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Comparative Pharmacology of Epibatidine: A Potent Agonist for Neuronal Nicotinic Acetylcholine Receptors

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SUMMARY

Pharmacological properties of the (+)- and (−)-isomers of synthetic epibatidine, exo-2-(6-chloro-3-pyridyl)-7-azabicyclo-[2.2.1]heptane, were compared with nicotine and acetylcholine on several subtypes of chicken and human nicotinic acetylcholine receptors (AChRs). Both isomers of epibatidine behaved as extremely potent full agonists on chicken ($\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, $\alpha7$, and $\alpha8$) and human ($\alpha3\beta2$, $\alpha3\beta4$, and $\alpha7$) neuronal AChRs expressed in *Xenopus* oocytes. Currents induced by epibatidine were effectively blocked by the nicotinic antagonists hexamethonium and mecamylamine. Apparent affinity was 100 to 1000-fold higher for epibatidine than for nicotine or acetylcholine. EC_{50} values ranged from 1 nM (for homomeric chicken $\alpha8$) to 2 μM (for homomeric chicken $\alpha7$). Epibatidine showed comparatively lower affinity for muscle-type AChRs from *Torpedo* and humans (EC_{50} values, 1.6 and 16 μM respectively). In binding assays, epibatidine was used on AChR subtypes immunoisolated from chicken brain and retina ($\alpha4\beta2$, $\alpha7$, and $\alpha8$), the human neuronal cell line SH-SY5Y ($\alpha3$ and $\alpha7$), *Torpedo* electric organ ($\alpha1\beta1\gamma\delta$), or the human rhabdomyosarcoma cell line TE671 ($\alpha1\beta1\gamma\delta$). Both isomers of epibatidine exhibited extremely high affinity for all neuronal AChRs tested, with K_i values ranging from 0.6 pM (human $\alpha3$ AChRs) to 0.6 μM (chicken $\alpha7$ AChRs). In contrast, epibatidine had lower affinity for *Torpedo* muscle-type AChRs (K_i ~5 μM). Racemic [^{3}H]epibatidine was an effective labeling reagent for human $\alpha3\beta2$ AChRs, exhibiting a K_D (0.14 nM) similar to the K_i values observed for unlabeled (+)-epibatidine (0.23 nM) or (−)-epibatidine (0.16 nM).

Epibatidine, an alkaloid obtained from skin extracts of the Ecuadoran poison frog *Epipedobates tricolor* (1), has proved to be 200-fold more effective than morphine in preventing nociception (2, 3). Nine chemical syntheses of epibatidine have been published (e.g., 4–7, for a review see Ref. 8). Epibatidine-induced analgesia is most likely mediated by AChRs because it is blocked by the selective nicotinic antagonist mecamylamine (2, 9, 10). Moreover, epibatidine effectively competes with nicotine and cytisine in rat brain preparations (2, 11, 12), it acts as a potent nicotinic agonist (12–15), and it has structural similarities with acetylcholine and nicotine (Fig. 1). Epibatidine analgesic activity resembles the analgesic activity that has been previously shown for nicotine (16, 17).

The physiological effects of epibatidine need to be correlated with specific AChR subtypes. AChRs are diverse in subunit compositions, pharmacological properties, and tissue distribution. To date, the gene family of vertebrate AChRs subunits includes 16 subunits: $\alpha1$–9, $\beta1$–4, γ, δ, and ϵ (for reviews see Refs. 18–20). Most α subunits are particularly associated with cholinergic ligand binding, but the structural subunits also contribute to ligand binding specificity. There are embryonic ($\alpha1\beta1\gamma\delta$) and adult ($\alpha1\beta1\epsilon\delta$) forms of muscle AChR, but it is unknown whether there are similar developmental changes in the subunit compositions of neuronal AChRs. Many potential subtypes of neuronal AChRs might be formed from combinations of the known subunits. Precise subunit compositions of most subtypes are unclear, although predominant subtypes of some regions are known. In brain, the predominant subtype with high affinity for nicotine is composed of $\alpha4$ and $\beta2$ subunits, whereas the predominant subtype with high affinity for αBgt contains $\alpha7$ subunits (21–22). In autonomic ganglia and retinae, the predominant AChRs contain $\alpha3$ subunits in combination with $\beta4$ or $\beta2$ and $\alpha5$ subunits (23–25). These AChRs have lower affinity for nicotine than do $\alpha4\beta2$ AChRs. Chicken retina contains a relative abundance of AChRs containing $\alpha8$ or both $\alpha7$ and $\alpha8$ subunits (21, 26). $\alpha8$ AChRs have lower affinity for αBgt than do $\alpha7$ AChRs or muscle AChRs, but they have much higher affinity for small cholinergic ligands than do $\alpha7$ AChRs (27).

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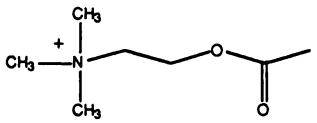
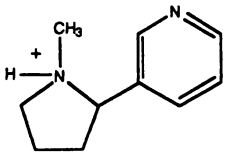
ABBREVIATIONS: αBgt, α-bungarotoxin; AChR, acetylcholine receptor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody.

Acetylcholine



Nicotine



Epibatidine

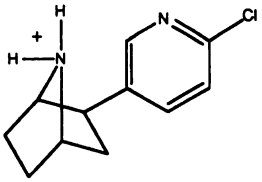


Fig. 1. Comparison of the structures of acetylcholine, nicotine, and epibatidine.

Recent studies indicate the presence of AChR subunits in some nonneuronal tissues (28, 29).

Different combinations of AChR subunits show significant functional diversity (for reviews see Refs. 18 and 30). Not only does sensitivity to agonists vary significantly between subunit combinations, but also the mode of action of a ligand may be changed from full agonist to partial agonist or antagonist (31, 32).

Therefore, to obtain an extended pharmacological profile for epibatidine, it is necessary to test its action on tissues that contain different AChR subtypes. Alternatively, it is possible to use as an assay recombinant AChRs with different combinations of the subunits in a heterologous expression system. Both approaches have limitations. A complication of testing different tissues containing AChRs is that many neuronal preparations contain more than one combination of the subunits, and the pharmacological profile obtained is an average of the properties of the different AChRs. A complication of studying the expression of combinations of subunit cDNAs is that the functional properties of these combinations do not always completely match those of the native AChRs. Nevertheless, the recombinant approach has the advantage of dealing with AChRs of known subunit compositions.

We report that both (+)- and (−)-isomers of synthetic epibatidine behave as potent agonists for several of the predominant subtypes of neuronal AChRs ($\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, and $\alpha8$) but exhibit lower affinity for muscle-type ($\alpha1\beta1\gamma\delta$) AChRs and for homomers of neuronal $\alpha7$ subunits when they are expressed in *Xenopus* oocytes. Epibatidine is a much more potent agonist than acetylcholine or nicotine on $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, and $\alpha8$ homomers. From binding studies with native and heterologously expressed AChRs, extremely high binding affinity of epibatidine for $\alpha3$-, $\alpha4$-, and $\alpha8$-containing AChRs was found in contrast to $\alpha7$- and $\alpha1$-containing AChRs. This will prove to be very valuable for research purposes. The recognition of those AChR subtypes with very high affinity and those with relatively low affinity for epibatidine will be useful for understanding its pharmacological effects in intact animals.

Materials and Methods

DNAs. Chicken $\alpha4$ and $\beta2$ (33) and $\alpha7$ and $\alpha8$ (21) and human $\alpha7$ (34), $\alpha1$ (35), and $\beta2$ (36) cDNAs were described previously. Human $\alpha3$ was cloned from a human brain library, and $\beta4$ was cloned from the human neuroblastoma cell line SH-SY5Y.[1] *Torpedo* $\alpha1$, $\beta1$, γ, and δ cDNAs were obtained through the generosity of Dr. Toni Claudio (Department of Cellular and Molecular Physiology, Yale University of Medicine, New Haven, CT). Genomic clones of chicken $\alpha3$, $\beta2$, and $\beta4$ were obtained through the generosity of Dr. Mark Ballivet (Department of Physiology, CMU, Geneva, Switzerland).

