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Erysodine, a competitive antagonist at neuronal nicotinic acetylcholine receptors

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Abstract

Erysodine, an erythrina alkaloid related to dihydro-β-erythroidine, was found to be a more potent inhibitor of [^{3}H]cytisine binding at neuronal nicotinic acetylcholine receptors but a less potent inhibitor of [^{125}I]α-bungarotoxin binding at muscle-type nicotinic acetylcholine receptors than dihydro-β-erythroidine. Erysodine was a competitive, reversible antagonist of (−)-nicotine-induced dopamine release from striatal slices and inhibited (−)-nicotine-induced $^{86}Rb^+$ efflux from IMR-32 cells. Erysodine was equipotent with dihydro-β-erythroidine in the dopamine release assay but 10-fold more potent in the $^{86}Rb^+$ efflux assay, suggesting differential subtype selectivity for these two antagonists. Erysodine, systemically administered to mice, entered the brain and significantly attentuated nicotine's hypothermic effects and its anxiolytic-like effects in the elevated plus-maze test. There was greater separation between antagonist and toxic doses for erysodine than for dihydro-β-erythroidine, perhaps because of erysodine's greater selectivity for neuronal receptors. In rats, erysodine prevented both the early developing decrease and the late-developing increase in locomotor activity produced by (−)-nicotine. The potent and competitive nature of erysodine's antagonism together with its ability to enter the brain after systemic administration suggest that erysodine may be a useful tool in characterizing neuronal nicotinic acetylcholine receptors.

Keywords: Nicotinic acetylcholine receptor antagonist; Erysodine; Dihydro-β-erythroidine; Pharmacokinetics

1. Introduction

Recent findings in molecular biology have revealed that nicotinic acetylcholine receptors in the brain are pentameric structures composed of α and β subunits. Genes coding for eight α (α_2–α_9) subunits and three β subunits (β_2–β_4) have been identified in neuronal tissue, and attempts to express these subunits in *Xenopus* oocytes have identified at least eight functional receptors (Deneris et al., 1991; Luetje and Patrick, 1991; Sargent, 1993; Elgoyhen et al., 1994). Currently, pharmacological probes for these receptor subtypes are limited, and tools that distinguish between the various neuronal nicotinic acetylcholine receptor subtypes as well as tools that distinguish between neuronal and muscle acetylcholine receptors would be useful in furthering our understanding of this complex receptor system (Williams et al., 1994).

Erythrina alkaloids have long been known for their curare-like effects. Lehman (1937) described the paralytic effects of alcohol extracts of *Erythrina americana* seeds on voluntary musculature. Subsequently, several alkaloids extracted from plants of the genus *Erythrina* were identified with curare-like properties, including dihydro-β-erythroidine (Fig. 1), which is among the most potent of the curare-like compounds isolated from Erythrina plants (Megirian et al., 1955). In addition to its curare-like effects, dihydro-β-erythroidine is a competitive antagonist at neuronal nicotinic acetylcholine receptors (Williams and Robinson, 1984). This property of dihydro-β-erythroidine has made it an important research tool, as it is the only competitive antagonist of neuronal nicotinic acetylcholine receptors in common use. In contrast to dihydro-β-erythroidine,

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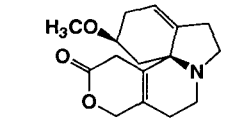
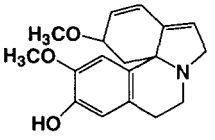


Dihydro – β - erythroidine



Erysodine

Fig. 1. Structures of dihydro-β-erythroidine and erysodine.

most neuronal nicotinic receptor antagonists do not bind with high affinity to the agonist binding site located on the α subunit of the nicotinic acetylcholine receptor (Kao et al., 1984), but rather are thought to interact with the ion channel itself, thus functioning as noncompetitive antagonists (Martin et al., 1989).

Although the effects of several erythrina alkaloids at muscle-type nicotinic acetylcholine receptors have been characterized, only the effects of dihydro-β-erythroidine have been characterized extensively at neuronal nicotinic acetylcholine receptors. In the current study, we investigated the effects of erysodine (Fig. 1), another erythrina alkaloid, at neuronal nicotinic acetylcholine receptors and now report that this compound appears to show greater selectivity than dihydro-β-erythroidine for neuronal as opposed to muscle-type nicotinic acetylcholine receptors.

2. Materials and methods

2.1. In vitro studies

Receptor binding

Membrane-enriched fractions were prepared from brains of male Sprague-Dawley rats (250–350 g) (Sasco, Madison, WI, USA) according to the method of Enna and Snyder (1975). Whole brains were homogenized in 15 volumes of 0.32 M sucrose and centrifuged at 1000 × *g* at 4° C for 10 min. Supernatants were centrifuged at 20000 × *g* for 20 min. The resulting P_2 pellets were homogenized with a Polytron (10 s at setting 7) in 15 volumes ice-cold H_2O and centrifuged at 8000 × *g* at 4° C for 20 min. The supernatant and loose buffy coat were centrifuged at 40000 × *g*. The pellet was washed in 15 volumes ice-cold H_2O and recentrifuged before storage at −80° C.

[^{3}H]Cytisine binding. [^{3}H]Cytisine binding was performed using a modification of the method of Pabreza et al. (1991). Membrane-enriched fractions were slowly thawed at 4° C and washed and resuspended in 30 volumes of assay buffer (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 50 mM Tris-Cl, pH 7.4, 4° C). Saturation isotherms were performed on aliquots of protein (100–200 μg), 0.05–6 nM [^{3}H]cytisine (30 Ci/mmol) and compounds to the final concentrations indicated. Samples were incubated in a final volume of 500 μl for 75 min at 4° C in duplicate. Non-specific binding was determined in the presence of 10 μM (−)-nicotine. Bound radioactivity was separated under vacuum onto No. 32 glass fiber filters (Schleicher and Schuell, Keene, NH, USA) using a Skatron filtration apparatus (Skatron, Sterling, VA, USA).

[^{125}I]α-Bungarotoxin binding. [^{125}I]α-Bungarotoxin binding was determined in membranes prepared from whole rat brain and from *Torpedo californica* electroplax.

α-Bungarotoxin binding to rat brain membranes was determined using a modification of the method of Marks et al. (1986). Rat brain membranes were resuspended in 15 volumes of assay buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM Hepes, pH 7.5). Aliquots containing 200 μg of tissue were added to a reaction mixture containing 1.9 nM [^{125}I]α-bungarotoxin (106 Ci/mmol) and the indicated concentrations of the test compounds in triplicate. Non-specific binding was determined in the presence of 1 μM unlabeled α-bungarotoxin. Binding was conducted at 37° C for 3 h. Bound radioactivity was isolated by rapid vacuum filtration onto No. 32 glass fiber filters (Schleicher and Schuell) using a Skatron filtration apparatus.

