5-Iodo-A-85380, an \(\alpha_4\beta_2\) Subtype-Selective Ligand for Nicotinic Acetylcholine Receptors

ALEXEY G. MUKHIN, DANIELA GÜNDISCH, ANDREW G. HORTI, ANDREI O. KOREN, GILLES TAMAGNAN, ALANE S. KIMES, JOANN CHAMBERS, D. BRUCE VAUPEL, SARAH L. KING, MARINA R. PICCIOTTO, ROBERT B. INNIS, and EDYTHE D. LONDON


Received September 29, 1999; accepted December 10, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

In an effort to develop selective radioligands for in vivo imaging of neuronal nicotinic acetylcholine receptors (nAChRs), we synthesized 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine (5-iodo-A-85380) and labeled it with \(^{125}\)I and \(^{123}\)I. Here we present the results of experiments characterizing this radiiodinated ligand in vitro. The affinity of 5-[\(^{125}\)I]iodo-A-85380 for \(\alpha_4\beta_2\) nAChRs in rat and human brain is defined by \(K_d\) values of 10 and 12 pM, respectively, similar to that of epibatidine (8 pM). In contrast to epibatidine, however, 5-iodo-A-85380 is more selective in binding to the \(\alpha_4\beta_2\) subtype than to other nAChR subtypes. In rat adrenal glands, 5-iodo-A-85380 binds to nAChRs containing \(\alpha_3\) and \(\beta_4\) subunits with 1/1000th the affinity of epibatidine, and exhibits 1/60th and 1/190th the affinity of epibatidine at \(\alpha_7\) and muscle-type nAChRs, respectively. Moreover, unlike epibatidine and cytisine, 5-[\(^{125}\)I]iodo-A-85380 shows no binding in any brain regions in mice homozygous for a mutation in the \(\beta_2\) subunit of nAChRs. Binding of 5-[\(^{125}\)I]iodo-A-85380 in rat brain is reversible, and is characterized by high specificity and a slow rate of dissociation of the receptor-ligand complex (\(t_{1/2}\) for dissociation \(\sim 2\) h). These properties, along with other features observed previously in in vivo experiments (low toxicity, rapid penetration of the blood-brain barrier, and a high ratio of specific to nonspecific binding), suggest that this compound, labeled with \(^{125}\)I or \(^{123}\)I, is superior to other radioligands available for in vitro and in vivo studies of \(\alpha_4\beta_2\) nAChRs, respectively.

Nicotinic acetylcholine receptors (nAChRs) are excitatory ligand-gated cation channels that are widely distributed in mammalian organisms, appearing in the central and peripheral nervous systems, neuromuscular junctions, and adrenal glands. The nAChR channel complex is composed of five protein subunits, which form a pore that is permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\) (Lindstrom, 1995; Holladay et al., 1997).

To date, \(\alpha\), \(\beta\), \(\gamma\), \(\delta\), and \(\epsilon\) subunits have been isolated and cloned from mammalian and avian tissues, with nine varieties of \(\alpha\) and four varieties of \(\beta\) subunits identified. The \(\alpha_1\), \(\beta_1\), \(\gamma\), \(\delta\), and \(\epsilon\) subunits form the neuromuscular junction receptor, the very first nAChR to be characterized. The other subunits (\(\alpha_2\–\alpha_9\) and \(\beta_2\–\beta_4\)) are found predominantly throughout the nervous system (Lindstrom, 1995; Holladay et al., 1997). This subunit diversity affords a large potential for a variety of nAChR subtypes, exhibiting distinct cation-conducting properties and pharmacological heterogeneity.

Based on binding properties and pharmacological sensitivity, major nAChR subtypes in mammalian brain can be categorized as \(\alpha\)-bungarotoxin-sensitive (\(\alpha_7\)) and \(\alpha\)-bungarotoxin-insensitive (e.g., \(\alpha_4\beta_2\)) (Lindstrom, 1995; Holladay et al., 1997). Accordingly, \(^{125}\)I-\(\alpha\)-bungarotoxin has been the radioligand of choice for in vitro characterization of the \(\alpha_7\) subtype of nAChR, whereas tritiated agonists, such as nico-
tine, acetylcholine, N-methylcarbamylcholine, cysteine, and epibatidine, have been used to study nAChRs of the latter group in vitro (Holladay et al., 1997). Of these ligands, epibatidine has the highest known affinity for α4β2 nAChRs, and outstanding in vitro binding characteristics (high specific-to-nonspecific binding ratio and slow kinetics of dissociation) (Dukat et al., 1993; Houghtling et al., 1995; Flores et al., 1996; Holladay et al., 1997; Stauderman et al., 1998; Xiao et al., 1998).

