PC computer with pClamp7 software. A 3-min wash between ACh applications produced reproducible control currents with no obvious desensitization. For responses in alkaloids (test responses), the perfusion fluid was stopped during alkaloid application for 3 min to conserve the compounds. Perfusion was started again 0.5 min before measuring responses to ACh in the presence of test alkaloids.

Control experiments in the Ca2+ chelator BAPTA were performed to minimize the activation of endogenous Ca2+-activated chloride channels in X. laevis oocytes. Those endogenous currents were prevented by BAPTA to be sure that they did not alter the pharmacological profiles of the tested alkaloids. Oocytes were loaded with BAPTA by incubating in a SOS solution containing BAPTA-AM (100 μM) for 4 h before recording, as described previously (Ibanez-Tallon et al., 2002). Otherwise, oocytes were incubated with a low-Ca2+ Ringer’s solution (82.5 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, 2 mM MgCl2, and 5 mM HEPES, pH 7.4) to depress the endogenous chloride conductance while we investigated the mechanism of receptor blockade by the alkaloids.

#### Data Analysis

The average peak amplitudes of three control responses to ACh (1 μM for αβ2 and αβ4 receptors; 100 μM for α7, α3β2, and α3β4 receptors) just preceding exposure to alkaloids were...
used to normalize the amplitude of each test response. These ACh concentrations were determined to be approximately 30% of the maximum effective concentrations (EC50), according to the ACh-concentration response curve (data not shown). Each data point of the concentration-response curve represents the average value ± S.E.M. of measurements from at least three oocytes.

Concentration-inhibition curves for alkaloids (agonists) were fitted by a nonlinear regression to the equation:  
\[ I = I_{\text{max}} - I_{\text{max}} N / (N + (I_{\text{EC50,high}} / X^{[\text{RH}]}) + (1 - a) / (1 + (I_{\text{EC50,low}} / X^{[\text{RL}]})^{n[H]})) \]

where \( I_{\text{max}} \) is the maximal normalized current response (in the absence of antagonist for inhibitory curves), \( a \) is the antagonist concentration, \( I_{\text{EC50}} \) is the antagonist concentration eliciting half-maximal current, and \( n[H] \) is the Hill coefficient. Curve fittings were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA). For antagonist efficacy curves, \( I_{\text{max}} \) was constrained to 1 and \( I_{\text{min}} \) was constrained to 0.

The ACh concentration-response curves for α4β2 nAChR receptors were fitted to the following two-component (double sigmoid) equation using Prism software (GraphPad Software, Inc.):  
\[ y = y_{\text{max}} [a1/(1 + (EC50\text{high}/X)^{n[H]}) + (1 - a1)/(1 + (EC50\text{low}/X)^{n[L]})] \]

where \( y \) is the percentage amplitude, \( a1 \) is the percentage of receptors in the high-affinity component, high and low refer to relative ACh sensitivity, \( x \) is the acetylcholine concentration, and \( n[L] \) is the Hill coefficient. Responses evoked by 1 mM ACh corresponded to 100% activation of the high- and low-affinity components. \( y_{\text{max}} \) is the maximal current amplitude (percentage), normalized by the amplitude of ACh (1 mM)-evoked currents in the absence of alkaloid. Moreover, fits to the concentration-response curve represent the average value of the concentration response curve (data not shown). Each data point of the concentration-response curve was fit by the F-statistic from the ratios of the minimum sums of squares of deviations.

**Statistical Analysis.** The significance of differences between two groups was assessed by Student’s t test, and the significance of differences between multiple groups were assessed by one-way analysis of variance followed by the Dunnett’s multiple range test. Values of \( P \) less than 0.05 were considered significant.

**Results**

The subtype selectivity of the frog alkaloids was investigated across five recombinant nAChRs (α4β2, α7, α3β2, α3β4, and α4β4) expressed in *X. laevis* oocytes. The oocytes were voltage-clamped, and application of 0.1 to 1000 μM ACh elicited inward currents. When the currents were elicited by the EC50 doses of ACh (see Data Analysis), α4β2 and α3β2 heteromeric receptors showed faster decay kinetics than β4-containing heteromeric receptors (α3β4 and α4β4), and currents through α7 homomeric receptors decayed very rapidly during the ACh application (Fig. 2, A and B). These features were consistent with previous observations (Alkondon and Albuquerque, 1993; Chavez-Noriega et al., 1997; Fenster et al., 1997). We also confirmed that the ACh responses in oocytes expressing α4β2 nAChRs were inhibited by 5 μM dihydroβ-erythroidine (DHβE), a competitive neuronal nAChR antagonist acting preferentially on non-α7 receptor) and α7 nAChRs were inhibited by 10 nM methyllycaconitine (MLA, an antagonist of α7-containing nicotinic receptors) (Fig. 2A). The α4β2 nAChR-mediated currents gradually recovered during a 25-min washout of DHβE, whereas α7 nAChR-mediated currents were completely recovered 15 min after removal of MLA.

