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Pseudophrynaminol: A Potent Noncompetitive Blocker of Nicotinic Receptor-Channels

Barbara Badio, H. Martin Garraffo, William L. Padgett, Nigel H. Greig and John W. Daly†*

LABORATORY OF BIOORGANIC CHEMISTRY, NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD 20892; AND *LABORATORY OF NEUROSCIENCES, NATIONAL INSTITUTE OF AGING, BALTIMORE, MD 21224, U.S.A.

ABSTRACT. (±)-Pseudophrynaminol inhibited carbamylcholine-elicited sodium-22 influx with an IC_{50} value of about 0.3 μM in both rat pheochromocytoma PC12 cells (ganglionic-type nicotinic receptor) and human medulloblastoma TE671 cells (neuromuscular-type nicotinic receptor). The inhibition in both cell lines appeared to be noncompetitive in nature. In rat cerebral cortical membranes, pseudophrynaminol had only low affinity (K_i 35 μM) for the agonist site on central nicotinic receptors at which [^{3}H]nicotine binds. Pseudophrynaminol, at 10 μM, had marginal effects on a variety of other central receptors, and even at 100 μM inhibited batrachotoxin-elicited sodium-22 influx in a synaptoneurosomal preparation by only 40%. It had no effect at 30 μM on acetylcholinesterase and was a weak inhibitor of butyrylcholinesterase. Thus, pseudophrynaminol appears to be a potent, rather specific, noncompetitive inhibitor of ganglionic and neuromuscular nicotinic receptor-channels. BIOCHEM PHARMACOL 53;5:671–676, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. pseudophrynamines; nicotinic receptors; physostigmine; noncompetitive blockers; acetylcholinesterase

Pseudophrynamines represent a novel class of alkaloids that are known in nature only from the skin of Australian myobatrachid frogs of the genus *Pseudophryne* [1, 2]. The structures of such pseudophrynamines are similar to those of the plant alkaloids eseroline and physostigmine (Fig. 1). The latter is well known as a slowly reversible inhibitor of acetylcholinesterase [3], but also causes a noncompetitive blockade of nicotinic receptor-channels [4], and has been proposed to modulate nicotinic receptor-channel opening [5]. The pseudophrynamine-class alkaloid pseudophrynaminol has now been synthesized and shown to be a potent noncompetitive blocker of nicotinic receptor-channels with similar potency at ganglionic and neuromuscular subtypes. It had no effect on acetylcholinesterase and was a weak inhibitor of butyrylcholinesterase.

MATERIALS AND METHODS

Materials

Pseudophrynaminol was synthesized from ω-*N*-methyltryptamine as follows: ω-*N*-methyltryptamine (6 mg) was treated with concentrated HCl (30 μL), and isoprene oxide (7.5 μL) was added at room temperature. After 2 min, the dark solution was treated with an aqueous saturated solution of $NaHCO_3$ (1 mL) and extracted with EtOAc (3 × 1 mL). After drying with anhydrous Na_2SO_4, the EtOAc was evaporated to yield a 5 mg residue that on GC-MS showed only two poorly separated peaks in a 1:9 ratio. The major component was pseudophrynaminol, by comparison of retention time and MS and Fourier-transformed infrared (FTIR) spectra with natural pseudophrynaminol. The minor component was an isomer of pseudophrynaminol having probably the *z* configuration at the double-bond position. The mixture of isomers, which was predominately (±)-pseudophrynaminol, was used for pharmacological investigation. Details of the synthesis will be published elsewhere.

[^{3}H]Nicotine (75.7 Ci/mmol) was from New England Nuclear (Boston, MA), ^{22}NaCl (0.2 mCi/mL) from Amersham Life Science (Arlington Heights, IL), (−)-nicotine ditartrate from Research Biochemicals International (Natick, MA), carbamylcholine from the Sigma Chemical Co. (St. Louis, MO), physostigmine from Merck & Co. (Rahway, NJ), and ω-*N*-methyltryptamine from Janssen Chimica (Geef, Belgium). Isoprene oxide was synthesized from isoprene as previously described [6]. Other compounds were from standard commercial sources.

