0026-895X/95/020280-08$3.00/0

MOLECULAR PHARMACOLOGY, 48:280–287 (1995).

Characterization of (±)-[^{3}H]Epibatidine Binding to Nicotinic Cholinergic Receptors in Rat and Human Brain

RICHARD A. HOUGHTLING, MARTHA I. DÁVILA-GARCÍA, and KENNETH J. KELLAR

Department of Pharmacology, Georgetown University School of Medicine, Washington, D. C. 20007

Received January 26, 1995; Accepted May 8, 1995

SUMMARY

Epibatidine is an alkaloid that was first isolated from the skin of the Ecuadoran frog *Epipedobates tricolor* by Daly *et al.* [*J. Am. Chem. Soc.* **102**:803–836 (1980)] and was found to have very high affinity for neuronal nicotinic receptors, where it acts as a potent agonist. Here we have measured and characterized the binding of (±)-[^{3}H]epibatidine to nicotinic receptors in rat brain. In rat forebrain homogenates, (±)-[^{3}H]epibatidine binds to two sites, with apparent affinities of 15 pM and 360 pM. Both of these binding sites have pharmacological profiles consistent with neuronal nicotinic receptors and a similar brain regional distribution. (±)-[^{3}H]Epibatidine also binds to sites in rat adrenal gland, suggesting that it can label a subtype of nicotinic receptor found in peripheral ganglia as well as the subtype that predominates in brain. In human cerebral cortex as well, (±)-[^{3}H]epibatidine binds two sites, one of which appears to have an affinity of <1 pM. We conclude that (±)-[^{3}H]epibatidine should be a very useful new tool for characterizing the properties and regulation of neuronal nicotinic receptors, including those not easily measurable with other radioligands.

EB, an azabicycloheptane alkaloid containing a chlorine moiety, was first isolated from the skin of the Ecuadoran frog *Epipedobates tricolor* by Daly *et al.* (2), who also elucidated its chemical structure (3). EB has since been synthesized by at least nine laboratories (4, 5) (for review, see Ref. 6).

The intense interest in EB stems at least in part from the discovery that it has very potent analgesic activity in mice that is not blocked by the opioid receptor antagonist naloxone (3, 7–10) but is blocked by the nicotinic receptor antagonist mecamylamine (7, 9, 10). Thus, EB appears to be a potent nicotinic agonist (7, 9). Consistent with this *in vivo* pharmacology, in brain homogenates EB does not compete effectively for opioid receptors (3, 7, 9) but does compete with very high affinity for nicotinic cholinergic receptors (7, 9, 11). In fact, the apparent affinity of EB for brain nicotinic receptors, measured in competition assays with (−)-[^{3}H]nicotine (9–11) or [^{3}H]cytisine (7, 12), is reported to be about 50 pM, making EB a ligand with one of the highest affinities known at neuronal nicotinic receptors. Interestingly, the two stereoisomers of EB are virtually equipotent, both in competition for binding at nicotinic receptors in brain homogenates and in their *in vivo* pharmacological effects (9–11).

Neuronal nicotinic receptors appear to be assembled from only two kinds of subunits, designated α and β. Rat and human brain express mRNA for at least six different α and three different β subunits (13–18) (for review, see Ref. 19), giving rise to the possibility for multiple subtypes of nicotinic receptors. Despite this potential diversity, the pharmacological characteristics and regional distribution of nicotinic receptors in rat and mouse brain measured with all known radiolabeled agonists, including [^{3}H]nicotine (20–23), [^{3}H]acetylcholine (22–24), *N*-[^{3}H]methylcarbamylcholine (25, 26), and [^{3}H]cytisine (27, 28), are virtually identical. This suggests that these ligands label the same subtype of nicotinic receptor in brain. In fact, studies using antibodies directed at specific nicotinic receptor subunits indicate that the receptors labeled by these agonists in rat brain are composed predominantly, if not exclusively, of α4 and β2 subunits (29, 30).

The high affinity of EB should make it an excellent probe with which to study neuronal nicotinic receptors and possibly to distinguish among different subtypes of neuronal nicotinic receptors that might exist in brain and peripheral tissues. Here we report the binding of [^{3}H]EB in rat and human brain and in rat adrenal gland. [^{3}H]EB binding in rat and human brain is best fit by a model for two binding sites, both of which have picomolar affinity for [^{3}H]EB and pharmacological characteristics of neuronal nicotinic receptors.

Materials and Methods

Drugs and reagents. [^{3}H]EB (56.5 Ci/mmol) was synthesized and supplied by DuPont-NEN (Boston, MA). Drugs were purchased

This work was supported by National Institutes of Health Grants DA06486, AG09973, and AG09884. M.I.D.-G. was supported by National Institutes of Health Fellowship DA05417. A preliminary report of part of this work has appeared (1).

ABBREVIATIONS: EB, (±)-epibatidine; DFP, diisopropyl fluorophosphate.

from Sigma Chemical Co. (St. Louis, MO), except for EB and dihydro-β-erythroidine, which were purchased from Research Biochemicals International (Natick, MA), and α-bungarotoxin, which was purchased from Biotoxins, Inc. (St. Cloud, FL).

Tissue preparation. Rat tissues were obtained from young, adult, male Sprague-Dawley rats. The forebrain was obtained by a single cut just behind the colliculi and excluded the cerebellum and medulla. In some experiments, specific brain regions and peripheral tissues were dissected. Normal human cerebral cortex was obtained from a brain bank. Transfected mouse L cell fibroblasts (M10 cells) that express α4/β2 nicotinic receptors under the control of a dexamethasone-inducible promoter (31) were provided by Dr. Jon Lindstrom (University of Pennsylvania Medical School). The cells were grown under the conditions described by Whiting *et al.* (31), in the absence or presence of 1 μM dexamethasone, for 3 or 4 days.