Radioligands developed for noninvasive in vivo imaging of α-bungarotoxin-insensitive nAChRs have exhibited shortcomings, such as poor subtype selectivity and high levels of nonspecific binding (Nyback et al., 1994). The radioligands [125I]IPH and [125I]IPH ((±)-exo-2-(2-[123I/125I]iodo-5-pyridyl)-7-azabicyclo[2.2.1]heptane), recently developed iodinated analogs of epibatidine, do not distinguish well between the α4β2 subtype and nAChRs containing α3 and β4 subunits (Davila-Garcia et al., 1997), much like epibatidine itself (Flores et al., 1996; Xiao et al., 1998). In contrast to α4β2 nAChRs, nAChRs containing α3 and β4 subunits, possibly in combination with α5 subunits, are distributed mostly in the peripheral nervous system and adrenal glands (Holladay et al., 1997). Therefore, high affinity for the latter receptors could contribute to the untoward cardiovascular effects of epibatidine and its analogs (Molina et al., 1997; Horti et al., 1997) and might limit the use of epibatidine-based compounds for imaging nAChRs in human subjects.

Recently, 3-(2S)-azetidinylmethoxy)pyridine (A-85380, Fig. 1) has been identified as a high-affinity nAChR ligand (Abreo et al., 1996). Subsequently, a chloro analog of A-85380, ABT-594 (Fig. 1), has been developed as a promising nonopioid analgesic having affinity for α4β2 nAChRs comparable to that of epibatidine, but lacking its toxicity (Bannon et al., 1998). In a search for improved radioligands suitable for noninvasive in vivo imaging of nAChRs, chemists in our group synthesized several halogenated analogs of A-85380 (Koren et al., 1998). Some of these compounds, particularly 5-iodo-A-85380 (Fig. 1), exhibited extremely high affinity for nAChRs in rat brain (Koren et al., 1998).

Initial evaluation of 5-[125I]iodo- and 5-[125I]iodo-A-85380 in vivo in mice (Musachio et al., 1998; Vaupel et al., 1998) and 5-[125I]iodo-A-85380 in rhesus monkey (Chefer et al., 1998) and baboon (Musachio et al., 1999) demonstrated that these radioligands readily crossed the blood-brain barrier, bound to cerebral nAChRs with high specificity, and had low toxicity. Here, we present an in vitro characterization of 5-[125I]iodo-A-85380, indicating that this ligand possesses excellent properties as a probe for studying the α4β2 nAChR subtype.

5-Iodo-A-85380 features high affinity for nAChRs, low nonspecific binding, slow dissociation from the receptor, and exceptionally high selectivity for the α4β2 subtype among the major mammalian nAChR subtypes. These properties, together with the results of in vivo studies with this ligand (Chefer et al., 1998; Musachio et al., 1998, 1999; Vaupel et al., 1998), suggest that 5-[125I]iodo-A-85380 may have exceptional potential as a radioligand for in vivo imaging of α4β2 nAChRs with single photon emission computed tomography.

**Materials.** 5-[125I]iodo-A-85380 and 5-[125I]iodo-A-85380 were prepared according to the literature procedures (Musachio et al., 1998; Horti et al., 1999). Specific activity of 5-[125I]iodo-A-85380 was determined as described previously (Horti et al., 1999). On the day of each synthesis, the specific activities of the three batches of 5-[125I]iodo-A-85380 used in these studies were 1550, 1980, and 2200 Ci/mmole, respectively. 125I-[a-Bungarotoxin, 125I-α-BTX, 100 Ci/mmole, [3H]cytisine (32 Ci/mmol), and [125I]Hlepipatidine (48 Ci/mmole) were obtained from New England Nuclear Corp. (Boston, MA). 5-Iodo-A-85380 was prepared by a published method (Koren et al., 1998). (±)-exo-2-(2-Iodo-5-pyridyl)-7-azabicyclo[2.2.1]heptane (IPH), a gift from Dr. Keller, was synthesized by Dr. J. L. Musachio at the Johns Hopkins University as described previously (Musachio et al., 1997). 3-(2S)-Azetidinylmethoxy)pyridine dihydrochloride (A-85380), α-bungarotoxin (α-BTX), (−)- and (±)-epibatidine, (−)-cytisine, (−)-epibatidine, (−)-cytisine, and (−)-apomorphine, and haloperidol were purchased from Research Biochemicals International (Natick, MA). Phystostigmine, disopropyl fluorophosphate (DFP), and all other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO). Male Fischer-344 and Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Rats, shipped at the age of 12 weeks, were housed in a temperature- and light-controlled vivarium for at least 2 weeks before being used for this study. Mice were generated by mating parents heterozygous for a mutation in the β2 nAChR subunit (Picciotto et al., 1995). Frozen Torpedo californica electric organ tissue was purchased from Marinus Inc. (Long Beach, CA). Frozen samples of postmortem tissue of human cerebral cortex (four subjects, 38 to 49 years of age, death from arteriosclerotic cardiovascular disease) were obtained from the Brain and Tissue Bank for Developmental Disorders (Baltimore, MD).