Expression of AChR subunits in Xenopus oocytes. Chicken and human cDNAs were cloned into a modified SP64T expression vector (37) with standard DNA-cloning procedures. cRNA was synthesized *in vitro* with the Megascript kit (Ambion, Austin, TX). Oocytes were prepared for injections as described in Colman (38) and injected with either 15 or 100 ng of cRNA per oocyte. Chicken $\alpha3$, $\beta2$, and $\beta4$ subunits were expressed by nuclear injections of 2 ng of genomic DNAs per oocyte. The oocytes were incubated in semisterile conditions at 18° in saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6) containing 5% heat-inactivated horse serum or, more recently, in 50% Leibovitz-15 media (GIBCO-BRL) buffered to pH 7.4 with 10 mM HEPES. Oocytes were incubated at 18° for 3–6 days before use.

Electrophysiological procedures and drug application. Currents in oocytes were measured with a standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725, Warner Instrument Corp.). Electrodes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ for the voltage electrode and 0.4–0.6 MΩ for the current electrode. All records were digitized (MacLab/2e interface and Scope software, AD Instruments), stored on a Macintosh IIcx computer, and analyzed with AXOGRAPH software (Axon Instruments).

The recording chamber was continually perfused at a flow rate of 10 ml/min with saline solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6. Application of the agonists was performed as described in detail previously (39). In summary, all agonists were applied with the use of a set of 2-mm glass tubes directed on the animal pole of the oocyte. Application was achieved by manual unclamping and clamping of a flexible tube connected to the syringe with the test solution. Typically, delay between beginning the application and the first deflection of the induced current was ~0.3 sec.

The Hill equation was fitted to the concentration-response dependencies with a nonlinear least-squares error curve fit method (KaleidaGraph, Abelbeck Software): $I(x) = I_{max}[x^n/(x^n+EC_{50}{}^n)]$, where $I(x)$ is current measured at the agonist concentration x, I_{max} the maximal current response at the saturating agonist concentration, EC_{50} is the agonist concentration required for the half-maximal response, and n is the Hill coefficient.

Cell cultures. SH-SY5Y cells were cultured in a 1:1 mixture of Ham's F12 nutrient mixture and Eagle's minimal essential medium containing 1% nonessential amino acids supplemented with 10% fetal calf serum in a 95% air/5% CO_2 humidified incubator at 37°. M10 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cell monolayers were washed

[1] R. Anand and J. Lindstrom, unpublished observations.

with phosphate-buffered saline and then scraped, pelleted in a microfuge in the cold, and stored at −80° until used.

mAbs. mAbs to α7 (mAb 306 and mAb 319) (21), α1, α3 (mAb 210) (40), α8 (mAb 305) (21), and β2 (mAb 290) (41) have been described previously. The mAbs were affinity purified with a protein G column.

Epibatidine. Epibatidine (oxalate salt) was synthesized at Merck Sharp & Dohme Research Laboratories (5, 6). Natural epibatidine is levorotatory as the free base but dextrorotatory as a salt (2, 6). In the present study, (+)- and (−)-epibatidine refer to rotation of the salts. Stocks of racemic [^{3}H]epibatidine were generous gifts of Dr. Ralph Loring (Department of Pharmaceutical Sciences, Northeastern University, Boston, MA) (42) or New England Nuclear, Inc. (Chadds Ford, PA). (−)-Nicotine tartrate and acetylcholine chloride were obtained from Sigma Chemical Co. (St. Louis, MO).

Solubilization of AChRs from SH-SY5Y cells, M10 cells, TE671 cells, and oocytes. Tissue was disrupted by brief vortexing in 5–7 volumes of lysis buffer (2% Triton X-100, 50 mM NaCl, 50 mM sodium phosphate buffer (pH7.5), 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 5 mM iodoacetamide). After 20 min of gentle rotation at 4°, the samples were centrifuged for 20 min at 4° in a microfuge, and the supernatants were used for subsequent binding studies.

Solubilization of AChRs from chick brains and retinae. Detergent extracts of AChRs from brains and retinae of embryonic day-18 chicks were prepared in the following manner. Brains and retinae were dissected from the chick and then frozen at −80°. The frozen tissues were thawed in 6 volumes of homogenization buffer (50 mM phosphate buffer, pH 7.5, 1 M NaCl, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 5 mM iodoacetamide) and then homogenized on ice for 2 min at 15,000 rpm with a Brinkman Polytron. After centrifugation at 50,000 rpm in a Beckman 50.2 Ti rotor for 30 min at 4°, the pellet was homogenized again in an equal volume of buffer (this time containing only 50 mM NaCl), and centrifuged again. The pellet was then extracted with 4 volumes of lysis buffer by brief homogenization with a Brinkman Polytron. The resuspended pellet was gently shaken for 2 hr at 4°. The mix was then centrifuged at 50,000 rpm in a Beckman 50.2 Ti rotor at 4° for 30 min, and the clear supernatant was used for subsequent experiments.

Immunoaffinity separation of α7 AChRs and α8 AChRs. Detergent extracts of chick brain were depleted of all α8-containing AChRs with purified mAb 305 coupled at 5 mg/ml of gel to Actigel ALD (Sterogene) according to the manufacturer's instructions. Extracts were incubated overnight at 4° with gentle agitation with mAb 305-coupled beads at a ratio of 1:5 (bead/extract). The beads were removed by brief centrifugation. The efficacy of depletion was then tested by solid radioimmunoassay with mAb 305-coated wells and 50 nM ^{125}I-αBgt. Similarly, retina extracts were depleted of all α7-containing AChRs with mAb 319-coupled beads [coupled at 5 mg/ml of gel to Affigel H2 (BioRad) according to the manufacturer's directions]. The mAb 319-coupled beads were then used for depletion in the same way as were the mAb 305-coupled beads. The extent of depletion of α7-containing AChRs was determined by solid-phase radioimmunoassays with mAb 319-coated wells and 20 nM ^{125}I-αBgt. Bound ^{125}I-αBgt was determined by gamma counting. Background binding was determined with microwells not coated with mAbs.

Binding assays. Binding assays were carried out by procedures similar to those we used previously (27). Briefly, solubilized AChRs were added to mAb-coated Immulon 4 microwells. After incubation overnight at 4°, the wells were rinsed three times with PBS-Tween 20 buffer. Various concentrations of epibatidine were added and incubated for 20 min before the addition of ^{125}I-αBgt (for overnight) or [^{3}H]nicotine (for 1 hr or overnight; similar results were obtained both ways). The assays were performed in duplicate in a total volume of 100 μl in the presence of ^{125}I-αBgt (0.1 nM for α1 AChRs, 2 nM for α7 AChRs, 20 nM for α8 AChRs) or [^{3}H]nicotine (5 nM for α4β2 and α3β2 AChRs, 20 nM for α3 AChRs). Background binding was determined with buffer rather than with solubilized AChRs. Normalized data were fitted to the Hill equation. Apparent K_I values were calculated with the Cheng-Prusoff equation.

Results and Discussion

Action of epibatidine on chicken AChRs. Fig. 2 gives representative responses induced by consecutive applications of increasing concentrations of epibatidine to an oocyte injected with chicken α4 and β2 mRNAs. Measurable responses were elicited by epibatidine in concentrations as low as 0.1 nM. Maximal responses were obtained at 100–300 nM. No responses were detected during epibatidine application on control noninjected oocytes.