The tissues were suspended in 50 mM Tris·HCl buffer (pH 7.4 at room temperature) and homogenized with a Brinkmann Polytron homogenizer. The homogenate was washed twice by centrifugation at 35,000 × *g* for 10 min in fresh buffer, and the final pellet was resuspended in fresh buffer.

Binding assays. Binding assays were carried out as described previously (1). Briefly, aliquots of tissue homogenates equivalent to 5 or 10 mg of tissue (300 or 600 μg of protein) were added to tubes containing Tris·HCl buffer, pH 7.4, and [3H]EB at the indicated concentrations and were incubated for 4 hr at 24°. Preliminary studies indicated that the binding reaction reached a steady state within 4 hr even at low concentrations of [3H]EB. Nonspecific binding was determined in tissues incubated in the presence of 300 μM (−)-nicotine hydrogen tartrate. In all assays, reactions were started by the addition of tissue. Specific binding was defined as the difference between total binding and nonspecific binding. In drug competition studies, drugs were dissolved in buffer and added at the indicated concentrations. When acetylcholine was used in competition studies, tissues were preincubated in buffer containing 1 mM DFP to inhibit cholinesterases. Incubations were terminated by vacuum filtration through Whatman GF/C filters, which were mounted on a Brandel cell harvester and pre-wet with 0.5% polyethylenimine to reduce binding to the filter (24). The filters were washed three times with 4-ml aliquots of buffer and then counted in a scintillation counter.

To maintain appropriate assay conditions for this very high affinity ligand (i.e., to avoid binding of >25% of the ligand), final assay volumes varied from 5 ml (for [3H]EB concentrations below 200 pM) to 2.5 ml (for [3H]EB concentrations between 200 pM and 1 nM) and to 0.25 ml (for [3H]EB concentrations above 1 nM). By varying the volume according to the [3H]EB concentration being measured, an adequate amount of free ligand was maintained without using an unnecessarily large amount of the ligand. Separate studies established that varying the volume of the assay did not significantly affect measured binding parameters. All assays were carried out in duplicate or triplicate.

Data analysis. Concentrations of free [3H]EB used in calculations were corrected for the amount bound to tissue, which was always <25% of the added ligand. Saturation and competition binding data were analyzed by nonlinear regression analyses (Accufit Saturation Two-Site and Accufit Competition programs; Beckman Instruments, Fullerton, CA). The data were fit to one-site and two-site models. The simpler model was accepted unless the two-site model gave a statistically better fit of the data ($p < 0.05$, by F test). Hill coefficients calculated from [3H]EB binding saturation and drug competition studies were analyzed statistically using Student's t test.

Results

[3H]EB binding constants. Binding of [3H]EB to sites in rat forebrain homogenates was measured over a concentration range of 1 pM to 15 nM. Over this concentration range, specific binding of [3H]EB was saturable and reached a plateau at approximately 5 nM (Fig. 1A). Measurements over this wide concentration range were possible because [3H]EB has very high affinity for binding sites in brain homogenates and because nonspecific binding, defined as binding remaining in the presence of 300 μM (−)-nicotine, was almost nonexistent at [3H]EB concentrations below 1 nM and remained low (<20% of total) even at the highest concentrations examined (Fig. 1A, *lower inset*). The Hill coefficients (n_H) of [3H]EB saturation binding curves (Fig. 1A, *upper inset*) were consistently less than 1 ($p < 0.001$), suggesting that specific binding of [3H]EB represents more than one class of binding site. Consistent with this, Scatchard plots of [3H]EB binding in rat forebrain were markedly curvilinear (Fig. 1B), and saturation binding data were fit best to a model for two binding sites, with K_d values of 15 pM and 360 pM (Fig. 1, legend). In these studies using rat forebrain, each site appeared to com-