A solid-phase binding assay was used to measure the binding of [^{125}I]α-bungarotoxin (106 Ci/mmol) to the muscle-type nicotinic acetylcholine receptor isolated from *Torpedo californica* electroplax (Chaturvedi et al., 1993). The wells of a 96-well microtiter plate (Immulon Removawells Strips, Dynatech, Chantilly, VA, USA) were coated with 0.5 μg of *Torpedo* membranes (ABS, Wilmington, DE, USA) in 50 mM $NaHCO_3$ buffer, pH 9.6, for 12 h at 4° C. Wells were then washed twice with phosphate-buffered saline (PBS) and quenched for 1 h with 5% bovine serum albumin. [^{125}I]α-Bungarotoxin (~ 1.9 nM/100 μl 10 mM phosphate buffer, pH 7.4/0.2% bovine serum albumin) was then added to the wells for 1 h. For competition experiments, increasing concentrations of competitor (50 μl) were added to wells in triplicate followed immediately by 50 μl of [^{125}I]α-bungarotoxin and incubated for 1 h. Non-specific binding was determined in the presence of 1 μM α-bungarotoxin. After incubation, wells were washed 5 times with PBS. Individual wells were placed in vials and radioactivity determined.

[^{3}H]Oxotremorine-M binding assay. [^{3}H]Oxotremorine-M (87 Ci/mmol) binding to the muscarinic acetylcholine receptor was performed in 20 mM Na_2PO_4 buffer, pH, 7.4 at 25° C for 45 min using a modification of the method of Birdsall et al. (1978). The assay mixture contained 100 μg of rat brain membranes per tube, 2 nM [^{3}H]oxotremorine-M and the indicated concentrations of compounds in triplicate. Non-specific binding was determined in the presence of 10 μM atropine. Radioactivity was isolated and radioactivity determined as described above.

Data analysis. In competition experiments, the drug concentration producing 50% inhibition (IC_{50}) of radioligand binding and the Hill coefficient (n_H) were determined from plots of log $(B_0 - B)/B$ versus log (concentration of drug), where B_0 and B are specific binding in the absence and presence of competitor, respectively using a four-parameter logistics program in RS/1 (Bolt Beranek and Newman, Cambridge, MA, USA). Inhibition constant (K_i) values were determined using the Cheng-Prusoff equation ($K_i = IC_{50}/1 + [L]/K_d$, where [L] = free radioligand concentration). Apparent dissociation constants ($K_{D'}$) were determined from saturation isotherms by non-linear regression analysis using Inplot (GraphPad, San Diego, CA, USA). Data were analyzed for both one-site and two-site fits using LIGAND (Munson and Rodbard, 1980). The $K_{D'}$ values were plotted versus the concentration of erysodine. Linear regression analysis was used to determine the x-intercept, which represents the inhibition dissociation constant (K_i) for erysodine.

Striatal dopamine release

Nicotinic acetylcholine receptor evoked release of [*ring*-2,5,6-^{3}H]dopamine (24.4 Ci/mmol) was measured in superfused rat striatal slices. Striata were dissected from two male Sprague-Dawley rats per experiment and sliced 0.35 × 0.25 mm by a McIlwain Tissue Chopper (Brinkman Instrument Co., Westbury, NY, USA). After two washes with Krebs-Hepes buffer (137 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 10 mM glucose, 15 mM Hepes-NaOH, pH 7.4, containing 10 μM pargyline and 10 μM ascorbic acid), slices were preincubated for 10 min at 37° C under 95%/5% O_2/CO_2. After replacing the buffer, slices were labeled with 100 nM [^{3}H]dopamine for 25 min in Krebs-Hepes at 37° C. Aliquots of slices were placed in 18 superfusion chambers of a Brandel SP2000 superfusion apparatus (Brandel, Gaithersburg, MD, USA). Following 47 min of washout, slices were exposed to agonist for 4 min with a flow rate of 0.6 ml/min. Antagonists, when present, were introduced 4 min prior to and during agonist exposure. Thirteen 2 min fractions were collected and counted in 5 ml of Ecolume. Tissue was recovered from superfusion chambers, solubilized with 1 ml of Solvable (DuPont-NEN, Boston, MA, USA) and counted in 15 ml of Ecolume.

Fractional release of [^{3}H]dopamine was calculated from radioactivity above baseline as a fraction of total radioactivity. Relative efficacies were calculated using the release evoked by 100 nM (−)-nicotine as a standard. EC_{50} values were determined by non-linear least-squares regression analysis using Inplot (GraphPad, San Diego, CA, USA) with data constrained to a competitive model. Data from 12 experiments were fitted by linear regression to obtain the pA_2 value and slope using the method of Arunlakshana and Schild (1959).

$^{86}Rb^+$ efflux

Cells of the IMR-32 human neuroblastoma clonal cell line (ATCC, Rockville, MD, USA) were maintained in a log phase of growth according to established procedures (Lukas, 1993). Experimental cells were seeded at a density of 500 000 cells/ml into a 24-well tissue culture dish. Plated cells were allowed to proliferate for at least 48 h before loading with 2 mCi/ml of $^{86}Rb^+$ (35 Ci/mmol) overnight at 37° C. The $^{86}Rb^+$ efflux assays were performed according to previously published protocols (Lukas and Bencherif, 1992) except serum-free Dulbecco's modified Eagle's medium was used during the $^{86}Rb^+$ loading, rinsing, and agonist-induced efflux steps. Responses were normalized to 100 μM (−)-nicotine because this is the concentration of (−)-nicotine that produces the maximum response (Lukas, 1993; Sullivan et al., 1994).

2.2. In vivo studies

Subjects

Male, CD-1 mice (30–35 g) and male, Wistar rats (250–300 g) obtained from Charles River Laboratories (Portage, MI, USA) were used in the behavioral experiments. All animals used in this study were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee. The mice were housed 14 to a cage and the rats were housed singly with free access to food and water in separate climate controlled facilities. All testing was conducted during the light portion of the 12 : 12 h light : dark cycle (lights on at 06:00 h).

Body temperature and locomotor activity

The effects of dihydro-β-erythroidine and erysodine on nicotine-induced reductions in body temperature and locomotor activity in mice were determined in separate experiments. In each case, the antagonist (0, 1.5, and 5 μmol/kg dihydro-β-erythroidine or 0, 3, 10, and 30 μmol/kg erysodine) was administered i.p. 5 min prior to (−)-nicotine (6.2 μmol/kg i.p.) or saline.

The mice were placed in a 41 × 41 cm open field 3–4 min after the nicotine challenge, and horizontal activity counts were recorded for 10 min using Digiscan activity monitors (Omnitech Electronics, Columbus, OH, USA). After the mice were removed from the activity monitors (approximately 15 min after the (−)-nicotine challenge), their body temperatures were measured using a rectal probe inserted 2 cm into the rectum (YSI TeleThermometer, Yellow Springs Instrument Co., Yellow Springs, OH, USA).