Ion Flux Assays in Cultured Cells

Rat pheochromocytoma PC12 cells were provided by Dr. G. Guroff (NIH, Bethesda, MD). Human medulloblastoma

† Corresponding author: Dr. John W. Daly, Bldg. 8, Rm. 1A17, NIH, Bethesda, MD 20892. Tel. (301) 496-4024; FAX (301) 402-0008.
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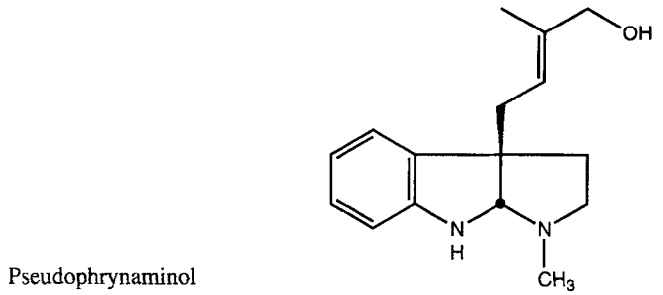
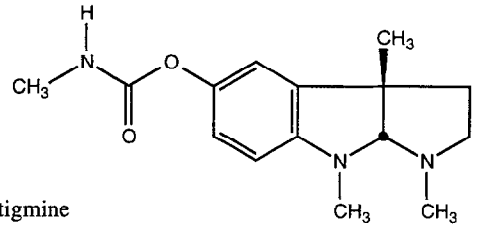
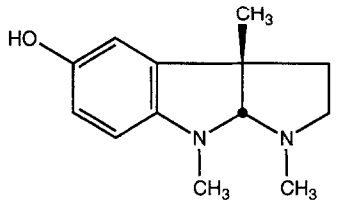


Pseudophrynaminol



(–)-Physostigmine



(–)-Eseroline

FIG. 1. Structures of pseudophrynaminol, (–)-physostigmine, and (–)-eseroline.

TE671 cells were from the American Type Culture Collection (Rockville, MD). The PC12 cells were grown in Dulbecco's modified Eagle's medium with 6% fetal bovine serum, 6% horse serum, and penicillin (100 U/mL) and streptomycin (100 mg/mL). The TE671 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were grown at 37° in an atmosphere enriched in CO_2.

Stimulation of sodium-22 influx was assayed in cultured cells as described previously [7]. Cells were plated in six-well culture plates (poly-D-lysine-coated) and cultured with [^{3}H]leucine-containing medium for 48 hr (PC12 cells) or 24 hr (TE671 cells). Medium was removed by aspiration, and 0.5 mL of the preincubation buffer (concentrations in mM: NaCl, 150; KCl, 5.4; $CaCl_2$, 2; HEPES/Tris, pH 7.4, 50; glucose, 5) was added at 22°. After 10 min, the preincubation buffer was replaced with the influx buffer (concentration in mM: NaCl, 5; KCl, 5.4; $CaCl_2$, 2; HEPES/Tris, pH 7.4, 50; glucose, 5; sucrose, 224; ouabain, 1) containing ^{22}NaCl (0.7 μCi) and either carbamylcholine or nicotine. Antagonists were present in both the preincubation and the influx buffers. After 2 min at 22°, the influx buffer was removed by aspiration, and the cells were washed three times with wash buffer (same composition as for the preincubation buffer). Cells were solubilized with 0.5 mL of 1% SDS in 0.5 N NaOH for 30–60 min and then pipetted into counting vials with 5 mL of Hydrofluor and 0.25 mL of 1 N HCl. Radioactivity (^{3}H, $^{22}Na^+$) was determined in a scintillation counter.

Nicotine Binding Assays in Rat Cerebral Cortical Membranes

Rat brains, obtained from Pel Freez Biological (Rogers, AK), were placed in ice-cold 50 mM Tris-HCl buffer (pH 7.4). Rat cerebral cortical tissue was removed and homogenized using a Polytron (setting 6, 10 sec). The homogenate was centrifuged for 15 min at 35,000 g at 4°. The pellet was washed once by suspension and recentrifugation in ice-cold Tris buffer. The final pellet was resuspended in ice-cold Tris buffer and stored at −70°. Aliquots were diluted to a concentration of 1–5 mg protein/mL. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard. [^{3}H]Nicotine receptor binding was assayed with rat cerebral cortical membranes as described previously [8, 9] in 20 mM HEPES buffer (pH 7.4), containing 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$. Each assay contained the test agent, a suspension of the rat cerebral cortical membranes (100 μL containing 200–300 μg protein), 200 μM diisopropyl fluorophosphate, and 2 nM [^{3}H]nicotine in a final volume of 0.5 mL. Each assay was for 120 min at 0–4° and was performed in triplicate. Nonspecific binding was determined with 10 μM nicotine. Binding assays were terminated by filtration through Whatman GF/B filters, using a Brandel M24R Cell Harvester (Brandel, Gaithersburg, MD). Filters were washed twice with 5 mL of ice-cold buffer and placed in scintillation vials with 5 mL of Hydrofluor scintillation fluid, followed by counting for tritium. The filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding.