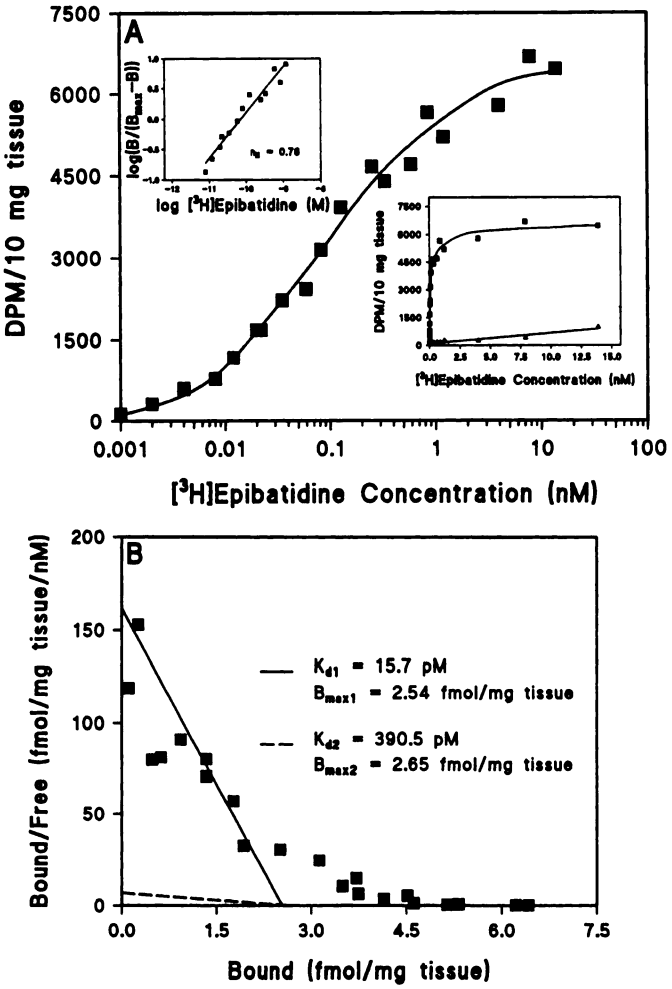

Fig. 1. Saturation binding of [3H]EB in rat forebrain membrane homogenates. A, Semilogarithmic plot of specific binding of [3H]EB (1 pM to 14 nM). *Upper inset*, Hill plot of specific binding. The Hill coefficient (n_H) from six such independent measurements was 0.73 ± 0.03 (mean ± standard error), which was significantly less than 1 ($p < 0.001$). *Lower inset*, binding curves showing specific binding (■) and nonspecific binding (▲). B, Scatchard plot of the specific binding shown in A. The K_d and B_{max} values shown were calculated from nonlinear regression analyses. The means ± standard errors (six experiments) for the K_d and B_{max} values were as follows: for site 1, 14.7 ± 3.8 pM and 2.5 ± 0.5 fmol/mg of tissue; for site 2, 360 ± 149 pM and 2.3 ± 0.4 fmol/mg of tissue, respectively.

prise approximately 50% of the total number of [^{3}H]EB binding sites and was equal to approximately 2.4 fmol/mg of tissue or 40 fmol/mg of protein (Fig. 1, legend).

At [^{3}H]EB concentrations of 22 pM and 360 pM, the half-times for association were approximately 20 min and 1 min, respectively, whereas the half-times for dissociation were approximately 3 hr and 2 hr, respectively (data not shown). The very slow rate of dissociation at 24° has impeded a thorough analysis of the kinetics of the binding reaction.

Pharmacology of [^{3}H]EB binding sites. Because of the possibility that [^{3}H]EB binds to two different sites in brain, the pharmacological profiles of the binding sites in rat forebrain were determined in studies in which drugs competed against either 21 pM or 335 pM [^{3}H]EB. Based on its two affinity constants (Fig. 1, legend), [^{3}H]EB at a concentration of 21 pM would be expected to occupy 58% of the higher affinity site but <10% of the lower affinity site, whereas at a concentration of 335 pM [^{3}H]EB would occupy nearly all of the higher affinity sites and 48% of the lower affinity sites.

In competition against 21 pM [^{3}H]EB, the rank order of potency for several well studied nicotinic agonists was cytisine > (−)-nicotine > acetylcholine[1] > carbachol (Fig. 2A; Table 1). The relative potencies of these drugs in competing for [^{3}H]EB binding sites are consistent with their relative affinities at brain nicotinic receptors radiolabeled by other nicotinic agonists (20–28). EB was approximately 10 times more potent than cytisine and 120 times more potent than (−)-nicotine in competing for binding (Fig. 2A; Table 1). (+)-Anatoxin-a, a freshwater cyanobacterium toxin (32) shown to be a high affinity agonist at neuronal nicotinic receptors (33, 34), was also quite potent at this site, competing with a potency similar to or slightly greater than that of (−)-nicotine (Fig. 2A; Table 1). The plant alkaloid (±)-anabasine competed with intermediate potency (Table 1).

The antagonists dihydro-β-erythroidine and curare competed effectively for binding against 21 pM [^{3}H]EB (Fig. 2B; Table 1); hexamethonium also competed, but with lower affinity (Fig. 2B; Table 1). In contrast, 2 μM α-bungarotoxin and 100 μM mecamylamine did not compete for [^{3}H]EB binding sites (Table 1); this is consistent with their lack of affinity for sites in brain labeled by other nicotinic agonists (20–27). The IC_{50} value of dihydro-β-erythroidine in competing for the higher affinity [^{3}H]EB binding sites was approximately 40 nM, which is comparable to its potency in competing for nicotinic sites in brain labeled by other agonists (23, 27, 28). In contrast, curare, with an IC_{50} value of 2 μM, appeared to be 10–20 times more potent in competing against [^{3}H]EB than against other radiolabeled agonists (for comparison, see Refs. 21–24, 27, and 28). Neither the cholinesterase inhibitor DFP nor the muscarinic antagonist atropine competed effectively for [^{3}H]EB binding sites (Table 1).

Nicotinic agonists (Fig. 3A; Table 1), as well as the antagonists dihydro-β-erythroidine and curare (Fig. 3B; Table 1), competed effectively for all of the binding sites in brain labeled by 335 pM [^{3}H]EB. In contrast, α-bungarotoxin and mecamylamine did not compete (Table 1). Again, neither DFP nor atropine affected [^{3}H]EB binding (Table 1). The rank order of potency of drugs in competing for sites labeled by 335 pM [^{3}H]EB was nearly identical to their rank order at the higher affinity site measured with 21 pM [^{3}H]EB, and again the IC_{50} of curare indicated that it was more potent in competing against [^{3}H]EB than against other radioligands.