The effects of erysodine on nicotine-induced changes in the locomotor activity of rats were also determined. In this experiment, erysodine (30 μmol/kg i.p.) was administered 5 min prior to (−)-nicotine (1.9 μmol/kg). Within 2 min of the second injection, the rat was placed in an open field (40 × 40 cm) and both horizontal activity and rearing were measured over six 10-min blocks with a photobeam activity system (San Diego Instruments, San Diego, CA, USA).

Elevated plus-maze

The elevated plus-maze, a pharmacologically validated measure of anxiety (Brioni et al., 1993; Pellow et al., 1985), was used to assess anxiolytic activity in mice. The plus-maze was constructed with gray Plexiglas and consisted of two open arms (17 × 8 cm) and two enclosed arms (17 × 8 × 15 cm) extending from a central platform (8 × 8 cm). The maze itself was placed on a platform which elevated it 39 cm above the floor. Light levels on the open and enclosed arms were similar (approximately 350 Lux). The animal's behavior was assessed using a video-camera mounted above the apparatus and a commercially available tracking system (Videomex, Columbus Instruments, Columbus, OH, USA). Both the time spent in the open arms (a measure of anxiolytic-like activity) and the total distance traveled during the test (a measure of general activity) were determined. (−)-Nicotine (0.62 μmol/kg i.p.) was administered 30 min before the session, and erysodine (30 μmol/kg i.p.) was administered 5 min prior to (−)-nicotine. All mice used were naive to the apparatus and were placed in the center of the maze at the beginning of the 5-min session.

Pharmacokinetic analysis

(−)-Nicotine, erysodine and dihydro-β-erythroidine were extracted from plasma and brain using a mixture of 0.1 ml of plasma or brain homogenates (prepared as described below) to 1.0 ml of buffer basified with 0.1–0.6 ml of 0.5 M K_2CO_3. This mixture was extracted with 5–8 ml of hexane/ethyl acetate (1:1) by vortexing and centrifuging at 8000 × *g* for 15 min at 18° C. The organic phase was back extracted with 0.3 ml of 0.02 N HCl by vortexing and centrifuging as indicated above. To prepare brain tissue homogenates, the brain was homogenized in 5 × weight volume of cold 1 N perchloric acid and centrifuged at 40000 × *g* for 20 min at 4° C. The supernatant was adjusted to pH 10.6 with 2 M K_2CO_3 and treated as described above. Traces of organic solvent in the acid extract from plasma and brain were removed in a fume hood for 1.5 h at room temperature without N_2 blowing or heating before analyses by high-performance liquid chromatography.

The analytical procedure consisted of injecting 5–50 μl of extract into a high-performance liquid chromatographic system (Hewlett Packard Model 1050, Naperville, IL, USA) fitted with a C_{18} reverse-phase column (15 × 0.46 cm, i.d.; ODS-AQ, 5 μm spherical particles, YMC) and a Coulochem II electrochemical detector (ESA, Bedford, MA, USA). The electrochemical detector was fitted with a conditioning cell (Model 5021) and an analytical cell (Model 5010) interfaced with Rainin integrator (Rainin, Woburn, MA, USA). Chromotography was accomplished isocratically at a flow rate of 1 ml/min using a mobile phase for (−)-nicotine consisting of acetonitrile, methanol and 20 mM K-PO_4 (10:10:80) with pH adjusted to 6.3 with tetramethyl ammonium hydroxide, for erysodine the mobile phase consisted of methanol and 50 mM K-PO_4 (40:60) with the pH adjusted to 6.4 while for dihydro-β-erythroidine the mobile phase contained methanol and 50 mM K-PO_4 (22:78) with the pH adjusted to 6.64. Corresponding calibration curves were run and sample values calculated using the Rainin Dynamax program. The sensitivity of the method was 0.5 ng/ml in plasma and 1.0 ng/g brain in brain tissue.

Compounds

Erysodine was extracted from erythrina seeds. Dihydro-β-erythroidine hydrobromide and oxotremorine sesquifumarate were obtained from Research Biochemicals International (Natick, MA, USA), and (−)-nicotine bitartrate salt was obtained from Sigma (St. Louis, MO, USA). [^{3}H](−)-Cytisine (30 Ci/mmol), [^{125}I]α-bungarotoxin (106 Ci/mmol), [^{3}H]oxotremorine-M (87 Ci/mmol), $^{86}Rb^+$ (35 Ci/mmol) and [*ring*-2,5,6-^{3}H]dopamine (24.4 Ci/mmol) were all obtained from DuPont-NEN (Boston, MA, USA). Erysodine was dissolved in distilled water for i.p. injections, and dihydro-β-erythroidine and (−)-nicotine were dissolved in 0.9% sterile saline solution. All drug doses are expressed in μmol/kg. Control injections consisted of vehicle (1 ml/kg for rats and 10 ml/kg for mice).

Statistical analysis

Data were analyzed by nonparametric Mann-Whitney *U*-tests or by analysis of variance (ANOVA) with post-hoc pairwise comparisons evaluated using Fisher's

Table 1
Cholinergic binding properties of erysodine and dihydro-β-erythroidine

	K_i (nM)			
	[^{3}H]Cytisine (rat brain)	[^{125}I]α-Bungarotoxin (rat brain)	[^{125}I]α-Bungarotoxin (*Torpedo* electroplax)	[^{3}H]Oxotremorine-M (rat brain)
Erysodine	5 ± 1.3	4000 ± 900	> 100000	> 100000
Dihydro-β-erythroidine	35 ± 3	9000 ± 1200	11000	> 100000

Values represent mean ± S.E.M., n = 3–5.

protected least-significant difference test (Statview II, Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. In vitro studies

Receptor binding

[^{3}H](−)-Cytisine has been shown to bind with high affinity to the $\alpha_4\beta_2$ subtype of nicotinic acetylcholine receptors, the major subtype in rodent brain accounting for greater than 90% of high-affinity (−)-nicotine binding sites (Flores et al., 1992; Whiting et al., 1992). Both erysodine and dihydro-β-erythroidine displaced [^{3}H](−)-cytisine in a concentration-dependent manner with respective K_i values of 5 ± 1 nM (n = 3) and 35 ± 3 nM (n = 3; Table 1). To investigate the mechanism underlying the inhibition of [^{3}H]cytisine binding by erysodine, saturation isotherms of [^{3}H]cytisine binding to rat brain membranes were performed in the presence of increasing concentrations of erysodine. Scatchard analysis of these data indicated that erysodine had no effect on the maximal density (B_{max}) of [^{3}H]cytisine binding sites but decreased the affinity (K_d) of the radioligand in a concentration-dependent manner (Fig. 2A). Linear regression analysis of the apparent dissociation constants ($K_{D'}$) for [^{3}H]cytisine as a function of the erysodine concentration (2.5–100 nM) was indicative of a competitive mechanism of inhibition (Fig. 2B). The K_i value for erysodine determined from this analysis was 6 ± 0.9 nM (n = 3).