Concentration dependence of the epibatidine-induced response of α4β2 AChRs is compared with the action of nicotine and acetylcholine (Fig. 2, *top right*). Nicotine and acetylcholine showed nearly the same potency in activating α4β2 AChRs, with EC_{50} values of ~0.4 μM, whereas epibatidine

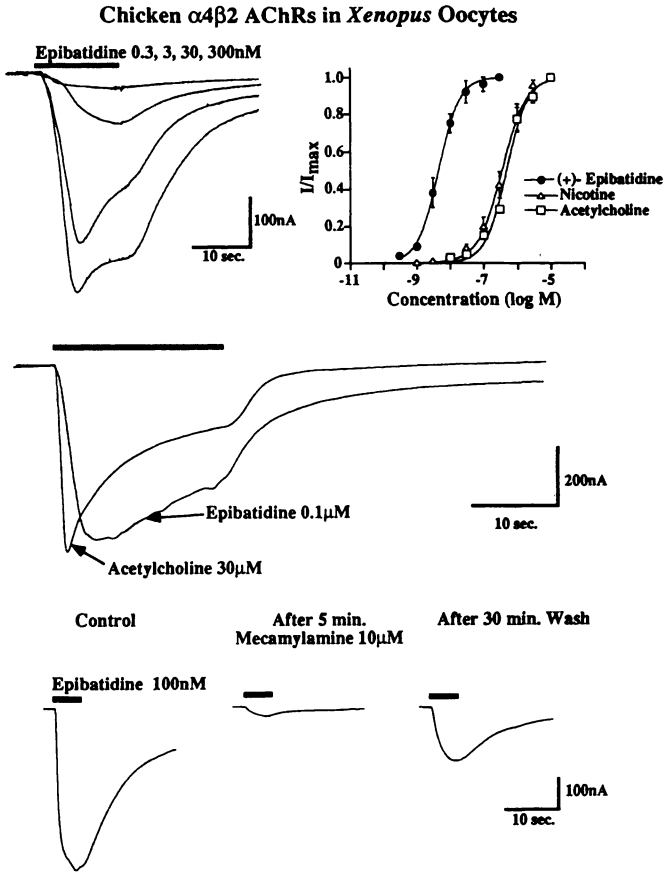


Fig. 2. Activation by epibatidine of chicken α4β2 nicotinic AChRs expressed in *Xenopus* oocytes. *Top left*, inward currents induced by the application of different concentrations of (+)-epibatidine on an oocyte 2 days after cytoplasmic injection of chicken α4 and β2 nicotinic subunit cRNAs (holding potential −50 mV). *Top right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine. Normalized currents are plotted against agonist concentrations. Averaged data (each point from three to five experiments) were fitted by the Hill equation (see Materials and Methods). *Middle*, time course of currents induced by saturating concentrations of the acetylcholine and epibatidine on the same oocyte held at −50 mV. Notice that the response induced by epibatidine exhibits significantly slower activation, desensitization, and inactivation. *Bottom*, inhibition of the epibatidine-induced current by the nicotinic antagonist mecamylamine. Three traces (before, after 5 min of mecamylamine application, and after 30-min washout) are shown.

was 2 orders of magnitude more potent (EC_{50} values for the agonists studied are listed in Table 1). Epibatidine behaved as a full agonist on $\alpha4\beta2$ AChRs because maximal currents induced by epibatidine had the same amplitude as maximal currents induced by acetylcholine (Fig. 2). Currents induced by epibatidine had a similar voltage dependence (i.e., strong inward rectification) as currents induced by nicotine or acetylcholine.

Kinetics of epibatidine currents differed significantly from the currents induced by acetylcholine and nicotine (Fig. 2, *middle*). Activation of AChRs by epibatidine was significantly slower, possibly reflecting the much lower concentrations of epibatidine used compared with acetylcholine (300-fold in Fig. 2, *middle*). Time from the beginning of the application of the saturating concentrations to the peak of the inward current was ~5 sec compared with 1 sec for nicotine and acetylcholine applied to the same oocyte. It takes ~0.3 sec for solution to the reach the oocyte (see Materials and Methods and Ref.). Maximal epibatidine responses exhibited much slower desensitization: during a 20-sec application, the current decreased only 30% compared with 65% for both acetylcholine and nicotine. After washout, AChRs stayed activated significantly longer, typically resulting in a long "tail" inward current that lasted up to 2 min after a 20-sec application of epibatidine. This might reflect the 200-fold greater affinity of epibatidine for $\alpha4\beta2$ AChRs compared with acetylcholine or nicotine [e.g., K_I epibatidine $= 2 \times 10^{-11}$ M (Table 2) versus 4×10^{-9} M for nicotine (34)].

Responses induced by epibatidine were inhibited by the noncompetitive channel-blocking antagonist mecamylamine (Fig. 2, *bottom*). Perfusion of the oocyte for 5 min with 10 μM mecamylamine caused almost complete block of the current with very slow partial (~30%) washout.

High potency of epibatidine as an agonist was reported previously for putative native $\alpha4\beta2$ AChRs in electrophysiological studies on rat hippocampal neurons (15) and by $^{86}Rb^+$ efflux studies on mouse thalamic synaptosomes (13). In both studies, epibatidine was shown to be a significantly more potent agonist than nicotine, with EC_{50} values in the low nanomolar range.

Epibatidine effectively inhibited [^{3}H]nicotine binding on M10 cells, a mouse fibroblast cell line permanently transfected with chicken $\alpha4\beta2$ AChR subunits (43) (Fig. 3, *top left*, and Table 2). The K_I value for epibatidine (19 pM) for chicken $\alpha4\beta2$ AChRs was close to the K_I for epibatidine (~40 pM) estimated for rat brain preparations by several groups (8, 13, 44). Epibatidine had 7-fold higher affinity than cytisine and a 200-fold higher affinity than nicotine for chicken $\alpha4\beta2$ AChRs as measured by Whiting *et al.* (42). The differences in the apparent affinities obtained electrophysiologically and in binding studies could be due to the increase in affinity of the AChR in the desensitized state, given the equilibrium conditions in binding studies.

Epibatidine behaved as an extremely potent agonist when applied to oocytes expressing chicken $\alpha3$ subunits with $\beta2$ or $\beta4$ subunits (Fig. 4). Currents induced by epibatidine acting on $\alpha3$ AChRs were blocked by the nicotinic antagonist hexamethonium (Fig. 4, *bottom*). Differences in the time course of currents induced by epibatidine for $\alpha3\beta2$ compared with $\alpha3\beta4$ (slow activation and and slow inactivation for $\alpha3\beta2$ versus fast activation and fast desensitization for $\alpha3\beta4$; Fig. 4, *top and middle*) were observed as well when these AChRs were activated by acetylcholine or nicotine.

(+)-Epibatidine did not discriminate between chicken $\alpha3\beta2$ and $\alpha3\beta4$ AChRs, whereas (−)-epibatidine was 4-fold less potent for $\alpha3\beta2$ AChRs than for $\alpha3\beta4$ AChRs (Fig. 4 and Table 1). Substitution for the $\beta2$ structural subunit in $\alpha3$-containing AChRs by the $\beta4$ subunit caused both a 2-fold decrease in the apparent affinity for nicotine (Fig. 4 and Table 1) and a 2-fold shift in intrinsic activity of nicotine from a partial agonist to a full agonist (Fig. 4). These results support the idea that in $\alpha3$ AChRs the time courses of activation, desensitization, and the apparent affinity for some ligands are determined by the β subunits (31, 32, 45).

The high apparent affinity for epibatidine activation of $\alpha3$-containing AChRs expressed in *Xenopus* oocytes that we observed correlates well with data reported by other groups who studied the effects of epibatidine on responses mediated by native nicotinic AChRs. $\alpha3$ AChRs are believed to be responsible for synaptic transmission in autonomic ganglia (23, 24). High potency of epibatidine as an agonist with low nanomolar EC_{50} values was reported for the responses me-

TABLE 1

Comparison of the agonist potency of (+)- and (−)-epibatidine, acetylcholine, and nicotine on nicotinic AChRs expressed in *Xenopus* oocytes

	EC_{50} (+)-Epibatidine	n_H	EC_{50} (−)-Epibatidine	n_H	EC_{50} (−)-Nicotine	EC_{50} Acetylcholine ACh
	μM		μM		μM	μM
Chicken AChRs						
$\alpha4\beta2$	0.0045 ± 0.0002	1.4	0.0042 ± 0.0003	1.5	0.35 ± 0.02	0.48 ± 0.04
$\alpha3\beta2$	0.017 ± 0.002	1.0	0.042 ± 0.005	1.2	200 ± 15.0[a]	4.1 ± 0.5
$\alpha3\beta4$	0.021 ± 0.003	1.2	0.009 ± 0.002	1.3	410 ± 35.0	53 ± 5.6
$\alpha7$	2.0 ± 0.1	1.8	2.2 ± 0.3	1.8	18 ± 0.5[c]	120 ± 7.4[c]
$\alpha8$	0.0011 ± 0.0001	1.4	0.0012 ± 0.0003	1.1	1.0 ± 0.1[c]	1.8 ± 0.1[c]
Human AChRs						
$\alpha3\beta2$	0.022 ± 0.003	1.6	0.13 ± 0.03	1.1	6.7 ± 0.5[a]	26 ± 0.3
$\alpha3\beta4$	0.073 ± 0.006	1.4	0.021 ± 0.007	1.8	110 ± 4.0	160 ± 5.0
$\alpha7$	1.2 ± 0.2	1.1	1.1 ± 0.2	1.3	40 ± 1.7[d]	79 ± 37.0[d]
$\alpha1\beta1\gamma\delta$[b]	7.3 ± 0.5	1.2	15.9 ± 2.2	1.4	ND	ND
Torpedo AChRs						
$\alpha1\beta1\gamma\delta$	1.6 ± 0.2	1.0	3.9 ± 0.2	1.2	250 ± 48.0	5.6 ± 0.5

[a] Partial agonist.
[b] α1 human, β1γδ *Torpedo*.
[c] From Ref. 31.
[d] From Ref. 26.