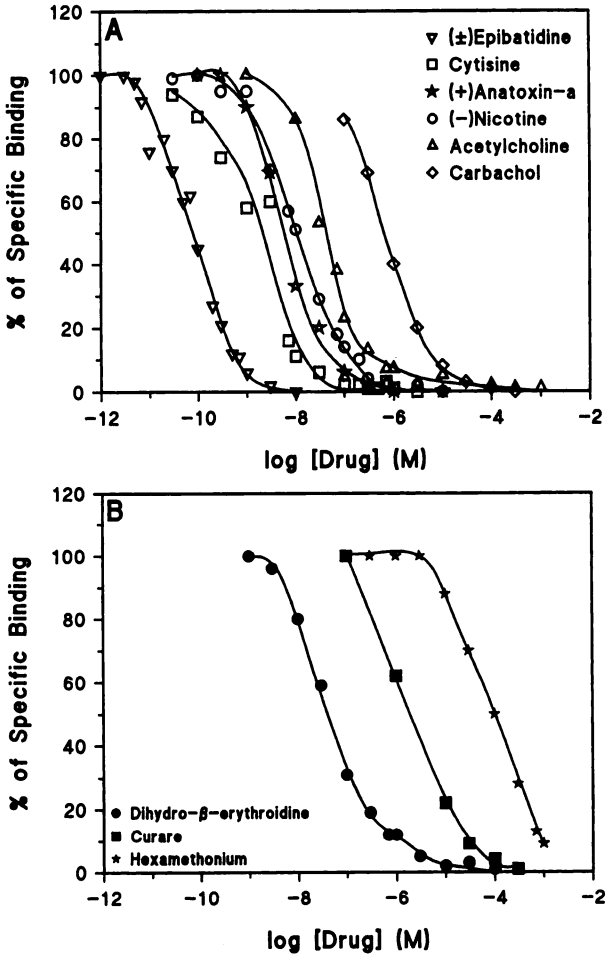

Fig. 2. Competition for nicotinic cholinergic binding sites in rat forebrain membranes labeled by 21 pM [^{3}H]EB. A, Competition by nicotinic agonists. B, Competition by nicotinic antagonists. When competition by acetylcholine was examined, tissues were preincubated with DFP to inhibit cholinesterases. Curves are representative of three to five independent measurements, except for the assay with hexamethonium, which was done once. The means ± standard errors of the IC_{50} and n_H values are shown in Table 1.

The Hill coefficients of several drugs, including (−)-nicotine, cytisine, dihydro-β-erythroidine, and curare, in competition with [^{3}H]EB were significantly less than 1 (Table 1). Nevertheless, none of the binding competition curves fit a model for two binding sites significantly better than a model for one site, so the simpler model was accepted for the present time.

Stereospecificity of [^{3}H]EB binding sites. The [^{3}H]EB used in this study was racemic, as was the unlabeled EB used in the competition studies shown in Figs. 2 and 3. Therefore, to determine whether the [^{3}H]EB binding sites distinguish between the two isomers of EB and whether differences in the binding affinities of the two isomers could account for the apparent second binding site for [^{3}H]EB, competition between (+)-EB and (−)-EB was compared at both the high affinity and low affinity [^{3}H]EB binding sites in brain. As shown in Fig. 4 and Table 1, no differences in the IC_{50} values of the stereoisomers of EB or the shapes of their binding

[1] Acetylcholine competition studies were carried out in the presence of DFP to inhibit cholinesterases.

TABLE 1

Competition by drugs for binding sites in rat forebrain homogenates labeled by 21 pM [^{3}H]EB or 335 pM [^{3}H]EB

IC_{50} values and Hill coefficients (n_H) were determined by nonlinear regression analyses. Values are the means ± standard errors of three to five independent measurements, except for those for (±)-anabasine and hexamethonium, which are values from a single experiment.

Drug	21 pM [^{3}H]EB		335 pM [^{3}H]EB	
	IC_{50}	n_H	IC_{50}	n_H
	nM		nM	
Agonists				
(+)-EB	0.061 ± 0.006	1.03 ± 0.04	0.34 ± 0.025	0.94 ± 0.03
(−)-EB	0.062 ± 0.009	0.92 ± 0.06	0.37 ± 0.014	0.97 ± 0.06
(±)-EB	0.078 ± 0.010	0.93 ± 0.06	0.39 ± 0.018	0.92 ± 0.04
Cytisine	0.9 ± 0.2	0.76 ± 0.05[a]	11.4 ± 0.5	0.74 ± 0.02[a]
(+)-Anatoxin-a	6.1 ± 1.2	1.02 ± 0.05	32.8 ± 8.3	0.75 ± 0.13
(−)-Nicotine	9.4 ± 0.2	0.73 ± 0.02[a]	55.3 ± 10.4	0.71 ± 0.05[a]
(+)-Nicotine	55.2 ± 39.0	0.78 ± 0.28	1,633 ± 227	0.93 ± 0.12
Acetylcholine	47.3 ± 15.9	0.87 ± 0.24	352.6 ± 71.6	1.24 ± 0.04
(±)-Anabasine	118.6	1.06	1,200	0.79
Carbachol	670.8 ± 142.6	0.87 ± 0.07	2,766 ± 970	1.01 ± 0.09
Antagonists				
Dihydro-β-erythroidine	39.0 ± 5.4	0.74 ± 0.04[a]	129.9 ± 21.2	0.78 ± 0.004[a]
Curare	2,025 ± 582	0.71 ± 0.07	4,500 ± 900	0.63 ± 0.05[a]
Hexamethonium	90,200	0.88	ND[b]	ND
α-Bungarotoxin	>2,000		>2,000	
Mecamylamine	>100,000		>100,000	
Other				
DFP	>100,000		>100,000	
Atropine	>100,000		>100,000	

[a] Hill coefficient significantly different from 1 ($p < 0.01$).
[b] ND, not determined.

curves were found at either binding site, indicating that the binding sites do not distinguish between the stereoisomers of EB. In contrast, the binding sites do discriminate between the stereoisomers of nicotine, with (−)-nicotine being approximately 6 times more potent at the higher affinity site and 30 times more potent at the lower affinity site (Fig. 4; Table 1).