In contrast to its activity at the $\alpha_4\beta_2$ nicotinic acetylcholine receptor subtype labeled by [^{3}H]cytisine, erysodine was approximately three orders of magnitude less potent (K_i = 4000 ± 900 nM, n = 3) in displacing [^{125}I]α-bungarotoxin binding from the α-bungarotoxin-sensitive nicotinic acetylcholine receptor subtype present in rat brain (Table 1). Dihydro-β-erythroidine (K_i = 9000 ± 1200 nM) was slightly less potent than erysodine in inhibiting [^{125}I]α-bungarotoxin binding to this subtype but was at least 10-fold more potent (K_i = 11000 nM) than erysodine (K_i > 100000 nM) in displacing the binding of [^{125}I]α-bungarotoxin to the $\alpha_1\beta_1\delta\gamma$ nicotinic acetylcholine receptor subtype found on *Torpedo* electroplax membranes (Table 1). Both erysodine and dihydro-β-erythroidine were found to display negligible affinity for muscarinic receptors.

[^{3}H]Dopamine release from rat striatal slices

The ability of erysodine and dihydro-β-erythroidine to inhibit nicotine (100 nM) evoked neurotransmitter

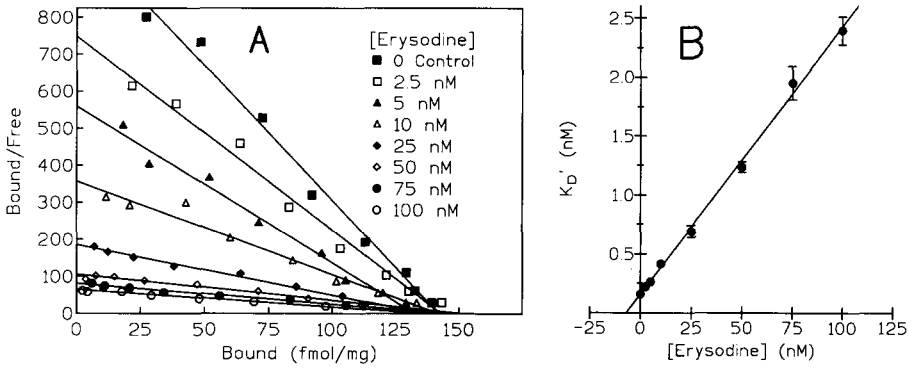


Fig. 2. Competitive inhibiton of [^{3}H]cytisine binding to rat brain membranes by erysodine. A: Scatchard plot representation of specific binding of eight concentrations of [^{3}H]cytisine as a function of erysodine concentration. Values represent the average of quadruplicates in one of three independent experiments. B: Kinetic plot of apparent dissociation constants for [^{3}H]cytisine as a function of erysodine concentration. The apparent dissociation constant, $K_{D'}$, was determined for each concentration of erysodine by non-linear regression analysis. The inhibition dissociation constant, K_i, for erysodine is represented by the negative of the x-intercept. This plot is one of three independent determinations.

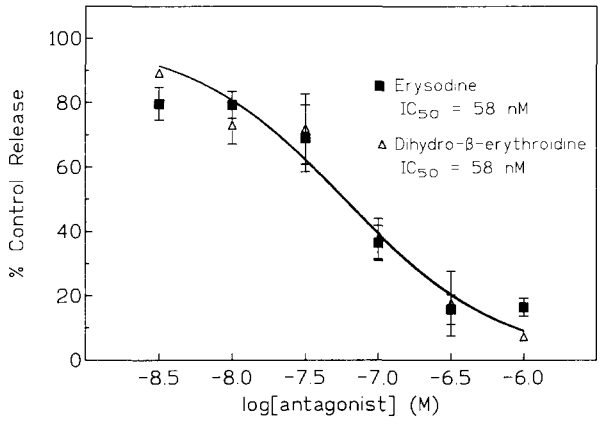


Fig. 3. Dose inhibition by erysodine and dihydro-β-erythroidine of 100 nM (−)-nicotine-evoked release of [^{3}H]dopamine from preloaded slices of rat striatum. Superfused slices were exposed to erysodine and dihydro-β-erythroidine concentrations prior to and during stimulation with the submaximal dose of (−)-nicotine. For each experiment, fractional percentage of evoked release for each sample was expressed as percentage of control fractional release by 100 nM (−)-nicotine (mean, 1.39%; range, 1.28–1.49%). Values represent mean ± S.E.M. of multiple determinations, $n = 3$–5.

release was investigated in rat striatal slices preloaded with [^{3}H]dopamine. Slices were exposed to antagonist 4 min prior to and during the stimulation with (−)-nicotine. Both agents inhibited nicotinic acetylcholine receptor-mediated release with identical potencies (erysodine, $IC_{50} = 58 \pm 3$ nM; dihydro-β-erythroidine, $IC_{50} = 58 \pm 5$ nM) (Fig. 3). The IC_{50} values are a measure of relative affinity, but do not properly reflect absolute affinity or give information concerning the mechanism of inhibition. To better determine the affinity of erysodine for the subtype(s) of nicotinic acetylcholine receptor mediating dopamine release from rat striatal slices, the effect of erysodine (10–320 nM) on concentration-response curves for (−)-nicotine-evoked [^{3}H]dopamine release was investigated. Erysodine caused a shift to the right of the response curve to (−)-nicotine, which was surmountable with increasing concentrations of (−)-nicotine (Fig. 4A). The K_d value, determined from dose ratios (EC'_{50}/EC_{50}), was: K_d = [erysodine]/(DR − 1) = 7.0 ± 0.1 nM ($n = 12$). Schild analysis of the erysodine competition for [^{3}H]dopamine release yielded a pA_2 of 8.08 ($K_B = 8.3$ nM) and a slope of 1.02 ± 0.07 ($n = 3$–5) (Fig. 4B). The linearity of the plot and the slope of unity are indicative of competitive, reversible antagonism.

$^{86}Rb^+$ efflux from IMR-32 cells

Both erysodine and dihydro-β-erythroidine inhibited (−)-nicotine-induced cation efflux from the human neuroblastoma cell line, IMR-32, in a concentration-dependent manner (Fig. 5). Erysodine ($IC_{50} = 7 \pm 2$ μM) was approximately 10-fold more potent than dihydro-β-erythroidine ($IC_{50} = 84 \pm 17$ μM) in this assay.