The responses mediated by recombinant nAChRs were blocked by most of the alkaloids used, as summarized in Table 1. When the oocytes expressing α4β2 nAChR were treated with indolizidine (−)-235B' (0.3 μM), the peak amplitude of the ACh-elicited currents was greatly decreased (Fig. 2B). This blocking effect was reversible within 10 min (data not shown). Compared with the efficacy on oocytes expressing the other receptor subtypes, (−)-235B' blocked the α4β2 receptor-mediated responses (IC50 = 74 nM, nH = 0.47 ± 0.04) with 6.0-fold higher sensitivity than blockade of α7 receptor-mediated responses (IC50 = 442 nM, nH = 0.48 ± 0.05), 40.5-fold higher than that of α3β2 receptor-mediated ones (IC50 = 3.0 μM, nH = 0.74 ± 0.05), 51.4-fold higher than that of α3β4 receptor-mediated ones (IC50 = 3.8 μM, nH = 0.47 ± 0.04).
To exclude the possibility that indolizidine (-)-235B' blocks nAChR signaling downstream, such as endogenous Ca\(^{2+}\)-activated chloride channels that could amplify the nAChR responses in *X. laevis* oocytes, BAPTA control experiments were performed. Oocytes were incubated with the membrane-permeable Ca\(^{2+}\) chelator, BAPTA-AM, before recording. Similar blocking actions of indolizidine (-)-235B' were observed in the BAPTA-AM–treated oocytes expressing α4β2 nAChRs. Compared with untreated oocytes, the peak amplitude of the currents elicited by ACh (1 μM) in the absence of (-)-235B' was 459 ± 46 nA (3 oocytes), and in the presence of 0.1 and 0.3 μM (-)-235B', the amplitudes were decreased to 58.0 ± 3.2 and 35.7 ± 6.8%, respectively. These results indicate that indolizidine (-)-235B' directly blocks α4β2 nAChRs.

Analysis of the α4β2 peak current amplitude revealed that the ACh concentration–response curves were not adequately described by a single Hill equation (Fig. 3A, dashed line, EC\(_{50}\) = 10.9 μM and n\(_H\) = 0.52). A significantly better fit was obtained with the sum of two Hill equations (Fig. 3A, continuous line), indicating the existence of a high- and low-affinity component (Zwart and Vijverberg, 1998; Coverton and Connolly, 2000; Buisson and Bertrand, 2001; Nelson et al., 2003; Almeida et al., 2004; Khiroug et al., 2004). The high-affinity coefficients were EC\(_{50,\text{high}}\) = 1.3 μM and n\(_{H,\text{high}}\) = 0.56, whereas the low-affinity coefficients were EC\(_{50,\text{low}}\) = 119 μM and n\(_{H,\text{low}}\) = 1.5. The fractions of high- and low-affinity components were 58.3 and 41.7%, respectively. In the presence of indolizidine (-)-235B' (0.1 μM), the ACh concentration–response curve shifted downward, and the responses to ACh at the maximal concentration (1 mM) did not reach the levels observed in the absence of (-)-235B' (Fig. 3A). These results indicate that indolizidine (-)-235B' is not acting as a competitive antagonist of α4β2 receptors. The concentration–response relationship for ACh in the presence of (-)-235B' was well fit by a single Hill equation (Fig. 3A, dashed line, EC\(_{50}\) = 8.8 μM and n\(_H\) = 0.44). When we applied the two-component model to the data, the fit obtained is shown as the continuous line in Fig. 3A, yielding the high-affinity coefficients of EC\(_{50,\text{high}}\) = 0.07 μM and n\(_{H,\text{high}}\) = 0.85 and the low-affinity coefficients of EC\(_{50,\text{low}}\) = 24.8 μM and n\(_{H,\text{low}}\) = 1.0. The fractions of high- and low-affinity components were 31.0 and 69.0%, respectively.