Distribution of [^{3}H]EB binding sites in brain. Two concentrations of [^{3}H]EB were used to measure the distribution of its binding sites in grossly dissected regions of rat brain. At a concentration of 44 pM, [^{3}H]EB would be expected to occupy about 74% of its high affinity binding sites and 11% of its lower affinty sites. At a concentration of 460 pM, it would occupy virtually all of the high affinity sites and approximately 56% of the lower affinity site. As shown in Fig. 5, the relative distributions of the higher affinity and lower affinity binding sites in rat brain were quite similar. In both cases, the thalamus had the highest number of sites, whereas the cerebral cortex, striatum, and midbrain had an intermediate number of sites and the medulla, cerebellum, and hippocampus had the lowest number of sites.

[^{3}H]EB also bound with high affinity in human cerebral cortex (Fig. 6A). Binding over a concentration range of 1 pM to 9 nM was fit best by a two-site model (Fig. 6B). In this tissue the density of sites was lower than in rat cortex but the dissociation constant of [^{3}H]EB at the higher affinity site (K_{d_1}) appeared to be even higher (Fig. 6B). However, it should be noted that, because the specific radioactivity of the ligand imposes a lower limit on the concentration of [^{3}H]EB that can be measured, we cannot reliably measure binding at concentrations below 1 pM with the currently available ligand. Therefore, the K_d of the higher affinity [^{3}H]EB binding site in human cerebral cortex reported here is an extrapolated value.

[^{3}H]EB binding sites outside of brain. We measured [^{3}H]EB binding sites in several tissues outside of the brain. Specific binding of [^{3}H]EB in rat adrenal gland was measurable at both the high and low concentrations of [^{3}H]EB (Fig. 5), with specific binding representing 50–70% of total binding. However, the amount of binding in adrenal gland was much lower than that in rat forebrain. In contrast to the adrenal gland, no specific binding of [^{3}H]EB was measurable in kidney, liver, or fat cells (data not shown).

[^{3}H]EB binding sites in M10 cells. To help address questions concerning the identity and nature of the [^{3}H]EB binding sites in brain, we measured binding of [^{3}H]EB to membranes from M10 cells. These transfected mouse L cell fibroblasts express nicotinic receptors composed of α4 and β2 subunits, under the influence of a dexamethasone-inducible promoter (31). In M10 cells that had not been induced by exposure to dexamethasone, [^{3}H]EB binding was very low (<4 fmol/mg of protein), whereas in induced cells the binding was approximately 86 fmol/mg of protein (Fig. 7). More importantly, saturation analysis of [^{3}H]EB binding to M10 cells under assay conditions identical to those used to measure binding in brain was best fit by a model for a single site, with a K_d of 4 pM (Fig. 7).

Discussion

The very high affinity and exceptionally low nonspecific binding of [^{3}H]EB have allowed measurements of its binding sites in brain over a very wide range of concentrations. Over the range of 1 pM to 15 nM, [^{3}H]EB saturation binding in rat forebrain homogenates had a Hill coefficient significantly less than 1 and was fit best by a model for two binding sites, with K_d values of 15 pM and 360 pM. These two sites appeared to be of equal density in rat forebrain homogenates. It should be noted, however, that with the currently used ^{3}H-labeled ligand and techniques it is not practical to test [^{3}H]EB con-

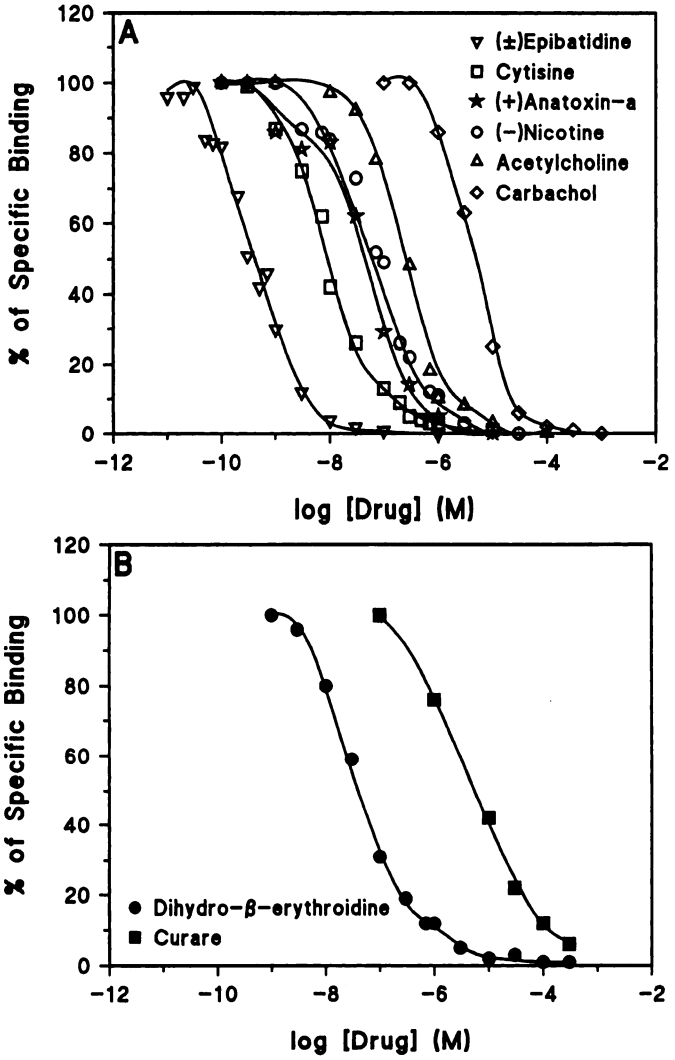

Fig. 3. Competition for nicotinic cholinergic binding sites in rat forebrain membranes labeled by 335 pM [^{3}H]EB. A, Competition by nicotinic agonists. B, Competition by nicotinic antagonists. When competition by acetylcholine was examined, tissues were preincubated with DFP to inhibit cholinesterases. Curves are representative of three to five such independent measurements. The means ± standard errors of the IC_{50} and n_H values are shown in Table 1.