3.2. In vivo studies

Temperature and locomotor activity

The effects of dihydro-β-erythroidine and erysodine on nicotine-induced reduction in body temperature and locomotor activity are shown in Figs. 6 and 7. Dihydro-β-erythroidine significantly attenuated the hypothermic effects of 6.2 μmol/kg (−)-nicotine (Fig. 6A). A two-way ANOVA revealed significant main effects for nicotine ($F(1,30) = 39.03$, $P < 0.0001$) and dihydro-β-erythroidine ($F(2,30) = 8.34$, $P < 0.005$) as well as a significant nicotine by dihydro-β-erythroidine interaction ($F(2,30) = 3.80$, $P < 0.05$). The interaction term and the dihydro-β-erythroidine main effect appear to be attributable to a dihydro-β-erythroidine blockade of nicotine's hypothermic effects, as dihydro-

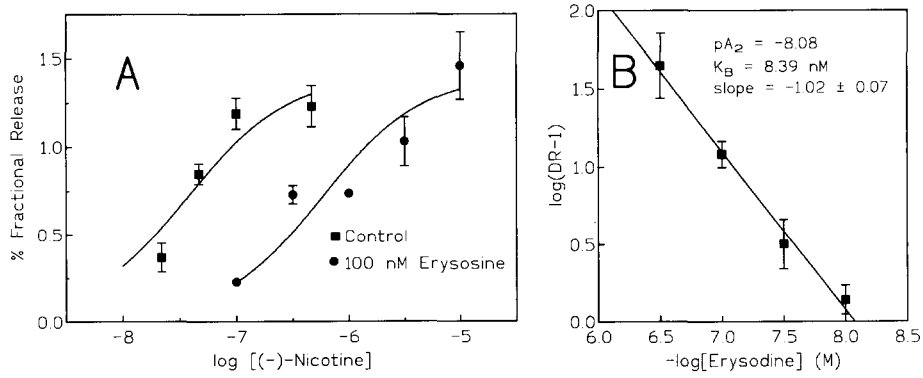


Fig. 4. Erysodine is a competitive antagonist of (−)-nicotine-evoked [^{3}H]dopamine release from rat striatal slices. A: Effect of 100 nM erysodine on concentration-response curves for (−)-nicotine-evoked release of [^{3}H]dopamine. The plot shown is a representative of 12 determinations. B: Schild plot of erysodine competition for (−)-nicotine-evoked release of [^{3}H]dopamine. The log of (dose ratio − 1) was plotted as a function of the negative log of erysodine concentration. The pA_2 was determined from the negative of the x-intercept. Each data point represents the mean ± S.E.M. of three separate determinations.

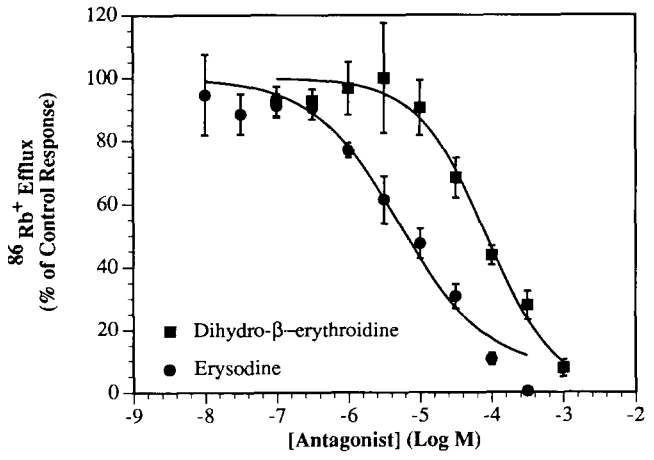


Fig. 5. Effect of erysodine and dihydro-β-erythroidine to inhibit nicotine-induced $^{86}Rb^+$ efflux from IMR 32 cells. IMR 32 cells that had been loaded with $^{86}Rb^+$ were exposed for 5 min to the concentration of antagonist indicated prior to the addition of 100 μM (−)-nicotine for 5 min. Typical values for specific and nonspecific $^{86}Rb^+$ efflux at K_{max} were 7500 and 2800 cpm, respectively, for cells loaded with 35000 cpm of $^{86}Rb^+$. All values were normalized as a percentage of the $^{86}Rb^+$ effluxed at 100 μM nicotine alone. Values are the mean ± S.E.M., $n = 5$.

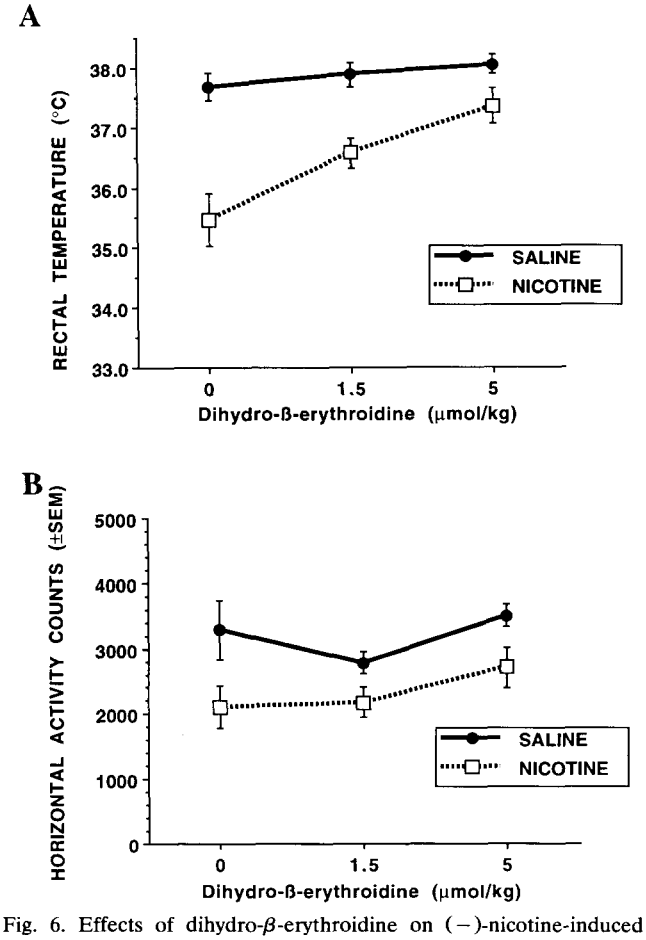


Fig. 6. Effects of dihydro-β-erythroidine on (−)-nicotine-induced hypothermia (A) and hypomotility (B) in mice. Dihydro-β-erythroidine was administered prior to 6.2 μmol/kg (−)-nicotine. Shown are mean values (± S.E.M.) for 6 animals per group.

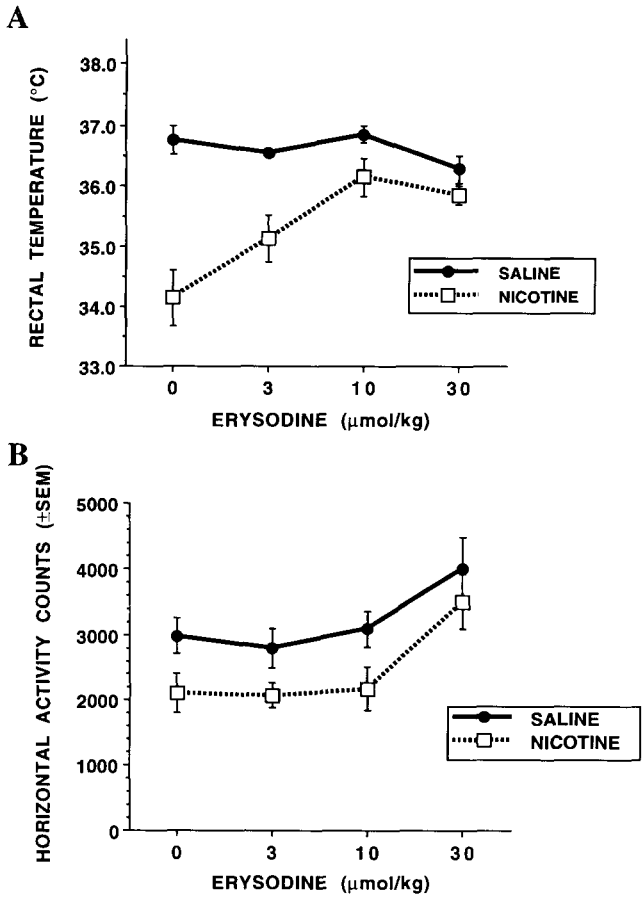


Fig. 7. Effects of erysodine on (−)-nicotine-induced hypothermia (A) and hypomotility (B) in mice. Erysodine was administered prior to 6.2 μmol/kg (−)-nicotine. Shown are mean values (± S.E.M.) for 7 animals per group.