To know the mode of the receptor blockade by (-)-235B', α4β2 receptors were activated by increasing concentrations of ACh (ranging from 10 nM to 1 mM) in the presence of (-)-235B' (0.1 μM). The ACh-elicited currents were measured in a lower external Ca\(^{2+}\) concentration (0.5 mM) to avoid the Ca\(^{2+}\)-activated chloride conductance. As shown in Fig. 3B, the relative blockade by (-)-235B' was larger at the higher concentrations of ACh. Thus, in the presence of the alkaloid (-)-235B' at a constant concentration (0.1 μM), the peak amplitudes of currents elicited by ACh at 0.1 and 100 μM were reduced by 32.8 ± 5.8 and 71.5 ± 3.4%, respectively; differences were statistically significant (P < 0.01, determined by unpaired Student's t test).

Moreover, the voltage dependence of the blocking effects of (-)-235B' on α4β2 currents was explored at different holding potentials (from −140 to +40 mV) in 20-mV steps. The currents were elicited by ACh (1 μM) in 0.5 mM Ca\(^{2+}\). As shown in Fig. 4A, the current–voltage relationship showed strong inward rectification for the ACh (1 μM)-elicited current at positive membrane potentials. Exposure to (-)-235B' (0.1 μM) induced a reduction of the peak amplitude of currents, and the magnitude of this reduction was more pronounced at hyperpolarized potentials. (-)-235B' blocked the α4β2 currents by 68 ± 6% at −140 mV and by only 38 ± 6% at −40 mV (Fig. 4B).

We also investigated whether (-)-235B' produces a use-dependent blockade of α4β2 receptors. When oocytes expressing α4β2 receptors were stimulated with short pulses of ACh (10 μM, 200 ms) every 8–10 s in 0.5 mM Ca\(^{2+}\), consistent, repeatable currents were obtained. Figure 5A shows a typical

### Table 1

Potency of blocking effects of frog skin alkaloids on *X. laevis* oocytes expressing recombinant nicotinic receptors

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>IC(_{50}) (95% CI)</th>
<th>α4β2 (1 μM ACh)</th>
<th>α7 (100 μM ACh)</th>
<th>α3β4 (100 μM ACh)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-235B'</td>
<td>0.07 (0.05, 0.11)</td>
<td>0.4 (0.3, 0.6)</td>
<td>3.8 (2.3, 6.1)</td>
<td>5–8</td>
<td></td>
</tr>
<tr>
<td>(-)-I'</td>
<td>6.0 (4.1, 8.8)</td>
<td>3.4 (1.7, 6.7)</td>
<td>&gt;10.0</td>
<td>3–5</td>
<td></td>
</tr>
<tr>
<td>(+)-II</td>
<td>16.8 (13.2, 21.3)</td>
<td>2.5 (1.8, 3.5)</td>
<td>14.7 (11.1, 19.5)</td>
<td>3–5</td>
<td></td>
</tr>
<tr>
<td>(-)-III</td>
<td>20.1 (11.7, 34.3)</td>
<td>1.8 (1.6, 2.1)</td>
<td>3.0 (2.2, 4.0)</td>
<td>3–7</td>
<td></td>
</tr>
<tr>
<td>(+)-6-epi-233A</td>
<td>&gt;30.0</td>
<td>&gt;30.0</td>
<td>15.1 (10.3, 22.3)</td>
<td>3–5</td>
<td></td>
</tr>
<tr>
<td>(-)-233A</td>
<td>4.5 (3.7, 5.5)</td>
<td>4.2 (3.6, 4.8)</td>
<td>14.1 (9.9, 20.2)</td>
<td>5–7</td>
<td></td>
</tr>
<tr>
<td>(-)-1-epi-207I</td>
<td>5.2 (4.2, 6.5)</td>
<td>0.6 (0.4, 0.9)</td>
<td>8.8 (4.7, 16.6)</td>
<td>3–5</td>
<td></td>
</tr>
<tr>
<td>(+)-207I</td>
<td>5.0 (2.5, 9.8)</td>
<td>3.4 (2.5, 4.6)</td>
<td>N.D.</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(+)-205B</td>
<td>13.5 (9.0, 20.2)</td>
<td>2.5 (1.6, 4.1)</td>
<td>11.3 (6.5, 19.8)</td>
<td>4–6</td>
<td></td>
</tr>
</tbody>
</table>