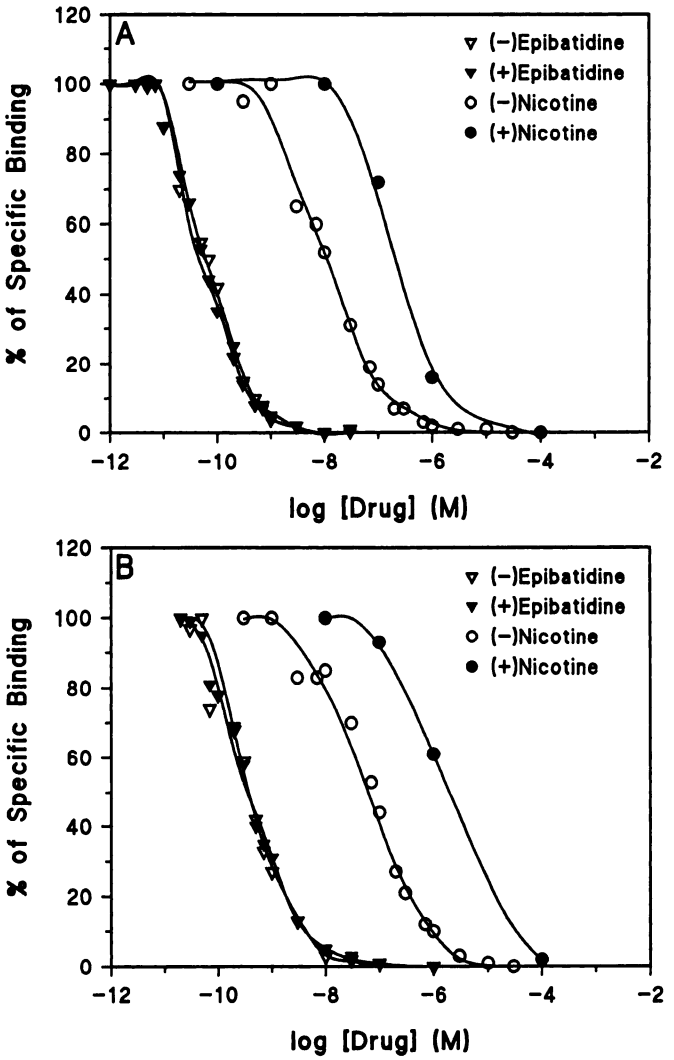

Fig. 4. Competition by the stereoisomers of EB and nicotine for nicotinic cholinergic binding sites in rat forebrain membranes labeled by 21 pM [^{3}H]EB (A) or 335 pM [^{3}H]EB (B). The means ± standard errors of the IC_{50} and n_H values are shown in Table 1.

centrations below 1 pM or to consider more complex models, such as those with three or more binding sites.

Both of the [^{3}H]EB binding sites measured here have pharmacological characteristics consistent with a neuronal nicotinic cholinergic receptor. Thus, all of the nicotinic agonists tested compete with high affinity for all of the sites labeled by [^{3}H]EB and, among nicotinic antagonists, dihydro-β-erythroidine, curare, and hexamethonium also compete effectively for [^{3}H]EB binding sites. Among all of the drugs tested, curare in particular, with IC_{50} values of 2 and 4.5 μM at the two [^{3}H]EB binding sites, appears to be much more potent competing against [^{3}H]EB than against any other radiolabeled agonist (e.g., compare with Refs. 22–24, 27, and 28).

The inability of α-bungarotoxin to compete for [^{3}H]EB binding sites indicates that neither site measured here represents the nicotinic receptor composed of α7 subunits, which has high affinity for α-bungarotoxin (35, 36). Unfortunately, competition by neuronal bungarotoxin could not be tested, because this toxin was unavailable at the time of these studies. The inability of the cholinesterase inhibitor DFP or the muscarinic antagonist atropine to compete indicates that [^{3}H]EB binding sites do not include cholinesterase enzymes or muscarinic receptors.

The rank orders of potency of drugs competing against 21 pM and 335 pM [^{3}H]EB were nearly identical. Furthermore, none of the binding competition curves fit a model for two sites better than a model for one site. One explanation for this may be that the higher affinity [^{3}H]EB binding site exerts a strong but artifactual influence on the apparent potency of drugs at the second site, as measured with the higher concentration of [^{3}H]EB. For example, the higher concentration of [^{3}H]EB used here occupies nearly all (96%) of the first (higher affinity) binding site as well as 48% of the second (lower affinity) site; consequently, if the forebrain homogenates used here contain nearly equal numbers of the two binding sites, the lower affinity sites would represent <33% of all nicotinic sites bound by 335 pM [^{3}H]EB. In addition, at the higher [^{3}H]EB concentration the competition curves for drugs competing at the higher affinity site would

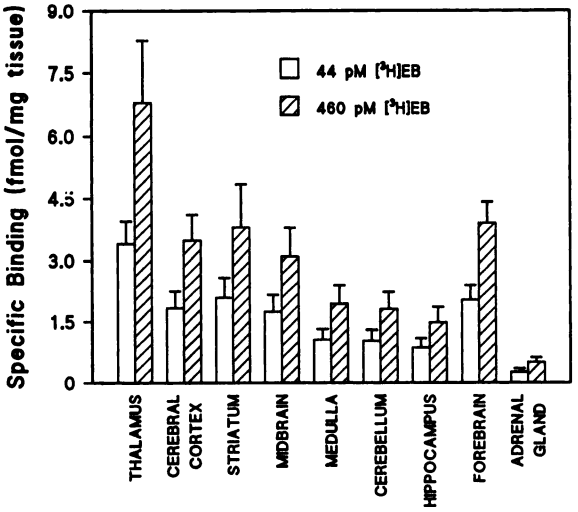

Fig. 5. Relative distribution of binding sites labeled by 44 pM and 460 pM [^{3}H]EB in homogenates from several areas of rat brain and adrenal gland. Values are the means ± standard errors from four to six independent measurements.