TABLE 2

Binding affinity of (+)- and (−)-epibatidine to native and expressed nicotinic AChRs

	K_i	
	(+)-Epibatidine	(−)-Epibatidine
	nM[a]	
Chicken		
$\alpha4\beta2$ AChRs from M10 cells	0.019 ± 0.0016	0.01 ± 0.0016
$\alpha7$ AChRs from chicken brain	590 ± 10	350 ± 40
$\alpha8$ AChRs from chicken brain		
Site 1	0.00023 ± 0.00004	
Site 2	24 ± 7.4	7.3 ± 0.3
Human		
$\alpha3\beta2$ AChRs expressed in oocytes	0.23 ± 0.58	0.15 ± 0.06
$\alpha3$ AChRs from SH-SY5Y cells	0.00059 ± 0.00013	0.031 ± 0.009
$\alpha7$ AChRs from SH-SY5Y cells	9.8 ± 2.1	3.1 ± 1.4
$\alpha1$ AChRs from TE671 cells	6.0 ± 2.1	12 ± 4.3
Torpedo		
$\alpha1$ AChRs from electric organ	3000 ± 190	5700 ± 140

[a] Each entry represents mean and standard error of at least three experiments. Competitive binding studies with immunoisolated AChRs used [^{3}H]nicotine for $\alpha3$ and $\alpha4$ AChRs and ^{125}I-αBgt for $\alpha1$, $\alpha7$, and $\alpha8$ AChRs as described in Materials and Methods. Estimates of affinity shown in this table used 30-min preincubations with epibatidine followed by either 1-hr incubation with [^{3}H]nicotine or overnight incubation with ^{125}I-αBgt. Overnight incubations with [^{3}H]nicotine gave similar results.

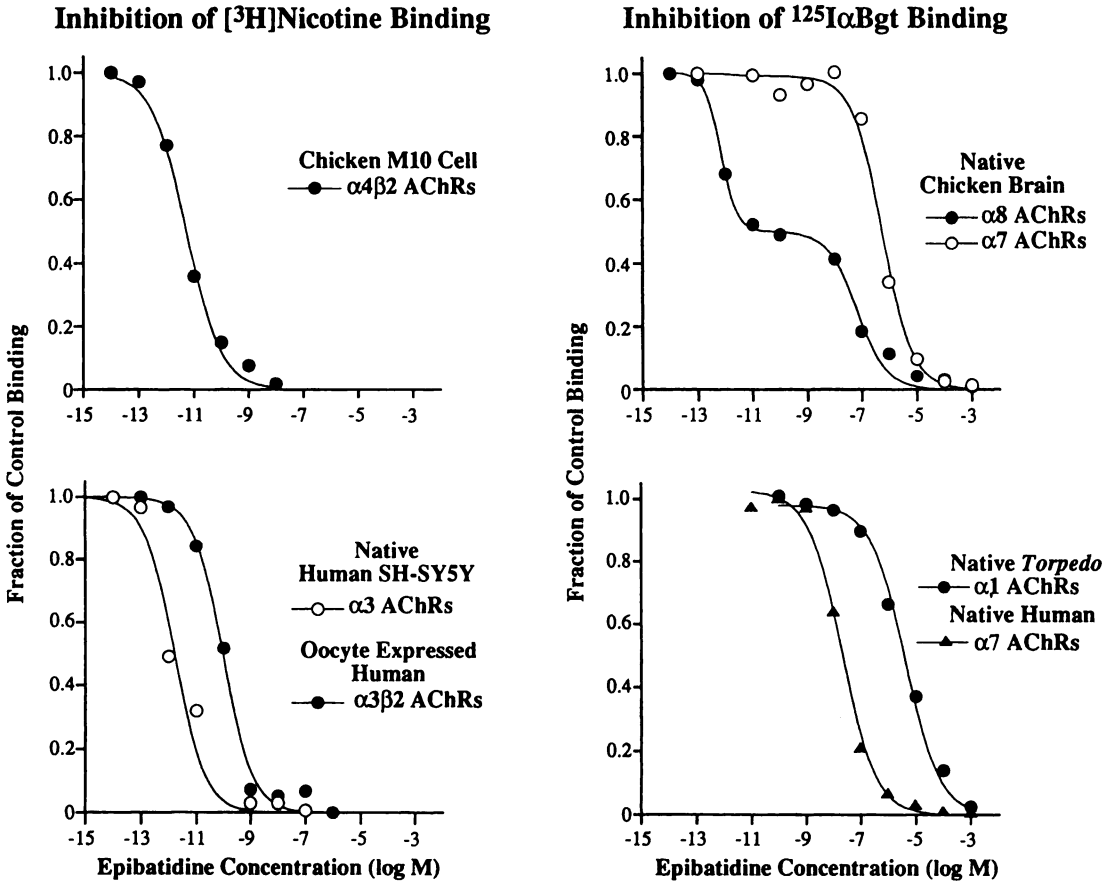


Fig. 3. Binding of epibatidine to expressed and native AChRs. *Two left panels*, inhibition of [^{3}H]nicotine binding. *Two right panels*, inhibition of ^{125}I-αBgt binding. *Top left*, M10 cell line, which expresses chicken $\alpha4\beta2$ AChRs (34). *Bottom left*, binding of epibatidine to human $\alpha3\beta2$ AChRs expressed in oocytes and native $\alpha3$ AChRs from the SH-SY5Y cell line. The data points shown represent values for [^{3}H]nicotine binding (at 5 nM [^{3}H]nicotine) in the presence of different concentrations of (+)-epibatidine normalized to the control value obtained in the absence of the epibatidine. *Top right*, binding of epibatidine to native $\alpha7$ and $\alpha8$ AChRs immunoisolated from chicken brain and retinae. *Bottom right*, binding of epibatidine to native human $\alpha7$ AChRs from the SH-SY5Y cell line, native human muscle-type AChRs from the TE671 cell line, and *Torpedo* AChRs expressed from cRNAs in oocytes. The data points shown represent values of the ^{125}I-αBgt binding (at 0.1 nM ^{125}I-αBgt for both muscle-type human AChRs and *Torpedo* AChRs, at 2 nM ^{125}I-αBgt for $\alpha7$, and at 20 nM ^{125}I-αBgt for $\alpha8$) in the presence of different (+)-epibatidine concentrations normalized to the control value obtained in the absence of epibatidine. Background was subtracted from all data presented. The $\alpha8$ AChR data were fitted with a Hill equation for two sites.