*IC\(_{50}\) (95% CI)*: 95% CI, 95% confidence intervals; N.D., not determined; n, number of data
*This compound will be designated as natural 223W.*
recording in one oocyte. In contrast, when (-)-235B' (0.1 μM) was superfused in the bath for 15 s preceding ACh application, then the responses to the repetitive pulses of ACh co-applied with (-)-235B' (0.1 μM) were progressively decreased in the same oocyte. Figure 5A). Figure 5B summarizes the results obtained during the first 64 s in different oocytes. We further studied whether the blocking effect of (-)-235B' (0.1 μM) depends on the frequency of the ACh pulses. Using a lower frequency (16-s interval) rather than 8-s interval of stimulation, we found a significant difference in the extent of the blockade of α4β2 currents at the two frequencies (Fig. 5B). At any time point tested, (-)-235B' produced a stronger blockade of the currents elicited by ACh pulses at the higher frequency (8-s interval) than at the lower frequency (16-s interval).

Next, we tested the effects of another type of the 5,8-disubstituted indolizidine analogs, 5-n-propyl-8-n-butylin-dolizidines I, II, and III, on the ACh-elicited currents in oocytes expressing recombinant nicotinic receptors. These three compounds are stereoisomers, and I has the same stereochemistry as (-)-235B' at the 5 and 9 positions (5,9-cis: 5,9Z), as shown in Fig. 1. The alkaloid I at 10 μM blocked the responses mediated by α4β2 receptors and α7 receptors similarly (Fig. 6A). In fact, these currents were blocked by this alkaloid in a concentration-dependent manner, with IC50 values of 6.0 μM for α4β2 currents and 3.4 μM for α7 currents (Fig. 6B, Table 1). In contrast, the indolizidines II and III were more potent at blocking the responses mediated by α7 receptors than α4β2 receptors (Table 1). In addition, the indolizidine I had a negligible effect on the α3β4 receptor responses at a high concentration (10 μM), whereas the responses through this receptor were substantially blocked by the indolizidine III (IC50 = 14.7 μM) and were potently blocked by the indolizidine I (IC50 = 3.0 μM).

Stereoselective pharmacological properties were also observed with the 5,6,8-trisubstituted indolizidines (+)-6-epi-223A and (-)-223A. The alkaloid (+)-6-epi-223A (10 μM) had a negligible effect on the ACh-elicited currents in oocytes expressing α4β2 or α7 receptors (Table 1), whereas (-)-223A (10 μM) blocked both the α4β2 and α7 receptor-mediated currents to a similar extent (Fig. 7A). (-)-223A blocked those
two receptors in a concentration-dependent manner with IC₅₀ values of about 4 μM (Fig. 7B, Table 1). The αβ4 receptor-mediated currents were also blocked by (-)-223A with an IC₅₀ value of 14 μM (Fig. 7, Table 1). This blocking effect was similar to that of (+)-6-epi-223A (IC₅₀ = 15 μM).

The 1,4-disubstituted quinolizidine (-)-1-epi-207I selectively blocked α7 receptor responses (IC₅₀ = 0.6 μM), and it did so with 8.7-fold higher sensitivity than blockade of α4β2 receptor responses (IC₅₀ = 5.2 μM) and 14.7-fold higher than that of α3β4 receptor responses (IC₅₀ = 8.8 μM) (Table 1). The alkaloid (+)-207I equally blocked the responses mediated by α4β2 receptors (IC₅₀ = 5.0 μM) and α7 receptors (IC₅₀ = 3.4 μM).

When the oocytes expressing α7 nAChRs were treated with 3 μM 8β-azacenacaphylene (+)-205B, which is the unnatural enantiomer, the peak amplitude of the ACh-elicited currents was greatly decreased, whereas the responses via α4β2 and α3β4 nAChRs were not strongly affected (Fig. 8A). The blocking effect of (+)-205B (3 μM) on α7 nAChRs was reversible within 10 min (data not shown). When the concentration-

**Discussion**

The bicyclic “izidine” alkaloids (indolizidines, quinolizidines, pyrrolizidines, and azabicyclo[5.3.0]decanes) from amphibian skin have been examined. The alkaloids are mainly from neotropical dendrobatid frogs, *Dendrobates* spp., and most of the bicyclic alkaloids are known to be disubstituted, such as 5,8- or 3,5-disubstituted indolizidines and 1,4-disubstituted quinolizidines (Daly et al., 1999). Previously, it has been shown that particular 5,8-disubstituted indolizidines, including (-)-235B, act as noncompetitive nicotinic antagonists and inhibit carbamylcholine-elicited $^{22}$Na⁺-influx via nAChR channels in PC-12 cells (Daly et al., 1991). In this study, we examined the selectivity of indoliz-
idines for multiple subtypes of recombinant nAChRs expressed in oocytes. In addition, we examined the effects of several synthetic compounds with structures of frog alkaloids. Among these compounds, we found that indolizidine (−)-235B was a highly potent blocker of α4β2 nAChRs (IC50 = 0.07 μM). (−)-235B seems to block via direct stoichiometry with α4β2 receptors, not through an indirect effect on the oocytes’ endogenous Ca2+-activated chloride channels.