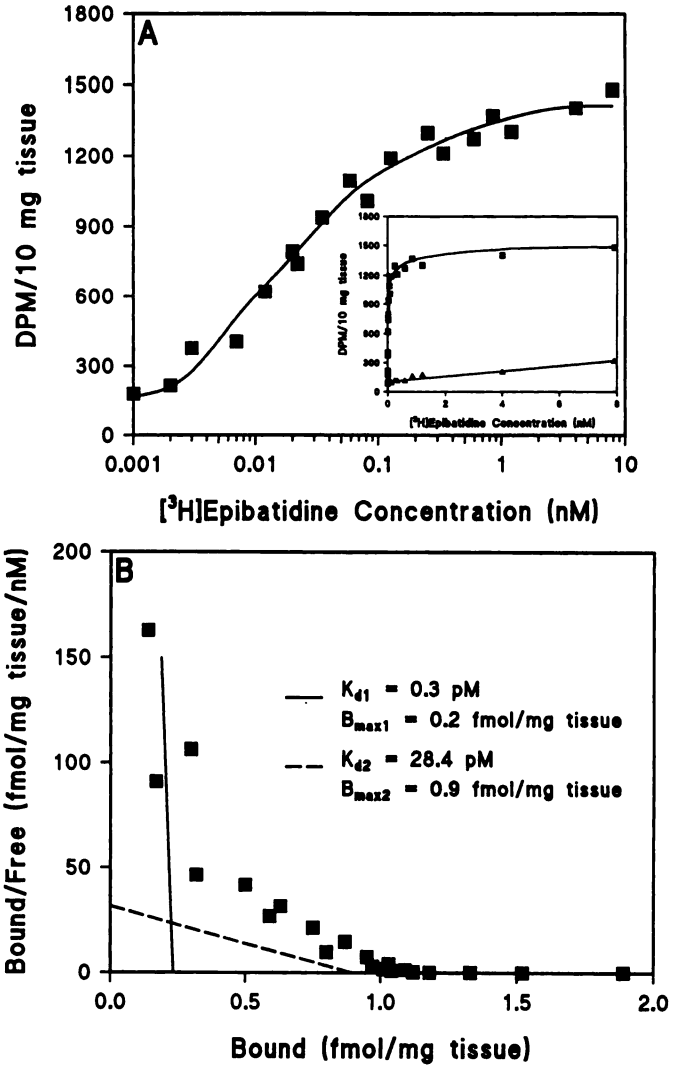

Fig. 6. Saturation binding of [^{3}H]EB in membrane homogenates from human cerebral cortex. A, Semilogarithmic plot of specific binding of [^{3}H]EB (1 pM to 9 nM). *Inset*, binding curves showing specific binding (■) and nonspecific binding (▲). B, Scatchard plot of the specific binding shown in A. The K_d and B_{max} values shown were calculated from nonlinear regression analysis.

be shifted to the right (37), approaching or converging with the curves for the lower affinity site. Nevertheless, the Hill slopes for several drugs, including cytisine, (−)-nicotine, dihydro-β-erythroidine, and curare, were significantly less than 1, suggesting that these drugs might distinguish between two or more [^{3}H]EB binding sites. A more accurate quantitative assessment of the pharmacology of the second [^{3}H]EB nicotinic site in brain might be achieved by masking the first site with a highly selective drug, once such a drug is identified.

The nicotinic receptor binding sites labeled by [^{3}H]EB do not distinguish between the (−)- and (+)-stereoisomers of EB. This lack of stereospecificity is somewhat unusual for ligand-receptor interactions, but it is consistent with the lack of stereospecificity of EB in competition against (−)-[^{3}H]nicotine for binding sites (9–11) and in its pharmacological effects *in vivo* and *in vitro* (8–10). This lack of stereospecificity probably indicates that the chiral center of EB does not participate in a crucial way in its binding to nicotinic receptors (9). On the other hand, the sites that bind [^{3}H]EB do discriminate clearly between the stereoisomers of nicotine and, consistent with the pharmacology of nicotinic receptors measured with other radioligands, (−)-nicotine is more potent than (+)-nicotine in competing with [^{3}H]EB at both binding sites.