All animal procedures performed at the National Institute on Drug Abuse Brain Imaging Center were approved by the National Institute on Drug Abuse Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals, as endorsed by the National Institutes of Health. All animal procedures performed at the Yale University School of Medicine were approved by the Yale Animal Care and Use Committee.

**Membrane Preparation.** After CO₂ euthanasia and decapitation of the rats, brains were removed and prepared as follows. Brain tissue used for binding studies was obtained by a single cut just behind the inferior colliculi to exclude the cerebellum and medulla. In some experiments, specific brain regions and the adrenal glands were isolated. Frozen samples of Torpedo californica electric organ and postmortem human cerebral cortical tissue were thawed at room temperature for 30 to 60 min before membrane preparation. Total membrane fractions from all tissues were isolated by homogenization of the respective tissue with a Brinkmann Polytron homogenizer in 10 to 20 volumes of a HEPES-salt solution (HSS), containing HEPES (pH 7.4, 15 mM), 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, and 1.8 mM CaCl₂, followed by centrifugation at 40,000g for 10 min. The pellets were washed twice with HSS through rehomogenization and centrifugation at the same settings. Three additional washings...
were performed in the case of the total membrane fraction from rat adrenal glands and *Torpedo californica* electric organ. Crude membrane fractions (P2) were isolated as described previously (Koren et al., 1998), and were stored in aliquots at −70°C for at least 16 h but not more than 4 weeks before use. On the day of assay, pellets were thawed, homogenized in 30 volumes of HSS, and centrifuged at 40,000g for 10 min. The resultant pellets were resuspended in a freshly prepared HSS and used for binding assays.

**Binding Assays.** Assays were carried out in HSS at 22°C unless otherwise specified. Incubations were performed in polyethylene tubes except for the assays with 125I-α-BTX, for which borosilicate glass tubes were used. The HSS for the studies of membranes from *T. californica* electric organ contained 0.1% of BSA. Nonspecific binding was determined in the presence of 300 μM (−)-nicotine except for the assays with 125I-α-BTX, for which 1 μM α-BTX was used instead (see the figure legends for other specific conditions for particular binding assays). Incubation was terminated by filtration through Whatman GF/B glass fiber filters, presoaked in 1% polyethyleneimine, using a Brandel 48-channel cell harvester. Filters were washed three times with 3-ml aliquots of a rinse buffer (50 mM ethyleneimine, using a Brandel 48-channel cell harvester. Filters were through Whatman GF/B glass fiber filters, presoaked in 1% polyethylenimine, using a Brandel 48-channel cell harvester. Filters were washed three times with 3-ml aliquots of a rinse buffer (50 mM Tris·HCl, pH 7.4). Details of the procedure were described previously (Perry and Kellar, 1995; Zoli et al., 1998). In each experiment, sections from three to six independent experiments performed on the same membrane preparations that were used for the competition assays. Results of the kinetic experiments were analyzed using semilogarithmic plots and linear regression analysis. The values of equilibrium constant of dissociation, Kd, obtained from the kinetic studies, were calculated by the equation Kd = f_diss/k_assoc, where k_diss and k_assoc are the dissociation and association rate constants, respectively.