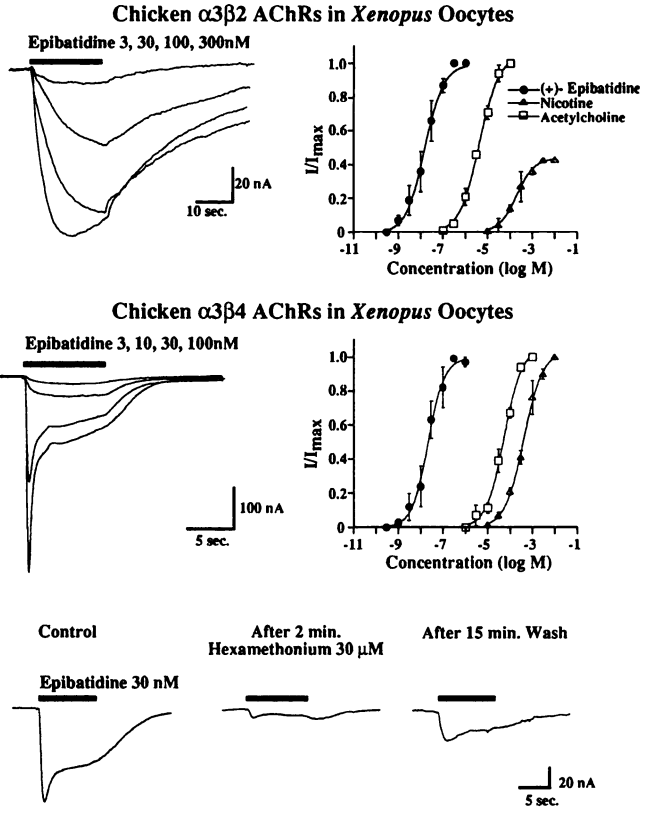


Fig. 4. Activation by epibatidine of chicken $\alpha3\beta2$ and $\alpha3\beta4$ AChRs expressed in oocytes. *Top left*, typical responses to (+)-epibatidine of $\alpha3\beta2$ AChRs. *Top right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on chicken $\alpha3\beta2$ AChRs. *Middle left*, typical responses to (+)-epibatidine of $\alpha3\beta4$ AChRs. *Middle right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on chicken $\alpha3\beta4$ AChRs. *Bottom*, inhibition of the currents induced by (+)-epibatidine in $\alpha3\beta4$ AChRs by the nicotinic antagonist hexamethonium. Traces obtained before, after 2 min of perfusion with 30 μM hexamethonium, and after 15-min washout are shown. Data for Fig. 4 were obtained at 2–5 days after intranuclear injection of chicken genomic cDNAs.

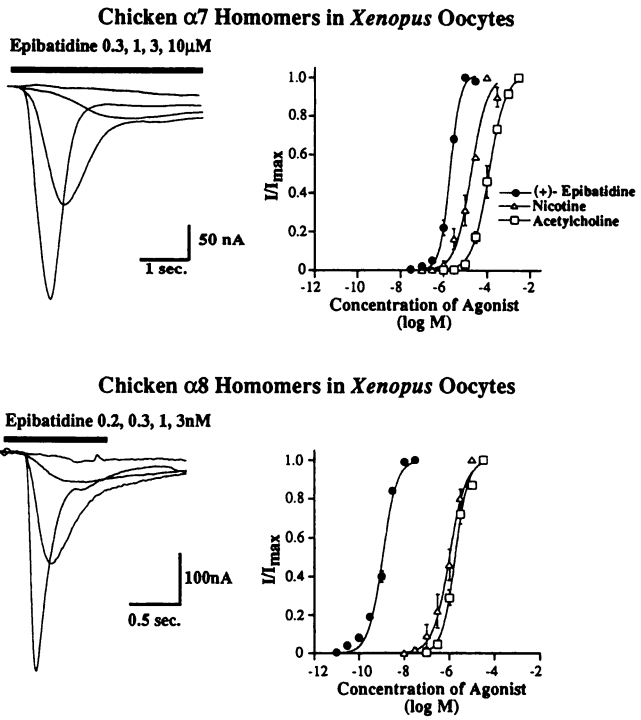


Fig. 5. Activation by epibatidine of chicken homomeric $\alpha7$ and $\alpha8$ AChRs expressed in oocytes. *Top left*, typical responses to (+)-epibatidine of $\alpha7$ homomers. *Top right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on chicken $\alpha7$ homomers. *Bottom left*, typical responses to (+)-epibatidine of $\alpha8$ homomers. *Bottom right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on chicken $\alpha8$ AChRs. Responses and dose-response curves were obtained under the conditions described in the legend of Fig. 2 at 2–5 days after cytoplasmic injection of the chicken $\alpha7$ and $\alpha8$ cRNAs.

diated by the native AChRs on isolated preparations of rat and guinea pig autonomic ganglia (46) and rat PC12 cells (9). *In situ*, epibatidine behaved as a potent ganglionic agonist, modulating ganglionic synaptic transmission and cardiorespiratory function in anesthetized rats when administered intravenously in very low doses (0.5–2 mg/kg) (47).

Epibatidine acted as a full agonist on chicken αBgt-sensitive homomeric $\alpha7$ and $\alpha8$ AChRs, producing rapidly desensitizing currents indistinguishable from those induced by acetylcholine or nicotine (Fig. 5). Half-maximum activation of $\alpha8$ homomers required a 2000-fold lower concentration of epibatidine than did activation of $\alpha7$ homomers (Table 1). For both homomers, epibatidine showed 10–1000-fold higher potency compared with nicotine or acetylcholine.

Selectivity of epibatidine for $\alpha8$ homomers over $\alpha7$ homomers was preserved in $\alpha8$- and $\alpha7$-containing native AChRs immunoisolated from chicken brain (Fig. 3 and Table 2). Epibatidine had relatively low affinity for $\alpha7$ AChRs (~1 μM). The difference between binding affinity (measured under equilibrium conditions when the AChRs presumably were in a desensitized state) and apparent affinity for activation of the $\alpha7$ homomers (obtained in nonequilibrium conditions) was minimal (Tables 1 and 2).

As shown previously, some nicotinic ligands recognize two pharmacologically distinct binding sites in native $\alpha8$ AChRs immunoisolated from chicken retina (27). Competitive inhibition of 20 nM ^{125}I-αBgt binding by epibatidine revealed two binding sites for $\alpha8$ AChRs immunoisolated from chicken brain, with the higher affinity site in the picomolar range and the lower affinity site in the 50 nM range (Fig. 3, *top right*, and Table 2). None of the sites match the apparent affinity obtained electrophysiologically for $\alpha8$ homomers, but both sites have significantly higher affinities compared with native $\alpha7$ AChRs.

Action of epibatidine on human and Torpedo AChRs. We tested epibatidine on human neuronal AChRs cloned from the cell line SH-SY5Y and expressed from cRNAs in *Xenopus* oocytes. Coinjection of human $\alpha3$ with $\beta2$ and $\beta4$ nicotinic subunit mRNAs resulted in very effective expression of functional acetylcholine-gated channels (Fig. 6). Both human $\alpha3\beta2$ and $\alpha3\beta4$ AChRs showed lower affinity for acetylcholine and higher affinity for nicotine compared with chicken $\alpha3\beta2$ and $\alpha3\beta4$ AChRs (Table 1 and Fig. 6). As was the case for chicken $\alpha3\beta2$ AChRs, nicotine behaved as a partial agonist for human $\alpha3\beta2$ AChRs, resulting in a maximum response to nicotine of ~50% of the maximum response induced by acetylcholine. Presence of the human $\beta4$ subunit caused nicotine to behave as a full agonist (Fig. 6), as was the case with chicken $\beta4$ subunits (Fig. 4). Epibatidine exhibited essentially the same potency on human $\alpha3$ AChRs compared with chicken $\alpha3$ AChRs. Currents induced by epibatidine