β-erythroidine significantly increased body temperature in (−)-nicotine-challenged mice ($F(2,15) = 7.88$, $P < 0.005$), but not in saline-challenged mice ($F(2,15) = 0.85$, $P > 0.4$). Significant nicotine-induced hypothermia was observed in mice pretreated with vehicle ($P < 0.005$) or with 1.5 μmol/kg of dihydro-β-erythroidine ($P < 0.005$), but not in mice pretreated with 5 μmol/kg of dihydro-β-erythroidine ($P > 0.05$). Erysodine produced similar effects on nicotine-induced hypothermia (Fig. 7A). In the erysodine experiment, a two-way ANOVA revealed significant main effects for nicotine ($F(1,48) = 42.26$, $P < 0.0001$) and erysodine ($F(3,48) = 4.86$, $P < 0.005$) and a significant nicotine by erysodine interaction ($F(3,48) = 6.08$, $P < 0.005$). As in the dihydro-β-erythroidine experiment, the nicotine by erysodine interaction term and the erysodine main effect appear to be due to an erysodine blockade of nicotine's hypothermic effects: erysodine increased body temperature in (−)-nicotine-challenged mice ($F(3,24) = 6.42$, $P < 0.005$), but not in saline-challenged mice ($F(3,24) = 1.97$, $P > 0.1$). Significant nicotine-induced hypothermia was observed in mice pre-

treated with vehicle ($P < 0.005$) or with 3 μmol/kg ($P < 0.005$) of erysodine but not in mice pretreated with 10 μmol/kg ($P > 0.05$) or 30 μmol/kg ($P > 0.1$) of erysodine. Although 10 μmol/kg erysodine significantly attenuated nicotine-induced hypothermia, it did not alter the hypothermic effects of the muscarinic agonist oxotremorine (0.26 and 0.79 μmol/kg; data not shown). In a separate experiment, the brain and plasma concentrations were determined for (−)-nicotine (6.2 μmol/kg) and the minimum doses of dihydro-β-erythroidine (5 μmol/kg) and erysodine (10 μmol/kg) required to produce significant attenuation of the hypothermic effect of 6.2 μmol/kg (−)-nicotine. In each case concentrations were measured at a time point corresponding to when body temperature had been assessed. Brain and plasma levels of (−)-nicotine were 240 ± 30 ng/g and 42 ± 4 ng/ml, respectively, while for dihydro-β-erythroidine the levels were 453 ± 35 ng/g and 18 ± 2 ng/ml respectively. In contrast, the brain and plasma levels for erysodine were much lower, 58 ± 8 and 21 ± 4, respectively (Table 2).

The effects of dihydro-β-erythroidine and erysodine on nicotine-induced decreases in locomotor activity in mice were less pronounced than the effects of these compounds on (−)-nicotine-induced hypothermia. In the dihydro-β-erythroidine experiment (Fig. 6B), the nicotine main effect was significant ($F(1,30) = 12.89$, $P < 0.005$), but the dihydro-β-erythroidine main effect ($F(2,30) = 2.31$, $P > 0.1$) and the dihydro-β-erythroidine by nicotine interaction ($F(2,30) = 0.50$, $P > 0.6$) were not. Thus, a clear blockade was not observed. In the erysodine experiment (Fig. 7B), the nicotine main effect was also significant ($F(1,48) = 9.98$, $P < 0.0001$) as was the erysodine effect ($F(3,48) = 6.65$, $P < 0.001$), but the interaction was not ($F(3,48) = 0.17$, $P > 0.9$). In this experiment, erysodine appeared to increase activity, but this effect was only significant in nicotine-treated animals ($F(3,24) = 4.74$, $P < 0.01$). Although there was no significant nicotine by erysodine interaction in this experiment, it should be noted that a tendency for (−)-nicotine to reduce locomotor activity was observed in vehicle-pretreated and mice treated with 3 or 10 μmol/kg of erysodine ($0.05 < P < 0.1$ in each case) but not in animals pretreated with 30 μmol/kg of erysodine ($P > 0.4$). In contrast, a nicotine-induced decrease in activity was noted in mice

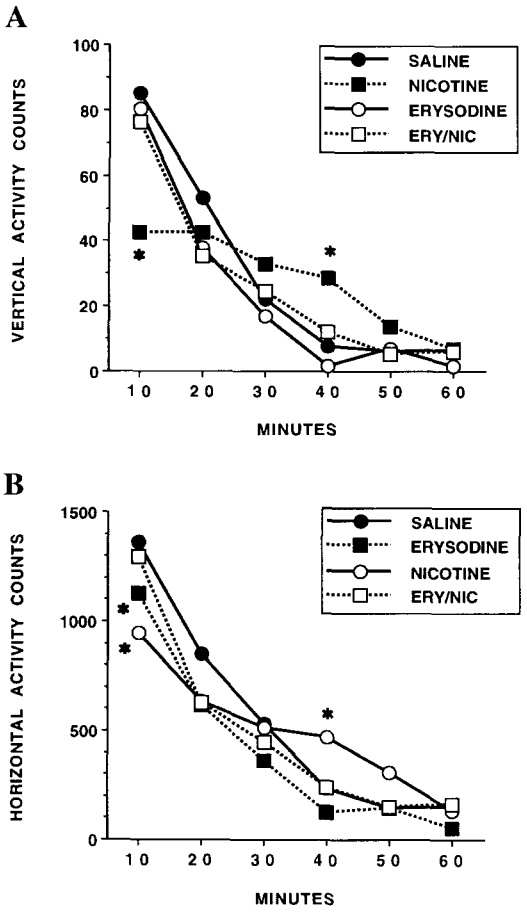


Fig. 8. Effects of erysodine (30 μmol/kg) on (−)-nicotine-induced suppression and stimulation of vertical activity (rearing) (A) and horizontal activity (B) of rats in an open field. Erysodine was administered 5 min prior to 1.9 μmol/kg (−)-nicotine. *Significantly different from control, $P < 0.05$ ($n = 8$ per group).

treated with the highest dose (5 μmol/kg) of dihydro-β-erythroidine tested ($P < 0.05$). Thus erysodine may have been somewhat more effective than dihydro-β-erythroidine in protecting against nicotine's locomotor effects in mice.