**In Vitro Autoradiography.** Sagittal slices (20 μm thick) at 0.4, 0.9, 1.4, 1.9, 3.9, and 4.2 mm lateral to midline from eight frozen Fischer-344 rat brains were obtained by sectioning in a cryostat at −20°C, and were thaw-mounted onto gelatin-coated slides. Sections were refrozen and kept frozen at −80°C until the day of the assay. Slices were preincubated for 20 min in 50 mM Tris·HCl buffer (pH 7.0) containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, and 1 mM MgCl2. Thereafter, they were incubated with 210 pM 5-[125I]iodo-A-85380 (specific activity 435 Ci/mmol) in the same buffer for 2 h at 25°C, then rinsed twice in ice-cold buffer for 5 min each and once in distilled water for 1 min. The slides were dried overnight in a vacuum desiccator. Nonspecific binding was assessed in adjacent slices incubated in 210 pM 5-[125I]iodo-A-85380 containing 10 μM nicotine bitartrate. Slices and appropriate 125I-standards were exposed to 3H-Hyperfilm for 5 weeks. Protein measurements were performed using a dye reagent kit (Bio-Rad, Richmond, CA) and BSA as a standard.

**Data Analysis.** Saturation binding data were subjected to Scatchard and linear regression analyses. Competition binding data were analyzed using nonlinear regression methods. Values of Kd were derived from the measured IC50 and Kd values for radioactive ligands using the Cheng-Prusoff equation Kd = IC50/(1 + IC50/F), where F is the concentration of unbound radioligand. The Kd values were obtained from three to six independent experiments performed on the same membrane preparations that were used for the competition assays.

**Results and Discussion**

**Kinetic and Equilibrium Binding Characteristics.** The specific binding of 5-[125I]iodo-A-85380, determined in rat brain membranes at 22°C and at a ligand concentration of 10 pM, reached one half the maximal (equilibrium) binding level in 67 ± 9 min (Fig. 2a). The binding was completely reversible and was characterized by a very slow dissociation (t½_diss = 132 ± 9 min) (Fig. 2b). The rate constants of association (k_assoc) and dissociation (k_diss) were (5.6 ± 1.4) × 10^−4/pM/min and (54 ± 6) × 10^−4/min, respectively. The Kd value, calculated as the ratio of k_diss to k_assoc, was 9.7 ± 1.8 pM.

Based on these data, subsequent equilibrium binding studies with 5-[125I]iodo-A-85380 were performed using a 4-h incubation at 22°C. At the lowest concentration of 5-[125I]iodo-A-85380 (ca. 1 pM) used in saturation studies, radioligand depletion of up to 30% was observed. To account for this depletion, the concentrations of free radioligand at equilibrium were calculated by reducing the concentration of total added radioactivity by the concentration of total bound radioactivity. The specific binding of 5-[125I]iodo-A-85380 in rat brain was saturable and was represented by a single population of binding sites over a radioligand concentration range of 1 to 500 pM (Fig. 3). The binding parameters (Kd and B_max) were 10.6 ± 0.3 pM and 29.5 ± 0.3 fmol/mg protein, respectively.
Similar binding characteristics were observed in four additional experiments performed on membranes from four independent preparations. The $K_i$ values in saturation studies with $5$-$[\text{^{125}I}]$iodo-A-85380 agreed with both the $K_i$ value of 9.7 pM derived from the kinetic experiments (Fig. 2) and the $K_i$ value of 11 pM obtained in our previous competition assays with ($\pm$)$[\text{^3H}]$epibatidine (Koren et al., 1998). The density of $5$-$[\text{^{125}I}]$iodo-A-85380 binding sites in rat forebrain was comparable to densities obtained using ($-$)$[\text{^3H}]$cytisine and ($-)$$[\text{^3H}]$nicotine (Lippiello and Fernandes, 1986; Pabreza et al., 1991; Flores et al., 1992), ligands that primarily label the $\alpha 4/\beta 2$ nAChR subtype in rat brain. Figure 3c depicts binding of $5$-$[\text{^{125}I}]$iodo-A-85380 in a postmortem sample of human brain cortex. As in rat brain, a single population of binding sites with a $K_i$ value of 11.6 ± 0.7 pM was observed. The density of binding sites in the human cortex was characterized by $B_{\text{max}} = 0.98 \pm 0.04$ pmol/g tissue, (53 ± 6 fmol/mg protein). This density was close to values obtained in studies of postmortem human cortex using ($\pm$)$[\text{^3H}]$epibatidine, ($-)$$[\text{^3H}]$nicotine, and ($-)$$[\text{^3H}]$cytisine (Silver et al., 1998).