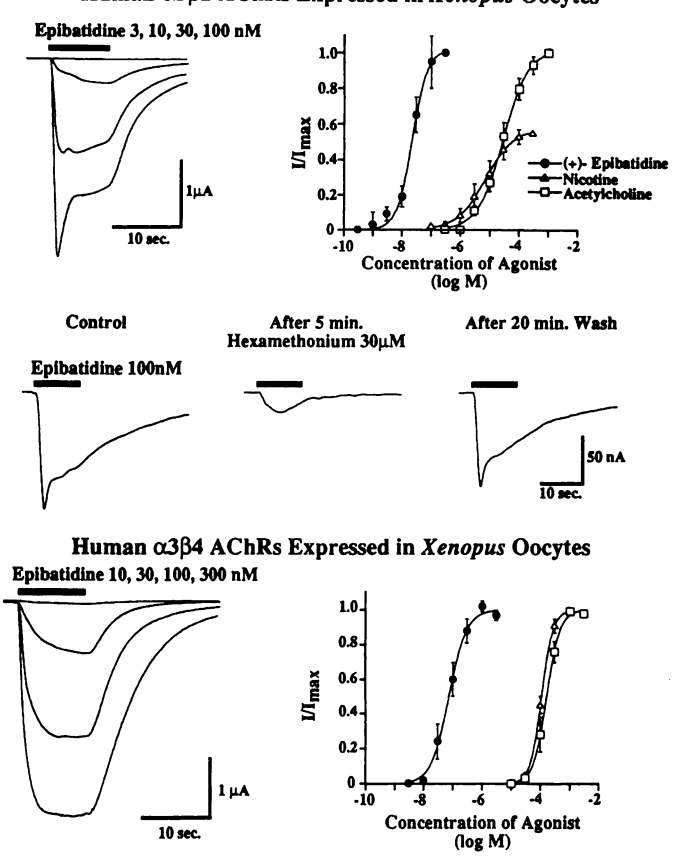


Fig. 6. Activation by epibatidine of human $\alpha3\beta2$ and $\alpha3\beta4$ AChRs expressed in oocytes. *Top left*, typical responses to (+)-epibatidine of $\alpha3\beta2$ AChRs. *Top right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on human $\alpha3\beta2$ AChRs. *Middle*, inhibition of currents induced by (+)-epibatidine acting on $\alpha3\beta2$ AChRs by the nicotinic antagonist hexamethonium. Traces before, after 5 min of perfusion by 30 μM of hexamethonium, and after 15-min washout are shown. *Bottom left*, typical responses to (+)-epibatidine of $\alpha3\beta4$ AChRs. *Bottom right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on human $\alpha3\beta4$ AChRs. Responses and dose-response curves were obtained under the conditions described in the legend of Fig. 2 at 2–5 days after cytoplasmic injection of human cRNAs.

were effectively blocked by the nicotinic antagonist hexamethonium (Fig. 6).

Differences in the time courses of responses induced by epibatidine were observed with human $\alpha3\beta2$ and $\alpha3\beta4$ AChRs (Fig. 6). Activation of $\alpha3\beta2$ AChRs was faster, with a characteristic peak that decayed to a relatively steady level within a few seconds. These responses of human AChRs contrast with those of chicken AChRs (Fig. 4) because chicken $\alpha3\beta2$ AChRs were activated and desensitized by epibatidine more slowly than were chicken $\alpha3\beta4$ AChRs. Differences between responses mediated through $\alpha3\beta2$ and $\alpha3\beta4$ subunits were described previously for rat AChRs (31, 45), and it was suggested that $\beta2$ and $\beta4$ subunits determine the time course of desensitization in $\alpha3$ AChRs.

Binding affinity of epibatidine for human recombinant $\alpha3\beta2$ AChR expressed in oocytes was estimated by both competitive inhibition of [^{3}H]nicotine binding and direct binding of racemic [^{3}H]epibatidine (Fig. 7). In the competitive inhibition experiments, the K_I values of (+)- and (−)-epibatidine for $\alpha3\beta2$ were 0.23 and 0.16 nM, respectively (Table 2). The

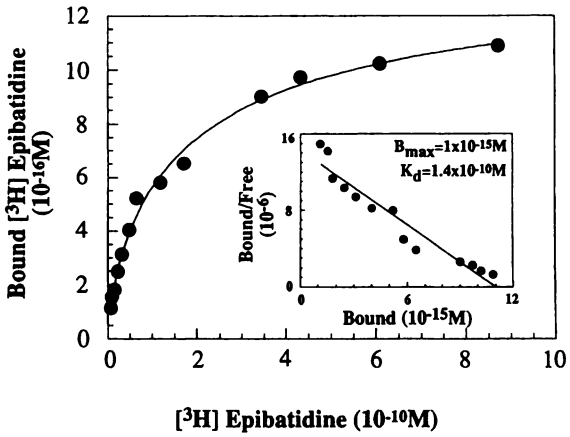


Fig. 7. Binding of racemic [^{3}H]epibatidine to recombinant human $\alpha3\beta2$ AChRs expressed in oocytes. The binding data are fitted to the Hill equation (K_I = 0.14 nM). *Inset*, Scatchard plot. These binding data reflect the results after a 24-hr incubation with [^{3}H]epibatidine.

K_D value of racemic [^{3}H]epibatidine was similar (0.14 nM). The addition of an equal amount of unlabeled epibatidine (with equal amounts of (+)- and (−)-isomers) to [^{3}H]epibatidine bound to $\alpha3\beta2$ at a saturating concentration (5.3 nM) reduced the amount of bound [^{3}H]epibatidine by 50%. This result demonstrates that the process of tritiation does not significantly alter the affinity of [^{3}H]epibatidine for $\alpha3\beta2$.

Native human $\alpha3$-containing AChRs immunoisolated from the SH-SY5Y cell line exhibited picomolar affinity for the (+)-isomer and 50-fold lower affinity for the (−)-isomer of epibatidine (Fig. 3 and Table 2). Binding affinity for (+)-epibatidine estimated for human recombinant $\alpha3\beta2$ AChRs expressed in oocytes was 400-fold lower compared with native $\alpha3$ AChRs (Fig. 3 and Table 2). Because SH-SY5Y cells are known to express $\alpha3$, $\alpha5$, $\beta2$, $\beta4$, and $\alpha7$ subunits (34, 48), this may indicate that the native human $\alpha3$ AChR from the SH-SY5Y cell line either contains a structural subunit other than $\beta2$ or contains additional subunits such as $\beta4$ and $\alpha5$ that change the pharmacological properties of the native AChRs.

Epibatidine activated human homomeric $\alpha7$ AChRs, resulting in responses indistinguishable in time course and voltage dependence from those induced by acetylcholine and nicotine (Fig. 8). Epibatidine appeared to be less potent on human $\alpha7$ homomers compared with $\alpha3\beta2$ and $\alpha3\beta4$ AChRs; however, it was 40–80-fold more potent on $\alpha7$ homomers compared with nicotine or acetylcholine, respectively.

In electrophysiological studies, human and chicken homomeric $\alpha7$ AChRs showed essentially the same EC_{50} values for both epibatidine isomers. In binding studies, however, human native $\alpha7$ AChRs immunoisolated from the SH-SY5Y cell line exhibited 50-fold higher affinity for (+)-epibatidine and 100-fold higher affinity for the (−)-isomer compared with chicken brain $\alpha7$ AChRs (Fig. 3 and Table 2).

Torpedo muscle-type AChRs showed lower binding affinity for epibatidine compared with neuronal-type AChRs (Table 2). The EC_{50} for activation of the *Torpedo* AChRs (1.6 μM for the (+)-isomer) (Table 1) surprisingly did not differ substantially from the K_I estimated by epibatidine inhibition of ^{125}I-αBgt binding (3 μM for the (+)-isomer) (Table 2). Epibatidine

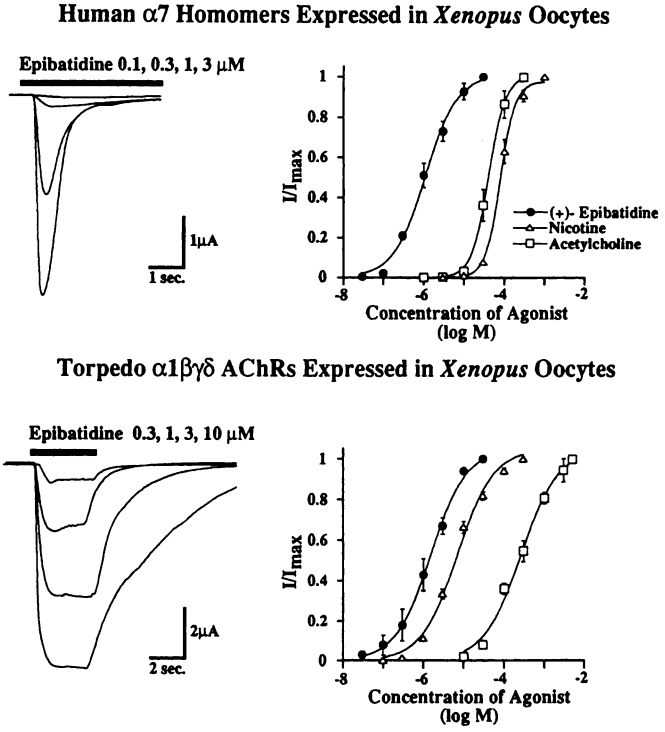


Fig. 8. Activation by epibatidine of human α7 homomers and muscle-type *Torpedo* α1β1δγ AChRs expressed in oocytes. *Top left*, typical responses to (+)- epibatidine of α7 homomers. *Top right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on human α7 homomers. *Bottom left*, typical responses to (+)-epibatidine of *Torpedo* α1β1γδ AChRs. *Bottom right*, dose-response curves for (+)-epibatidine, acetylcholine, and nicotine on the *Torpedo* AChRs. Responses and dose-response curves were obtained under the conditions described in the legend of Fig. 2 at 2–5 days after cytoplasmic injection of cRNAs.

is a more potent agonist for *Torpedo* AChRs than is acetylcholine (by 3.5-fold) or nicotine (by 160-fold) (Fig. 7 and Table 1). Substitution of the human α1 subunit for the *Torpedo* α1 subunit in combination with *Torpedo* β1, γ, and δ subunits resulted in formation of new functional AChRs with slightly lower apparent affinity for epibatidine (Table 2).