Ion Flux Assays in Synaptoneurosomes

Guinea pig cerebral cortical synaptoneurosomes were obtained as previously described [10]. Briefly, the cortex of one brain was homogenized in 10 mL of sodium-free buffer (pH 7.4) in a glass–glass homogenizer (five strokes). The composition of the sodium-free buffer was as follows (concentrations in mM): choline chloride, 130; HEPES (adjusted to pH 7.4 with 50 mM Tris), 50; glucose, 5.5; $MgSO_4$, 0.8; KCl, 5.4. The suspension was centrifuged at 1000 g for 10 min, the supernatant was decanted, and the pellet was reconstituted in 35 mL of buffer. The suspension was filtered first through two layers of nylon material (100 mesh) and then through Millipore filters (LCWP-047, 10 μm pore size). After centrifugation for 10 min at 1000 g, the new pellet was reconstituted in an appropriate volume of fresh sodium-free buffer.

Sodium-22 flux into the synaptoneurosomal preparation

was based on a method previously described for synaptosomes [11]. Aliquots of synaptoneurosomal suspensions (containing 200–300 mg of protein) were preincubated for 10 min at 37° in 100 μL of incubation buffer containing 1 μM batrachotoxin alone or with 100 μM pseudophrynaminol. Incubation buffer consisted of fresh sodium-free buffer containing 1 mg/mL bovine serum albumin. The $^{22}NaCl$ (0.2 μCi) was then added in 150 μL of an influx buffer (concentrations in mM: NaCl, 2.66; HEPES/Tris, pH 7.4, 50; choline chloride, 128; KCl, 5.4; $MgSO_4$, 0.8; glucose, 5.5; ouabain, 5; and 1 mg/mL bovine serum albumin) containing 1 μM batrachotoxin alone or with 100 μM pseudophrynaminol. Influx of $^{22}Na^+$ was stopped after 10 sec by addition of 3 mL of cold wash buffer (concentrations in mM: HEPES/Tris, pH 7.4, 5; choline chloride, 163; $MgSO_4$, 0.8; $CaCl_2$, 1.8; and 1 mg/mL bovine serum albumin). Samples were collected on a Gelman filter (GN-6, 0.45 μm pore size) and washed twice with 3 mL of wash buffer. Filters were dissolved in Filtron-X (National Diagnostics, Sommerville, NJ) for liquid scintillation counting. The specific uptake of $^{22}Na^+$ was determined by subtracting nonspecific uptake obtained in the presence of 5 μM tetrodotoxin from total uptake.

Inhibition of Cholinesterases

Activity of acetylcholinesterase and butyrylcholinesterase was determined with freshly prepared human red blood cells or plasma, respectively, in 0.1 M Na_3PO_4 buffer (pH 8.0), using the spectrophotometric method of Ellman *et al.* [12], as modified by Atack *et al.* [13]. The ability of the compounds to inhibit the enzymatic degradation of the specific substrates [acetyl-(β-methyl)thiocholine for acetylcholinesterase and S-butylrylthiocholine for butyrylcholinesterase] was determined over the concentration range of 0.3 nM to 30 μM. Inhibitory activity was calculated as an IC_{50}, which is defined as the concentration (μM) required to inhibit 50% of the enzyme activity of the cholinesterase. The compounds were analyzed in duplicate in four separate assays.