Nonspecific binding of $5$-$[\text{^{125}I}]$iodo-A-85380 was proportional to the concentration of the radioligand (data not shown) and, at a concentration of 100 pM (approximately 10 times the $K_i$ value), constituted ca. 10% of total binding. Much of this value was attributable to binding of the radioligand to filter material. True nonspecific binding to tissue typically did not exceed 5% of total binding.

As seen in Table 1, the binding affinity of $5$-$[\text{^{125}I}]$iodo-A-85380 in rat brain membranes was moderately sensitive to variations in temperature during the incubation period. Thus, increasing the incubation temperature from 4–37°C resulted in a modest increase in the $K_i$ value (from 9.9 ± 0.8 to 20 ± 2 pM, respectively). It should be emphasized that incubation at 4°C required an extended incubation time (24 h) to reach equilibrium. The $4$-h incubation time routinely used seemed to be insufficient to reach equilibrium at this temperature and resulted in an inaccurate $K_i$ value of 15.5 ± 0.9 pM ($n = 2$). On the other hand, when incubating at 22°C, increasing the duration beyond 4 h (up to 18 h) did not produce significant changes in the observed $K_i$ or $B_{\text{max}}$ values (data not shown). This observation suggests that the $4$-h incubation time was sufficient to reach equilibrium at $22^\circ C$ and that neither the radioligand nor the receptor protein underwent degradation under the assay conditions used.

**Competition Studies.** In competition assays with $5$-$[\text{^{125}I}]$iodo-A-85380, affinities for nAChRs in rat brain for six well-characterized nicotinic agonists and four nicotinic antagonists (Table 2) fell into an order that was consistent with that previously observed in assays using other radioligands for $\alpha$-bungarotoxin-insensitive nAChRs (Pabreza et al., 1991; Decker et al., 1995; Houghtling et al., 1995). Compounds that did not effectively inhibit binding of $5$-$[\text{^{125}I}]$iodo-A-85380 (Koren et al., 1998). The density of $5$-$[\text{^{125}I}]$iodo-A-85380 (130 pM) was more than 10-fold higher than its $K_i$ value. Because more than 90% of the binding sites were occupied by the radioligand at this concentration, the results obtained effectively characterized the entire population of $5$-$[\text{^{125}I}]$iodo-A-85380 binding sites in rat forebrain. In all cases where $K_i$ values were determined, the pseudo-Hill coefficient values were close to 1. These findings support our previous conclusion that in the rat forebrain, over the concentration range used, $5$-$[\text{^{125}I}]$iodo-A-85380 labels a homog-

![Fig. 3. Scatchard plots of $5$-$[\text{^{125}I}]$iodo-A-85380 binding data obtained from saturation studies in P2 membrane fraction of Fischer-344 rat brain (A), in total membrane fraction of Sprague-Dawley rat brain (B), and in total membrane fraction of postmortem tissue of human cerebral cortex (C). Rat brain membranes (18–25 $\mu$g of protein) or human cortical membranes (28–30 $\mu$g of protein) were incubated for a total volume of, respectively, 1 or 0.5 ml with 1 to 500 pM $5$-$[\text{^{125}I}]$iodo-A-85380 for 4 h at $22^\circ C$. Data were analyzed as described in Experimental Procedures. Each point represents the mean from three or four replicates (S.E.M. < 7%). A, pooled data from two independent experiments performed on the same membrane preparation. Four additional experiments were performed on membranes from two separate membrane preparations. The $K_i$ and $B_{\text{max}}$ values (mean ± S.E.) obtained from these six experiments were $10.6 \pm 0.3$ pM and $160 \pm 25$ fmol/mg protein ($3.5 \pm 0.6$ pmol/g tissue), respectively. B, results of a single experiment. Similar binding characteristics were observed in four additional experiments performed on membranes from four independent preparations. The $K_i$ and $B_{\text{max}}$ values (mean ± S.E.) obtained from these five experiments were $10.0 \pm 0.2$ pM and $178 \pm 6$ fmol/mg protein ($3.9 \pm 0.2$ pmol/g tissue), respectively. C, results of a single experiment. Similar results were obtained in three additional experiments performed on membranes of human postmortem cortical tissue obtained from different subjects. The $K_i$ and $B_{\text{max}}$ values (mean ± S.E.) obtained from all four experiments were $11.6 \pm 0.7$ pM and $53 \pm 6$ fmol/mg protein ($0.98 \pm 0.04$ pmol/g tissue).
enaous population of agonist binding sites associated with α-bungarotoxin-insensitive nAChRs, presumably the α4β2 subtype.