The concentration-response relationship for ACh on α4β2 nAChRs was best fit by the sum of two Hill equations. This finding is consistent with previous reports, indicating that there are at least two different populations of α4β2 nAChRs with different affinities for ACh when ectopically expressed in X. laevis oocytes (Zwart and Vijverberg, 1998; Covernton and Connolly, 2000; Khiroug et al., 2004) and in HEK293 cell lines (Buisson and Bertrand, 2001; Nelson et al., 2003; Almeida et al., 2004). It has been proposed that these different properties are attributable to different subunit stoichiometries: the high-affinity nAChR is thought to be (α4)2(β2)3, whereas the low-affinity nAChR is (α4)3(β2)2 (Nelson et al., 2003).

The blockade of α4β2 currents by (−)-235B was more pronounced as the ACh concentrations increased (from 10 nM to 100 μM). If the low-affinity population of α4β2 receptors were predominant compared with the high-affinity population in the present condition, selective blockade of the low-affinity receptors might explain the stronger effect of (−)-235B on the responses to higher concentrations of ACh. In fact, the populations were almost equal between the high-affinity component (58.3%) and the low-affinity component (41.7%) based on the analysis of the ACh concentration-response curve (Fig. 3A). Furthermore, we estimate that activation of the high- and low-affinity receptors contributed to 58% and 42%, respectively, of the peak currents elicited by 1 μM ACh. The alkaloid (−)-235B (0.1 nM-10 μM) blocked the ACh-elicited (1 μM) currents, and the concentration-response relationship for (−)-235B was well described by a single sigmoid curve (Fig. 2C), without requiring a double sigmoid, indicating that (−)-235B did not significantly differentiate between the high- and low-affinity receptors. Thus, the mode of action of (−)-235B seems to be independent of these receptor populations. Because the probability of the α4β2 nAChR-channel opening increases as the ACh concentration increases, (−)-235B blocks the receptor-channels that are more frequently open at higher ACh concentrations by entering and occluding the open-channel pores. In general, open channel blockers are known to exhibit both a voltage-dependent and a use-dependent inhibition of nAChRs (Buisson and Bertrand, 1998; Pintado et al., 2000). Consistent with that expectation, we observed that (−)-235B exerted stronger inhibition of the α4β2 currents at hyperpolarized potentials. In addition, the α4β2 currents elicited by repetitive ACh pulses were progressively inhibited by (−)-235B in a use-dependent manner. Therefore, the mode of action of (−)-235B is likely to be open channel blockade.

The sensitivity of indolizidine (−)-235B for α4β2 receptors was comparable with that of the best characterized antago-

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**Fig. 7. Inhibitory effects of indolizidine (−)-223A on ACh-induced currents in X. laevis oocytes expressing recombinant nicotinic receptors.** Currents were recorded in voltage-clamp mode at −60 mV. A, representative traces showing the ACh-elicited currents in the absence and presence of (−)-223A (10 μM). Horizontal bars indicate the period of perfusion with ACh for 5 s. Concentrations of ACh used were 1 μM for α4β2 receptors and 100 μM for α7 and α3β4 receptors. For test responses, oocytes were preincubated with (−)-223A for 3 min and then exposed to ACh with (−)-223A. Vertical scale bars represent 1 μA for α4β2 currents, and 0.5 μA for α7 and α3β4 currents. B, concentration-response curves for (−)-223A on recombinant nicotinic receptors. Current responses to ACh in the presence of (−)-223A in each oocyte were normalized to the ACh responses (control responses) recorded in the same oocytes. Values represent the mean ± S.E.M. for five to seven separate experiments.