Human muscle-type α1 AChRs from the TE671 cell line exhibited nanomolar binding affinity for both isomers of epibatidine (Table 2).

Conclusions. The present study summarizes properties of (+)- and (−)-isomers of the alkaloid epibatidine as an agonist and ligand for different subtypes of native and recombinant nicotinic AChRs. Epibatidine behaves as an extremely potent agonist for several subtypes of chicken and human neuronal nicotinic AChRs, whereas it has rather moderate affinity for muscle-type AChRs.

For all chicken and human combinations of nicotinic AChR subunits functionally tested in oocytes, epibatidine showed much higher apparent affinity than did acetylcholine or nicotine, being able to activate most of them (except α7 homomers) in low nanomolar concentrations. Both isomers of epibatidine have more than 10-fold higher affinity (EC_{50} ~4 nM) for chicken α4β2 AChRs than does the potent alkaloid anatoxin (EC_{50} ~50 nM), which is produced by the cyanobacterium *Anabaena flos aqua* (49, 50). Thus, epibatidine is the most potent nicotinic agonist so far described.

The high potency of both epibatidine enantiomers that we observed for α4β2, α3β2, and α3β4 recombinant AChRs expressed in *Xenopus* oocytes correlates well with data obtained in functional studies on native putative α4β2 AChRs and α3 AChRs (9, 13–15). Lower potency for expressed αBgt-sensitive neuronal α7 AChRs and muscle α1β1γδ AChRs also parallels data on native putative α7 and muscle-type AChRs (8, 13, 15).

Comparison of different nicotinic AChR subunit combinations expressed in oocytes reveals clear differences between their sensitivity to the classic nicotinic agonists acetylcholine and nicotine. Chicken α4β2 AChRs have high affinity for both agonists (EC_{50} <1 μM). Recombinant AChRs formed from chicken α3β2 and α3β4 subunit combinations or human α3β4 subunit combination have low affinity for nicotine (EC_{50} >100 μM). For both chicken and human recombinant α3β4 AChRs, nicotine behaves as partial agonist. Human and chicken recombinant α7 homomeric AChRs and recombinant α3β4 AChRs show rather low affinity for acetylcholine.

In both functional and binding studies, most AChRs showed only moderate selectivity between (+)- and (−)-isomers of epibatidine. However, (+)-epibatidine had substantially higher affinity for native chick α8-containing and native human α3-containing AChRs.

High affinity for neuronal nicotinic AChRs as well as high potency for gating of these AChRs position epibatidine in the ranks of the most powerful tools for binding and functional studies of the native and recombinant nicotinic AChRs. Recent studies on analgesic, locomotor, hypothermic, cardiovascular, and metabolic activity of epibatidine (9, 10, 44, 45, 50) indicate the potential importance of this compound for further exploration of the physiological roles of central nicotinic AChRs, as well as for possible application of related compounds in clinical studies and therapy of pathological conditions that involve disorders of central neuronal nicotinic AChR function (51, 52).

References

1. Spande, T. F., H. M. Garraffo, M. W. Edwards, H. J. C. Yeh, L. Pannel, and J. W. Daly. Epibatidine: a novel (chloropyridyl)azabicycloheptane with potent analgesic activity from Ecuadoran poison frog. *J. Am. Chem. Soc.* **114**:3475–3478 (1992).
2. Qian, C. G., T. C. Li, T. Y. Shen, L. Libertinegarahan, J. Eckman, T. Biftu, and S. Ip. Epibatidine is a nicotinic analgesic. *Eur. J. Pharmacol.* **250**: R13-R14 (1993).
3. Badio, B., and J. W. Daly. Epibatidine, a potent analgesic and nicotinic agonist. *Mol. Pharmacol.* **45**:563–569 (1994).
4. Corey, E., J. Loh, S. AchyothaRao, D. Daley, and S. Sarshar. Stereocontrolled total synthesis of (+)- and (−)-epibatidine. *J. Org. Chem.* **58**:5600–5602 (1993).
5. Fletcher, S. R., R. Baker, M. S. Chambers, S. C. Hobbs, and P. J. Mitchell. The synthesis of (+)- and (−)-epibatidine. *J. Am. Chem. Soc.* **15**:1216–1218 (1993).
6. Fletcher, S. R., R. Baker, M. S. Chambers, R. H. Herbert, S. C. Hobbs, S. R. Thomas, H. M. Verrier, A. P. Watt, and R. G. Ball. Total synthesis and determination of the absolute-configuration of epibatidine. *J. Org. Chem.* **59**:1771–1778 (1994).
7. Huang, D. F., and T. Y. Shen. A versatile total synthesis of epibatidine and analogs. *Tetrahedron Lett.* **34**:3251–3254 (1993).
8. Broka, C. Synthetic approaches to epibatidine. *Med. Chem. Res.* **4**:449–460 (1994).
9. Badio, B., H. Garraffo, T. Spande, and J. Daly. Epibatidine: discovery and definition as a potent analgesic and nicotinic agonist. *Med. Chem. Res.* **4**:440–448 (1994).
10. Damaj, M., S. Tucker, M. Aceto, and B. Martin. Analgesic activity of epibatidine and its enantiomers in a chemical model of pain in mice. *Med. Chem. Res.* **4**:483–492 (1994).
11. Dukat, M., M. Damaj, W. Glassco, D. Domas, E. May, B. Martin, and R. Glennon. Epibatidine: a very high affinity nicotinic-receptor ligand. *Med. Chem. Res.* **4**:131–139 (1994).
12. Sullivan, J. P., M. W. Decker, J. D. Brioni, D. Donnely-Roberts, D. J.

Anderson, A. W. Bannon, C. Kang, P. Adems, M. Piattoni-Kaplan, M. J. Buckley, M. Gopalakrishnan, M. Williams, and S. P. Arneric. (±)-Epibatidine elicits a diversity of *in vitro* and *in vivo* effects mediated by nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* **271**:624–631 (1994).