Regional Distribution in Brain. To test our hypothesis that 5-[125I]iodo-A-85380 labels α4β2 nAChRs, we investigated distribution of the radioligand binding in the rat brain using both in vitro binding assays and autoradiography. In all brain regions studied, 5-[125I]iodo-A-85380 binding was characterized by interaction with a single population of homogenous binding sites (Scatchard plots not shown) with $K_d$ values close to 11 pM (Fig. 4). The average of $K_d$ values from all regions studied was 11.0 ± 0.2 pM. These constants closely agreed with the $K_d$ and $K_i$ values observed for the whole rat forebrain (Fig. 3, Table 2). The regional distribution of binding sites (Fig. 4) closely matched that of the α4β2 nAChR subtype measured previously in rat brain using (–)-[3H]cytisine and (±)-[3H]epibatidine (Pabreza et al., 1991; Houghtling et al., 1995); with the highest

TABLE 2

Inhibition of 5-[125I]iodo-A-85380 binding by nAChR and non-nAChR ligands

Rat brain P2 membrane fractions (10–11 μg of protein) were incubated in a total volume of 0.2 ml with 130 pM 5-[125I]iodo-A-85380 and 9 to 11 concentrations of competitors for 4 h at 22°C and analyzed as described in Experimental Procedures. The inhibition constants ($K_i$ values) were calculated by the Cheng-Prusoff equation from measured IC$_{50}$ values using a $K_d$ value of 10 pM for 5-[125I]iodo-A-85380 binding. In all assays, the pseudo-Hill coefficients ($n_H$) did not differ significantly from 1. Data represent mean ± S.E. obtained from four to six experiments per compound. Experiments were performed in duplicate.

![Fig. 4. Distribution of 5-[125I]iodo-A-85380 in Fischer-344 rat brain. The individual brain structures obtained from ten animals were pooled and total membrane fractions from each region were isolated as described in Experimental Procedures. Membrane samples from each brain region were incubated in a total volume of 0.25 ml with 1 to 500 pM 5-[125I]iodo-A-85380 for 4 h at 22°C. For all brain regions, Scatchard analyses produced data consistent with homogeneous populations of binding sites with similar $K_d$ values. Gray ($B_{max}$ values) and open ($K_d$ values) columns represent means obtained from three to four saturation assays performed on membranes from two separate preparations. For all regions studied, S.E.M. values were less than 10% except for the hypothalamus, where S.E.M. = 25%. Cb, cerebellum; Cx, frontal cortex; F-Br, forebrain; Hipp, hippocampus; Hyp, hypothalamus; SC, superior colliculus; Str, striatum; Th, thalamus.](image-url)
Procedures) previously described techniques (Bougis et al., 1986; Arneric et al., 1994). These techniques use $^{125}$I-$\alpha$-bungarotoxin and membrane fractions isolated either from the rat brain (for the $\alpha_7$ subtype) or from T. californica electrophlax (for muscle-type nAChRs). In our experiments, $^{125}$I-$\alpha$-bungarotoxin bound to a single population of binding sites in each of the two membrane fractions, exhibiting $K_d$ values of $1.5 \pm 0.2$ nM ($n = 3$) in rat brain and $2.3 \pm 0.3$ nM ($n = 3$) in electrophlax, consistent with published data (Zeghloul et al., 1988; Quik et al., 1996).

To complete the study on nAChR subtype selectivity, we developed an assay using (+)-[3H]epibatidine and a membrane fraction from rat adrenal glands to estimate the affinity of 5-iodo-A-85380 for nAChRs containing $\alpha_3$ and $\beta_4$ subunits. This assay was based on a previous study, which showed that (+)-[3H]epibatidine, in addition to its high affinity for $\alpha_4\beta_2$ nAChRs in rat brain (Houghtling et al., 1995), bound to cells stably expressing receptors of the $\alpha_3\beta_4$ subtype (Stauderman et al., 1998; Xiao et al., 1998) and to membranes from rat adrenal glands (Houghtling et al., 1995; Flores et al., 1997). Results of studies with bovine adrenals (Criadó et al., 1997; Wenger et al., 1997) and cultured rat pheochromocytoma cells (PC12) (Rogers et al., 1992; Henderson et al., 1994) suggested that the adrenal glands were rich in nAChR subtype(s) containing $\alpha_3$ and $\beta_4$