When the 30 μmol/kg dose of erysodine was tested in rats, a more reliable blockade of nicotine's locomotor effects was observed. The pattern of effects was similar for both horizontal activity and rearing (Fig. 8). Analysis of these data by a three-way repeated measures ANOVA with erysodine and nicotine as the

Table 2
Mouse brain and plasma levels of (−)-nicotine, erysodine and dihydro-β-erythroidine following administration of behaviorally effective doses

Compound	Brain level (ng/g)	Plasma level (ng/ml)
(−)-Nicotine (6.2 μmol/kg i.p.)	240 ± 30 (6)	42 ± 4 (6)
Erysodine (10 μmol/kg i.p.)	58 ± 8 (5)	21 ± 4 (5)
Dihydro-β-erythroidine (5 μmol/kg i.p.)	453 ± 35 (5)	18 ± 2 (5)

Values are means ± S.E.M. at 15 min after injection for (−)-nicotine and 20 min after injection for erysodine and dihydro-β-erythroidine (corresponding to the time at which body temperature was measured in a separate set of mice). Numbers in parentheses represent the number of individual animals evaluated.

between-group measures and time as the repeated measure revealed that 1.9 μmol/kg (−)-nicotine had a biphasic effect on rearing (nicotine by time interaction $F(5,140) = 7.72$, $P < 0.0001$) and horizontal activity (nicotine by time interaction $F(5,140) = 4.27$, $P < 0.005$). In both cases this interaction was a reflection of a nicotine-induced decrease during the first 10 min and a nicotine-induced increase during the later portion of the session, with a maximal increase noted at 40 min. A significant three-way interaction between erysodine, nicotine, and time was also found for both rearing ($F(5,140) = 3.45$, $P < 0.01$) and horizontal activity ($F(5,140) = 5.86$, $P < 0.0001$). To evaluate the nature of this interaction, separate one-way ANOVAs were conducted at the time points where nicotine produced its maximal suppressive (10 min) and stimulatory (40 min) effects. Erysodine, by itself, did not significantly affect rearing at either of these time points but significantly reduced the effects of nicotine in each case ($P < 0.05$). Erysodine reduced horizontal activity somewhat ($P < 0.05$) at 10 min but prevented the suppressive effect of (−)-nicotine when it was administered prior to nicotine ($P < 0.01$). At the 40 min time point, erysodine, by itself, did not significantly affect horizontal activity but prevented the stimulatory effect of (−)-nicotine ($P < 0.05$). Determination of brain levels of erysodine in a separate set of rats revealed comparable brain concentrations at these two time points (280 ± 31 ng/g brain ($n = 3$) at 10 min and 306 ± 45 ng/g brain ($n = 3$) at 40 min). These concentrations are similar to the (−)-nicotine levels produced by the injection of (−)-nicotine (1.9 μmol/kg) used in this experiment (318 ± 42 ng/g brain; $n = 3$). Plasma levels of (−) nicotine at 10 min (49 ± 2 ng/ml) and 40 min (27 ± 1.3 ng/ml) were much lower than the corresponding brain levels. In contrast, the brain:plasma ratio for erysodine was 1.5 at 10 min and 1.0 at 40 min.

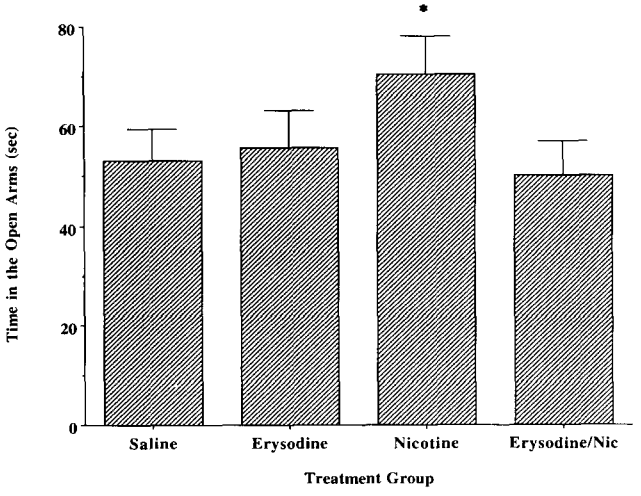


Fig. 9. Effects of erysodine (30 μmol/kg) on (−)-nicotine's anxiolytic-like effects in the elevated plus-maze. Shown is the mean (+S.E.M.) time mice spent on the open arms during the 5 min test. Erysodine was administered 5 min prior to 0.62 μmol/kg (−)-nicotine. *Significantly different from saline group and erysodine/nicotine group, $P < 0.05$ ($n = 8$–14 per group).

Elevated plus-maze

The effects of 30 μmol/kg erysodine on nicotine's anxiolytic-like effects in the elevated plus-maze test were also assessed (Fig. 9). In this experiment, erysodine by itself did not affect the amount of time spent in the open arms of the apparatus, whereas (−)-nicotine (0.62 μmol/kg) significantly increased time spent on the open arms ($P < 0.05$; Mann-Whitney U-test). Pretreatment with erysodine, however, blocked this anxiolytic-like effect of (−)-nicotine (nicotine vs. erysodine/nicotine, $P < 0.05$; Mann-Whitney U-test). The significant effect of (−)-nicotine on time spent in the open arms of the plus-maze was not accompanied by an effect on distance traveled during the maze exploration (nicotine vs. saline, $P > 0.9$; Mann-Whitney U-test). Brain and plasma concentrations of erysodine determined in a subset of these mice killed immediately after testing on the elevated plus-maze were 92.2 ± 13.6 ng/g and 30.5 ± 5.3 ng/ml respectively.

4. Discussion

In vitro studies revealed erysodine to be a potent competitive ligand at nicotinic acetylcholine receptors with a different pattern of selectivity than that displayed by dihydro-β-erythroidine. In receptor binding experiments, erysodine had 7-fold greater affinity for the high-affinity nicotine binding site labeled by [^{3}H](−)-cytisine than did dihydro-β-erythroidine. This site appears to correspond to the $\alpha_4\beta_2$ subtype of nicotinic acetylcholine receptor, the major high-affinity (−)-nicotine binding site in rodent brain (Flores et al., 1992; Whiting et al., 1992). Thus, erysodine would appear to display greater affinity for the $\alpha_4\beta_2$ nicotinic acetylcholine receptor subtype than does dihydro-β-erythroidine. In contrast, erysodine had only slightly greater affinity than dihydro-β-erythroidine for neuronal nicotinic acetylcholine receptors labeled by α-bungarotoxin (presumably the α_7 subtype) and displayed 10-fold lower affinity for the muscle-type nicotinic acetylcholine receptor expressed in *Torpedo* electroplax. Neither compound bound appreciably to muscarinic acetylcholine receptors.