13. Sullivan, J., C. Briggs, D. Donnelly-Roberts, J. Brioni, R. Radek, D. McKenna, J. Campbell, S. Arneric, M. Decker, and A. Bannon. (±)-Epibatidine can differentially evoke responses mediated by putative subtypes of nicotinic acetylcholine receptors (nAChRs). *Med. Chem. Res.* **4**:502–516 (1994).
14. Loring, R., T. McHugh, J. McKay, and X. Zhang. Epibatidine: a potent desensitizing agonist of neuronal nicotinic receptors in the chick nervous system. *Med. Chem. Res.* **4**:517–527 (1994).
15. Alkondon, M., and E. Albuquerque. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. III: Agonist actions of the novel alkaloid epibatidine and analysis of type II current. *J. Pharmacol. Exp. Ther.* **274**:771–782 (1995).
16. Aceto, M. D., H. Awaya, B. R. Martin, and E. L. May. Antinociceptive action of nicotine and its methiodide derivatives in mice and rats. *Br. J. Pharmacol.* **79**:869–876 (1983).
17. Yang, C. Y., W. H. Wu, and V. K. Zbuzek. Antinociceptive effect of chronic nicotine and nociceptive effect of its withdrawal measured by hot-plate and tail-flick in rats. *Psychopharmacol. Ser. (Berl.)* **106**:417–420 (1992).
18. Sargent, P. B. The diversity of neuronal nicotinic acetylcholine receptors. *Ann. Rev. Neurosci.* **16**:403–443 (1993).
19. Lindstrom, J. Nicotinic acetylcholine receptors, in *CRC Handbook of Receptors* (A. North, ed.). CRC Press, Boca Raton, Florida, 153–175 (1994).
20. Elgoyhen, E. B., O. Johnson, J. Boutler, D. Vetter, and S. F. Heinemann. $\alpha 9$: an acetylcholine receptor with novel pharmacological properties expressed in rat chochlear hair cells. *Cell* **79**:705–715 (1994)
21. Schoepfer, R., W. G. Conroy, P. Whiting, M. Gore, and J. Lindstrom. Brain α-bungarotoxin binding protein cDNAs and mAbs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. *Neuron* **5**:35–48 (1990).
22. Whiting, P. J., R. Schoepfer, W. G. Conroy, M. J. Gore, K. T. Keyser, S. Shimasaki, F. Esch, and J. M. Lindstrom. Expression of nicotinic acetylcholine receptor subtypes in brain and retina. *Mol. Brain Res.* **10**:61–70 (1991).
23. Corriveau, R. A., and D. K. Berg. Coexpression of multiple acetylcholine receptor genes in neurons: quantification of transcripts during development. *J. Neurosci.* **13**:2662–2671 (1993).
24. Vernallis, A. B., W. G. Conroy, and D. K. Berg. Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. *Neuron* **10**:451–464 (1993).
25. Britto, L. R., D. E. Hamassaki-Britto, E. S. Ferro, K. T. Keyser, H. J. Karten, and J. M. Lindstrom. Neurons of the chick brain and retina expressing both α-bungarotoxin-sensitive and α-bungarotoxin-insensitive nicotinic acetylcholine receptors: an immunohistochemical analysis. *Brain Res.* **590**:193–200 (1993).
26. Keyser, K. T., L. R. Britto, R. Schoepfer, P. Whiting, J. Cooper, W. Conroy, P. A. Brozozowska, H. J. Karten, and J. Lindstrom. Three subtypes of α-bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. *J. Neurosci.* **13**:442–454 (1993).
27. Anand, R., X. Peng, J. J. Ballesta, and J. Lindstrom. Pharmacological characterization of α-bungarotoxin-sensitive acetylcholine receptors immunoisolated from chick retina: contrasting properties of $\alpha 7$ and $\alpha 8$ subunit-containing subtypes. *Mol. Pharmacol.* **44**:1046–1050 (1993).
28. Tarroni, P., F. Rubboli, B. Chini, R. Zwart, M. Oortgiesen, E. Sher, and F. Clementi. Neuronal-type nicotinic receptors in human neuroblastoma and small-cell lung carcinoma cell lines. *FEBS Lett.* **312**:66–70 (1992).
29. Minna, J. D. The molecular biology of lung cancer pathogenesis. *Chest* **103**:449S–456S (1993).
30. Role, L. W. Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. *Curr. Opin. Neurobiol.* **2**:254–262 (1992).
31. Luetje, C. W., and J. Patrick. Both α- and β-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J. Neurosci.* **11**:837–845 (1991).
32. Papke, R. L., and S. F. Heinemann. Partial agonist properties of cytisine on neuronal nicotinic receptors containing the $\beta 2$ subunit. *Mol. Pharmacol.* **45**:142–149 (1994).
33. Schoepfer, R., P. Whiting, F. Esch, R. Blacher, S. Shimasaki, and J. Lindstrom. cDNA clones coding for the structural subunit of a chicken brain nicotinic acetylcholine receptor. *Neuron* **1**:241–248 (1988).
34. Peng, X., M. Katz, V. Gerzanich, R. Anand, and J. Lindstrom. Human $\alpha 7$ acetylcholine receptor: cloning of the $\alpha 7$ subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional $\alpha 7$ homomers expressed in Xenopus oocytes. *Mol. Pharmacol.* **45**:546–554 (1994).
35. Schoepfer, R., M. Luther, and J. Lindstrom. The human medulloblastoma cell line TE671 expresses a muscle-like acetylcholine receptor: cloning of the α-subunit cDNA. *FEBS Lett.* **226**:235–240 (1988).
36. Anand, R., and Lindstrom, J. Nucleotide sequence of the human nicotinic acetylcholine receptor $\beta 2$ subunit gene. *Nucleic Acids Res.* **18**:4272 (1990).
37. Melton, D., P. Krieg, M. Rebagliati, T. Maniatis, K. Zuin, and M. Green. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing SP6 promoter. *Nucleic Acids Res.* **12**:7035–7056 (1984).
38. Colman, A. Translation of eucaryotic messenger RNA in *Xenopus* oocytes, in *Transcription and Translation: A Practical Approach* (B. D. Hames and S. J. Higgins, eds.). Oxford, UK, IRL Press, 271–302 (1984).
39. Gerzanich, V., R. Anand, and J. Lindstrom. Homomers of $\alpha 8$ and $\alpha 7$ subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. *Mol. Pharmacol.* **45**:212–220 (1994).
40. Tzartos, S., L. Langeberg, S. Hochschwender, L. W. Swanson, and J. Lindstrom. Characteristics of monoclonal antibodies to denaturated *Torpedo* and to native calf acetylcholine receptors: species, subunit and region specificity. *J. Neuroimmunol.* **10**:235–253 (1986).
41. Whiting, P. J., and J. M. Lindstrom. Characterization of bovine and human neuronal nicotinic acetylcholine receptors using monoclonal antibodies. *J. Neurosci.* **8**:3395–3404 (1988).
42. McKay, V., J. Lindstrom, and R. Loring. Determination of nicotinic receptor subtypes in chick retina using monoclonal antibodies and ^{3}H-epibatidine. *Med. Chem. Res.* **4**:528–537 (1994).
43. Whiting, P., R. Schoepfer, J. Lindstrom, and T. Priestley. Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. *Mol. Pharmacol.* **40**:463–472 (1991).
44. Houghtling, R., M. Davila-Garcia, S. Hurt, and K. Kellar. (^{3}H)-Epibatidine binding to nicotinic cholinergic receptors in brain. *Med. Chem Res.* **4**:538–546 (1994).
45. Cachelin, A. B., and R. Jaggi. β Subunits determine the time course of desensitization in rat $\alpha 3$ neuronal nicotinic acetylcholine receptors. *Pflueg. Arch. Eur. J. Physiol.* **419**:579–582 (1991).
46. Bonhaus, D., K. Bley, C. Broka, D. Fontana, E. Leung, R. Lewis, A. Shieh, and E. Wong. Characterization of the electrophysiological, biochemical, and behavioral actions of epibatidine. *J. Pharmacol. Exp. Ther.* **3**:1199–1203 (1995).
47. Fisher, M., D. Hangfu, T. Shen, and P. Guynet. Epibatidine, an alkaloid from the poison frog epipedobates tricolor, is a powerful ganglionic depolarizing agent. *J. Pharmacol. Exp. Ther.* **2**:702–707 (1994).
48. Lukas, R. J., S. A. Norman, and L. Lucero. Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastoma clonal line. *Mol. Cell. Neurosci.* **4**:1–12 (1993).
49. Swanson, K. L., C. N. Allen, R. S. Aronstam, H. Rapoport, and E. X. Albuquerque. Molecular mechanisms of the potent and stereospecific nicotinic receptor agonist (+)-anatoxin-a. *Mol. Pharmacol.* **29**:250–257 (1986).
50. Thomas, P., M. Stephens, G. Wilkie, M. Amar, G. G. Lunt, P. Whiting, T. Gallagher, E. Pereira, M. Alkondon, and E. X. Albuquerque. (+)-Anatoxin-a is a potent agonist at neuronal nicotinic acetylcholine receptors. *J. Neurochem.* **60**:2308–2311 (1993).
51. Levin, E. D., and J. A. Rosecrans. Promise of nicotine-based treatments. *Drug Dev. Res.* **31**:1–2 (1994).
52. Whitehouse, P. J. Cholinergic therapy in dementia. *Acta Neurol. Scand. Suppl.* **149**:42–45 (1993).

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