**Table 3**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_i$ at nAChR Subtype, nM (Ratio to $K_i$ at $\alpha_4\beta_2$)</th>
<th>$\alpha_4\beta_2$</th>
<th>$\alpha_3\beta_4x$</th>
<th>$\alpha_7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-IPH</td>
<td>0.027 $\pm$ 0.004 (1)</td>
<td>0.11 $\pm$ 0.04 (4)</td>
<td>30 $\pm$ 3 (1,100)</td>
<td>6.5 $\pm$ 0.1 (240)</td>
</tr>
<tr>
<td>(+)-Epibatidine</td>
<td>0.005 $\pm$ 0.001 (1)</td>
<td>0.049 $\pm$ 0.02 (6)</td>
<td>4.0 $\pm$ 0.5 (500)</td>
<td>7.5 $\pm$ 0.5 (900)</td>
</tr>
<tr>
<td>(+)-Nicotine</td>
<td>0.84 $\pm$ 0.13 (1)</td>
<td>100 $\pm$ 20 (120)</td>
<td>130 $\pm$ 10 (150)</td>
<td>1,000 $\pm$ 100 (1,200)</td>
</tr>
<tr>
<td>(+)-Cytisine</td>
<td>0.15 $\pm$ 0.02 (1)</td>
<td>54 $\pm$ 9 (300)</td>
<td>260 $\pm$ 20 (1,400)</td>
<td>190 $\pm$ 20 (1,100)</td>
</tr>
<tr>
<td>A-85380</td>
<td>0.017 $\pm$ 0.002 (1)</td>
<td>14 $\pm$ 2 (800)</td>
<td>17 $\pm$ 2 (1,000)</td>
<td>320 $\pm$ 20 (19,000)</td>
</tr>
<tr>
<td>5-Iodo-A-85380</td>
<td>0.010 $\pm$ 0.001 (1)</td>
<td>51 $\pm$ 5 (5,000)</td>
<td>250 $\pm$ 20 (25,000)</td>
<td>1,400 $\pm$ 200 (140,000)</td>
</tr>
</tbody>
</table>
subunits as well as α7 subtype, but expressed few, if any, receptors of the α4β2 subtype. Binding assays with (±)-[^3]H]epibatidine using rat adrenal gland membranes demonstrated a single population of binding sites (data not shown) with a $K_d$ value of $55 \pm 5$ pM ($n = 3$). (±)-[^3]H]Epibatidine binding to the rat adrenal gland membranes at a radioligand concentration of 0.5 nM was not blocked (data not shown) by α-bungarotoxin at concentrations as high as 10,000 times its affinity ($K_i$ = 1 nM) at α7 nAChRs (Quik et al., 1996). This observation suggests that, at conditions used for the competition assays, the binding of (±)-[^3]H]Epibatidine in rat adrenal glands does not reflect interactions with the α7 subtype. In light of the above-cited reports, the present data are consistent with the view that[^3]H]Epibatidine binds to nAChRs containing α3 and β4 subunits. Nonetheless, we cannot exclude the possibility that some portion of binding could reflect interactions with nAChRs including some other subunits (e.g., α5).

Results of the competition assays for different nAChR ligands/subtypes are summarized in Table 3. It is notable that the affinity of 5-ido-A-85380 for the α4β2 receptor exceeded its affinities for other major mammalian nAChR subtypes by three to five orders of magnitude. In this regard, 5-ido-A-85380 is vastly superior to all α4β2-specific nAChR ligands known to date, including (−)-cytisine, which has long been the ligand of choice for characterizing the α4β2 subtype. The high affinity of 5-ido-A-85380 for the α4β2 nAChR subtype measured in competition assays with (±)-[^3]H]Epibatidine was confirmed in additional assays with (−)-[^3]H]Cytisine, which yielded a nearly identical $K_i$ value of 10.5 ± 0.7 pM ($n = 3$), and was consistent with results from binding assays with radiolabeled 5-ido-A-85380 (Figs. 2 and 3), which provided $K_i$ values of 9.7 to 10.6 pM. It should be noted that the exceptionally high α4β2-subtype selectivity of 5-ido-A-85380 is consistent with previous studies on interactions of the structurally related compounds, A-85380 (Sullivan et al., 1998) and ABT-594 (Bannon et al., 1998), with the α4β2, α7, and muscle-type nAChRs.