Erysodine proved to be a potent antagonist of nicotine-mediated effects in vitro. Erysodine antagonized nicotine-induced dopamine release with an IC_{50} equivalent to that found with dihydro-β-erythroidine (58 nM). Characterization of erysodine's antagonism in this assay using Schild analysis was indicative of competi-

tive, reversible antagonism. In the $^{86}Rb^{+}$ flux assay in IMR-32 cells, erysodine was 10-fold more potent than dihydro-β-erythroidine in preventing nicotine-induced cation flux. Thus, dihydro-β-erythroidine is less potent in antagonizing nicotinic acetylcholine receptor-mediated cation efflux in IMR-32 cells, which is thought to reflect activation of the $\alpha_3\beta_4$ subtype (Lukas, 1993), and relatively more potent in antagonizing nicotinic acetylcholine receptor-mediated (−)-nicotine-evoked release of [^{3}H]dopamine from striatal slices. Since there is evidence to support a role for the $\alpha_3\beta_2$ nicotinic acetylcholine receptor subtype in [^{3}H]dopamine from striatal slices (Grady et al., 1992), our results with dihydro-β-erythroidine are consistent with the observation that dihydro-β-erythroidine is relatively weak at inhibiting activation of the $\alpha_3\beta_4$ subunit combination expressed in *Xenopus* oocytes relative to its effects at other subunit combinations, including $\alpha_3\beta_2$ and $\alpha_4\beta_2$ (Luetje and Patrick, 1991; Luetje et al., 1994). Moreover, the results of the in vitro functional experiments in the current study would suggest that erysodine has a greater affinity for the $\alpha_3\beta_4$ subtype than does dihydro-β-erythroidine but that these compounds may have similar affinities for the $\alpha_3\beta_2$ subtype.

In vivo results also support the characterization of erysodine as a nicotinic acetylcholine receptor antagonist. Erysodine and dihydro-β-erythroidine were both effective in preventing nicotine-induced hypothermia in mice; and consistent with its poor affinity for muscarinic acetylcholine receptors, erysodine did not prevent oxotremorine-induced hypothermia. Comparisons across experiments suggest that dihydro-β-erythroidine may have been slightly more potent than erysodine in blocking nicotine-induced hypothermia. No significant nicotine effect was observed in mice treated with 5 μmol/kg of dihydro-β-erythroidine, whereas a comparable reduction in the nicotine effect required 10 μmol/kg of erysodine. This would appear to be inconsistent with our finding that erysodine has greater affinity for high-affinity nicotinic binding sites in vitro, and could be suggestive of subtype selectivity. It is more likely, however, that this apparent discrepancy between the in vitro and in vivo results can be explained by marked differences in the pharmacokinetic profiles of erysodine and dihydro-β-erythroidine in mice. Both compounds readily entered the brain after systemic administration, but unlike erysodine, dihydro-β-erythroidine appeared to be concentrated in the brain at the time point at which body temperature was measured. Injection of the lowest dose of dihydro-β-erythroidine that significantly attenuated nicotine-induced hypothermia produced a brain concentration 8- to 9-fold higher than that produced by the minimum effective dose of erysodine. Thus, the ratio of brain concentrations achieved with the minimum effective antagonist doses of these antagonists is in close agreement with their relative in vitro affinities for the high-affinity (−)-nicotine binding site.

It is notable that the margins of safety between the minimum effective antagonist dose and doses producing toxicity are quite different for erysodine and dihydro-β-erythroidine. A dose of 10 μmol/kg of dihydro-β-erythroidine (twice the minimally effective blocking dose) was fatal in 4 out of 4 mice tested, whereas no obvious toxicities were noted with 30 μmol/kg of erysodine (3 times the minimally effective antagonist dose). Lethality after dihydro-β-erythroidine appeared to be associated with breathing difficulties, and the greater toxicity of this compound relative to erysodine may be related to the greater affinity of dihydro-β-erythroidine for muscle-type nicotinic acetylcholine receptors found in the receptor binding studies.

Neither compound was particularly potent in blocking nicotine's locomotor effects in mice. Dihydro-β-erythroidine did not appear to attenuate nicotine's effects at any dose tested, and although erysodine was somewhat effective, its tendency to increase activity by itself complicates the interpretation of this effect. In general, the modest effects of these compounds on nicotine-induced changes in locomotor activity resemble those reported for mecamylamine, a nicotinic cholinergic channel blocker, which has been reported to block nicotine's temperature effects somewhat more effectively than its locomotor activity effects in mice (Collins et al., 1986).

In contrast to the results with mice, erysodine was clearly effective in antagonizing nicotine's effects on open field behavior in rats. Thus, in rats erysodine prevented both nicotine's initial reduction of rearing behavior and its later stimulation of rearing. Pharmacokinetic analysis revealed comparable concentrations of erysodine in brain tissue at these two time points, concentrations that were higher than those achieved in the mouse studies, which may account for the more pronounced antagonist effects observed in rats. These results are consistent with previous studies in rats where both the early suppression and the later stimulation of activity produced by (−)-nicotine are blocked by the noncompetitive antagonist mecamylamine and by central administration of the long-lasting nicotinic acetylcholine receptor antagonist, chlorisondamine (Clarke, 1984; Decker and Majchrzak, 1993).

As previously observed (Brioni et al., 1993), (−)-nicotine increased the time spent in the open arms of the plus maze, an effect that was not accompanied by a change in locomotor activity. An increase in open-arm exploration is also produced by clinically effective anxiolytic agents, such as diazepam, clonazepam, and ethanol (Brioni et al., 1993; Pellow et al., 1985) and based on these findings is interpreted as an anxiolytic-like effect. In the current study, erysodine, by itself, had no effect on plus-maze exploration, but it did

prevent the anxiolytic-like effect of (−)-nicotine. The anxiolytic-like effect of (−)-nicotine can also be antagonized by the centrally active noncompetitive antagonists mecamylamine and chlorisondamine but not by the peripheral antagonist, hexamethonium (Brioni et al., 1993). Thus, nicotine's anxiolytic-like effects on this test appear to be mediated by central nicotinic acetylcholine receptors.

The results from both in vitro and in vivo studies suggest that erysodine is a potent, competitive antagonist at neuronal-type nicotinic acetylcholine receptors and that it readily enters the brain after parenteral administration. The compound has higher affinity for the predominant neuronal-type nicotinic acetylcholine receptor than dihydro-β-erythroidine but lower affinity than dihydro-β-erythroidine for muscle-type nicotinic acetylcholine receptors. This significantly enhanced ratio between affinity for neuronal relative to muscle nicotinic acetylcholine receptors may explain why the in vivo safety profile observed with systemic administration of erysodine appears to be superior to that observed with dihydro-β-erythroidine. This finding, coupled with evidence from in vitro functional studies that erysodine and dihydro-β-erythroidine have different subtype selectivities, may make erysodine an important tool for further functional characterization of the rapidly expanding array of nicotinic acetylcholine receptors.

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