The relative distributions of the two [^{3}H]EB binding sites in rat brain, measured here with 44 pM and 460 pM [^{3}H]EB, are similar to each other and to the distribution of nicotinic sites measured with other radiolabeled nicotinic agonists (22, 23, 27). Studies with antibodies directed at the subunits of nicotinic receptors have shown that the predominant receptor with high affinity for (−)-[^{3}H]nicotine and [^{3}H]cytisine in rat brain is composed of α4 and β2 subunits (29, 30). In M10 cells, which presumably express only α4/β2 nicotinic receptors, [^{3}H]EB binds with a K_d of 4 pM, which is similar to the K_d of the higher affinity site in rat brain. Therefore, it is almost certain that at least one of the [^{3}H]EB binding sites measured here in rat brain is an α4/β2 nicotinic receptor (and the same may be true in human brain). However, an important question concerns the identity of the second binding site; that is, does it represent a second nicotinic receptor or a second site on the same receptor? The high affinity α4/β2 nicotinic receptor in brain contains two α4 subunits and three β2 subunits (17, 18). The sites to which agonists bind are located on the α subunits of nicotinic receptors; thus, it is possible that the two different [^{3}H]EB binding sites represent the two individual α subunits. If this were the case, however, the two α4 subunits might be expected to have the same affinity for agonists, because with this 2:3 stoichiometry the α4 subunits would have identical flanking subunits (either β2 and β2 or β2 and α4) and thus would be mirror images of each other. Furthermore, although it is possible that the binding of [^{3}H]EB to the first α4 subunit might influence its affinity at the second α4 subunit, either by steric effects or by a conformational change in the receptor protein, the binding of all other radiolabeled agonists fits a one-site model (21–27, 38). Finally, under binding assay conditions identical to those used for brain, [^{3}H]EB binding in M10 cells, which express

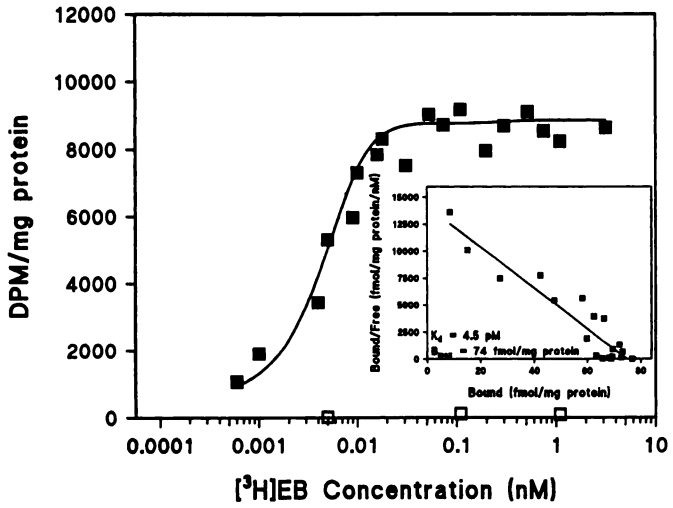

Fig. 7. Saturation binding of [^{3}H]EB to membrane homogenates of M10 cells. A semilogarithmic plot of specific binding of [^{3}H]EB (1 pM to 3 nM) to homogenates from cells that had been grown in the absence (□) or presence (■) of 1 μM dexamethasone for 4 days, to induce the expression of α4/β2 nicotinic receptors, is shown. *Inset*, Scatchard plot of the specific binding of [^{3}H]EB. Binding curves were best fit to a model for a single site, with a K_d of 4.1 ± 0.3 pM and a B_{max} of 86 ± 22 fmol/mg of protein (three experiments).

only the α4/β2 receptor, fits best to a one-site model. This strongly indicates that the second binding site in brain is not a second site on or intrinsic to the α4/β2 receptor.

The simplest explanation for the two [^{3}H]EB binding sites in brain, of course, is that the second site represents binding to a second nicotinic receptor subtype. In this regard, it is important to note that [^{3}H]EB clearly binds to sites in rat adrenal gland membranes, which, based on measurements in rat PC-12 cells (39, 40), are thought to contain receptors composed of α3 or α5 subunits but not α4 or α2 subunits. Moreover, in the human neuroblastoma cell line IMR-32, which has been shown to contain α3 subunits (41) but so far not α4 subunits, [^{3}H]EB binds with very high affinity, whereas [^{3}H]cytisine binding is nearly absent.[2] Finally, McKay *et al.* (42) recently reported that [^{3}H]EB binds to sites in chick retina that can be immunoprecipitated with an antibody directed against an α3 subunit. The affinity of EB for this site was reported to be 160 pM (42). Additional studies will be required to determine the identity of the second [^{3}H]EB binding site in rat brain and, if it is a second nicotinic receptor subtype, to determine its subunit composition.

In human cerebral cortex also, [^{3}H]EB appears to bind to two sites, one of which appears to have an affinity that we estimate to be <1 pM. Although this is a highly extrapolated value, it should be noted that EB was recently reported to have an affinity in the femtomolar range at a neuronal nicotinic receptor in human SHSY-5Y cells (43).

In conclusion, [^{3}H]EB is a new radioligand with exceptionally high affinity for neuronal nicotinic receptors. Its potential for measurements of more than one subtype of neuronal nicotinic receptor should make it very useful for characterizing and comparing the pharmacological properties, densities, and regulation of neuronal receptors in brain, peripheral ganglia, and neuronal cell lines. Furthermore, EB has the potential to be iodinated with ^{125}I, which would allow assessment of nicotinic receptors in tissues with very low receptor densities and/or at ligand concentrations well below 1 pM. In addition, [^{3}H]EB labels nicotinic receptors in mouse brain *in vivo* (44); thus, because of its extremely low nonspecific binding EB, if appropriately labeled with ^{18}F or ^{123}I, could be an excellent ligand for imaging and quantifying nicotinic receptors in human brain *in vivo*.

[2] M. I. Dávila-García, R. A. Houghtling, and K. J. Kellar, unpublished observations.

Acknowledgments

We thank Dr. Stephen D. Hurt of DuPont-NEN for making [^{3}H]EB available and Dr. Jon Lindstrom for generously providing the M10 cells.

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Send reprint requests to: Kenneth J. Kellar, Department of Pharmacology, Georgetown University School of Medicine, Washington, DC 20007.