The high selectivity of 5-ido-A-85380 for the α4β2 nAChR subtype was additionally confirmed by in vitro autoradiographic studies of the β2-knockout mouse brain. In brain from the wild-type mouse, distribution of 5-[125]Iido-A-85380 binding resembled that of [125]IIPH (Fig. 6), and the known pattern of distribution of α4β2 nAChRs. Unlike the case of the wild-type mouse, 5-[125]Iido-A-85380 did not exhibit binding in any brain region of mice homozygous for a mutation in the β2 subunit of nAChRs. Unlabeled were the medial habenula and interpeduncular nucleus (Fig. 6), which were labeled with [125]IIPH (Fig. 6), and which were labeled previously with [3]H]Epibatidine and [3]H]Cytisine in mice that had a mutation in the β2 subunit (Zoli et al., 1998). As shown previously (Perry and Kellar, 1995), the medial habenula and interpeduncular nucleus contain substantially higher densities of binding sites for [3]H]Epibatidine than for [3]H]Cytisine. Taken together, the results of studies of nAChRs in the medial habenula and interpeduncular nucleus suggest the presence of at least two distinct types of nAChRs in these regions. Of these types, only one, namely, that containing β2 subunit (presumably, the α4β2 nAChR subtype), can be labeled with 5-[125]Iido-A-85380. Thus, 5-[125]Iido-A-85380 appears to be more selective than either epibatidine or, more importantly, cytisine, which has been accepted heretofore as the most selective high-affinity ligand for the α4β2 nAChR subtype.

Available data do not rule out the possibility that 5-ido-A-85380 has high affinity for subtypes other than α4β2, which are far less abundant in mammalian brain. The investigation of such a possibility is a subject for future studies. Nonetheless, taking into consideration recent unpublished findings (K. J. Kellar, personal communication) that the parent compound, A-85380, has picomolar affinities toward several β2-containing nAChR subtypes (including the α3β2 subtype) but only nanomolar affinities toward β4-containing nAChRs (including α4β4), it is reasonable to assume that 5-ido-A-85380 would follow the same pattern. Additionally, taken together with the above-cited results from Dr. Kellar’s laboratory, the present observation of low affinity of A-85380 toward nAChRs in rat adrenal glands suggests that these receptors do not contain β2 subunits and are represented by α3β4x nAChRs, where x may or may not represent another subunit, e.g., α5. In this regard, it is noteworthy that the ratios of affinity for the α4β2 subtype to affinity for α3β4x nAChRs, derived in the present work for (±)-epibatidine, cytisine, (−)-nicotine, and A-85380 (Table 3), closely matched recently published data obtained for the same compounds using rat brain membranes (α4β2 nAChRs) and a cell line stably expressing α3β4 nAChRs (Xiao et al., 1998).

In summary, the present results demonstrate that 5-ido-A-85380 is an excellent ligand for studying nAChRs. It features extremely high affinity, slow dissociation from the receptor-ligand complex, high specific-to-nonspecific binding...
5-Iodo-A-85380, an α4β2 Subtype-Selective nAChR Ligand

ratio, and exceptionally high selectivity for the α4β2 nAChR subtype. Furthermore, the ability to produce 5-[125I]iodo-A-85380 with a specific activity of up to 2200 Ci/mmol makes it possible to detect nAChRs in the femtomolar range.

Recent in vivo studies with 5-[123I]iodo-A-85380 in the mouse (Musachio et al., 1998; Vauple et al., 1998) and rhesus monkey (Chefer et al., 1998) and baboon (Musachio et al., 1999) demonstrated that this radioligand readily crosses the blood-brain barrier, specifically accumulates in the brain regions enriched with the α4β2 nAChRs, and exhibits low toxicity. These data, together with the results of the present in vitro characterization of 5-iodo-A-85380, suggest that radiolabeled with 123I, this compound would be particularly promising for noninvasive imaging of nAChRs with single photon emission computed tomography in both animals and humans.

Acknowledgments

We thank Cindy Ambroz (National Institute on Drug Abuse Brain Imaging Center) for preparing this manuscript and excellent administrative support, and Louis Amici (Yale University) for technical assistance.

References


Send reprint requests to: Edythe D. London, Brain Imaging Center, National Institute on Drug Abuse, 5500 Nathan Shock Dr., Baltimore, MD 21224. E-mail: elondon@traucer.org