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**Fig. 8. Inhibitory effects of 8β-azaacencaphyline (+)-205B on ACh-induced currents in X. laevis oocytes expressing recombinant nicotinic receptors.** Currents were recorded in the voltage-clamp mode at −60 mV. Concentrations of ACh used were 1 μM for α4β2 receptors and 100 μM for α7 and α3β4 receptors. For test responses, oocytes were preincubated with (+)-205B for 3 min and then exposed to ACh with (+)-205B. A, representative traces showing the ACh-elicited currents in the absence and presence of (+)-205B (3 μM). Horizontal bars indicate the period of perfusion with ACh for 5 s. Vertical scale bars represent 1 μA for α4β2 receptor and 0.5 μA for α7 and α3β4 receptors. B, concentration-response curves for (+)-205B on recombinant nicotinic receptors. Current responses to ACh in the presence of (+)-205B in each oocyte were normalized to the ACh responses (control responses) recorded in the same oocytes. Values represent the mean ± S.E.M. for four to six separate experiments.
nist of α4β2 nAChRs, DHβE, observed in X. laevis oocytes expressing human α4β2 receptors (IC50 = 0.11 μM (Chavez-Noriega et al., 1997). Moreover, (-)-235B selectively blocked α4β2 receptors more effectively than α3β2 receptors (40.5-fold) or α3β4 receptors (51.4-fold). These pharmacological properties are also comparable with DHβE. However, the selectivity for α4β2 receptors over α7 or α4β4 receptors was different between (-)-235B and DHβE: the rank order of potency of (-)-235B was α4β2 > α7 > α3β2 > α3β4 > α4β4, whereas that of DHβE is α4β4 > α4β2 > α3β2 > α3β4 > α4β7 (Chavez-Noriega et al., 1997). It may be possible to develop even more potent and selective ligands on the basis of the structure of indolizidine (-)-235B.

The structure of natural 5,6,8-trisubstituted indolizidine (-)-223A has recently been revised from (+)-6-epi-223A (Toyooka et al., 2002). It is interesting that (-)-223A but not (+)-6-epi-223A exhibited blocking effects on α4β2 and α7 receptors. These results suggest that the alkaloid (-)-223A may bind to the nAChRs in a stereoselective manner. We also examined the stereoselective pharmacological properties of three stereoisomers, 5,8-disubstituted indolizidines I, II, and III. The indolizidine I has a 5,9-cis (5,9Z) structure, whereas the other two compounds have 5,9-trans (5,9E) structures. Because the indolizidine I exhibited a greater sensitivity to α4β2 receptors than the indolizidines II and III, the 5,9-cis (5,9Z) structure may be important for binding to α4β2 receptors with high affinity. Indeed, indolizidine (-)-235B that possesses the 5,9-cis (5,9Z) structure was a potent blocker of α4β2 receptors, as mentioned above. To test this hypothesis, it would be necessary to examine the effect of the (-)-235B stereoisomer with a 5,9-trans (5,9E) structure on α4β2 receptors in a future study.

The alkaloid (+)-205B has a unique tricyclic structure of 8b-azaacacenaphylene ( Daly et al., 1999). No other alkaloids from amphibian skin are known to belong to this class. The absolute stereochemistry of this alkaloid was determined by the total synthesis (Toyooka et al., 2003). The alkaloid (+)-205B produced a selective inhibition of α7 receptors over α4β2 or α3β4 receptors. We also found the high selectivity (8.7-fold) of 1,4-disubstituted quinolizidine (-)-1-epi-207I for α7 receptors over α4β2 receptors, which is greater than the α7 selectivity (5.4-fold) of (+)-205B. Therefore, we suggest that a novel class of nicotinic antagonists may be developed based on the structure of these compounds.

In conclusion, we found that indolizidine (-)-235B is a potent noncompetitive blocker of α4β2 nAChRs, and its specificity is comparable with that of the competitive antagonist DHβE. We also found that quinolizidine (-)-1-epi-207I and tricyclic (+)-205B are selective blockers of α7 nAChRs. It should also be noted that some of the alkaloids used in this study exhibited subtype-selective blockade of nAChR in a stereoselective manner. These results suggest that it may be possible to obtain novel, potent, and subtype-selective blockers of nicotinic receptors by synthesizing stereoisomers of natural alkaloids. Thus, it is anticipated that the approach based on the frog alkaloids can provide lead compounds for the future design of drugs to treat cholinergic disorders in the central nervous system.

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References


Zwart R and Vijverberg HP (1998) Four pharmacologically distinct subtypes of α4β2
nicotinic acetylcholine receptor expressed in Xenopus laevis oocytes. Mol Pharma-
col 54:1124–1131.

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