RESULTS

(±)-Pseudophrynaminol inhibited carbamylcholine-elicited sodium-22 influx in PC12 cells, containing a ganglionic-type nicotinic receptor (Fig. 2A), and in TE671 cells, containing a neuromuscular-type nicotinic receptor (Fig. 2B). (±)-Pseudophrynaminol was of equal potency (IC_{50} ~ 0.3 μM) at both the ganglionic-type and the neuromuscular-type nicotinic receptors. Pseudophrynaminol was orders of magnitude more potent than either (−)-physostigmine or (−)-eseroline at both subtypes of nicotinic receptor (Table 1). The inhibition of nicotine-elicited sodium-22 influx by pseudophrynaminol in both cell lines appeared to be noncompetitive, since the inhibitory effect of (±)-pseudophrynaminol was not overcome with increasing concentrations of nicotine (Fig. 3A and B).

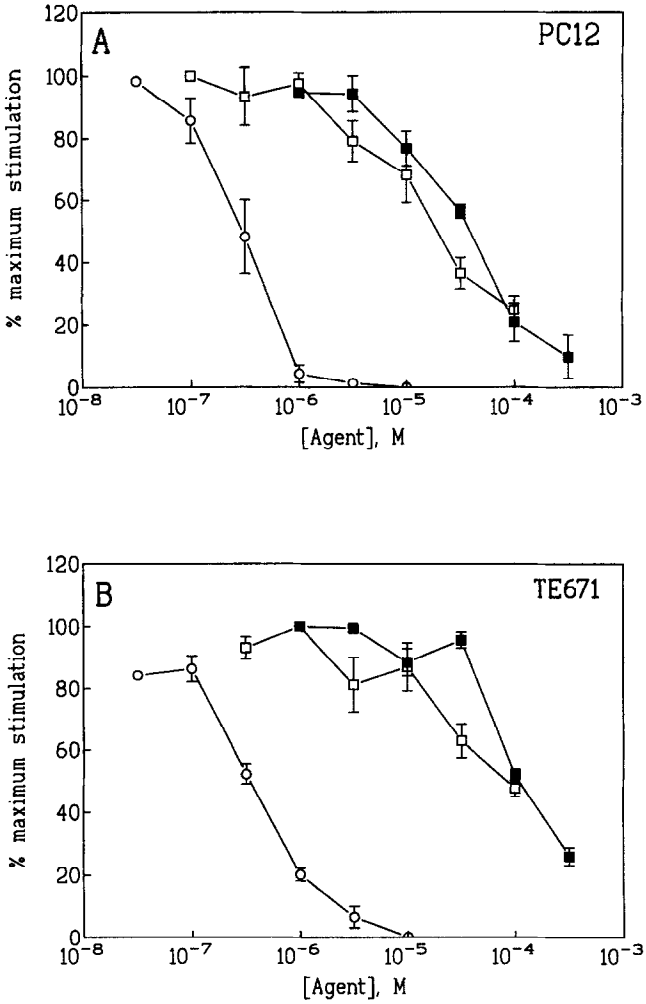


FIG. 2. Inhibition of carbamylcholine-elicited sodium-22 influx in (A) PC12 cells and (B) TE671 cells. Influx assays were as described in Materials and Methods with the following alkaloids: (±)-pseudophrynaminol (○); (−)-physostigmine (□); and (−)-eseroline (■). Each value is reported as a percentage of stimulation obtained with 2 mM carbamylcholine and is the mean ± SEM (N = 3). Typically, 2 mM carbamylcholine-elicited sodium-22 influx resulted in 500–600 cpm/10,000 PC12 cells and 3000–4000 cpm/10,000 TE671 cells. Basal sodium-22 influx was 50–100 cpm/10,000 PC12 cells and 200–400 cpm/10,000 TE671 cells.

As would be expected for noncompetitive blockers, (±)-pseudophrynaminol, (−)-physostigmine, and (−)-eseroline were very weak competitive inhibitors of [^{3}H]nicotine binding, with IC_{50} values greater than 100 μM. The K_i values ranged from 35 μM for (±)-pseudophrynaminol to greater than 100 μM for (−)-physostigmine (Table 1). In addition, (±)-pseudophrynaminol at 10 μM was assayed in the NIMH NOVA Screen (NOVA SCREEN, Hanover, MD). At this concentration, which is 30-fold higher than the IC_{50} for blockade of nicotinic receptor-channels (Table 1), (±)-pseudophrynaminol caused only about a 50% inhibition of binding of ligands to α_1- and α_2-adrenergic, H_2-histaminergic, and opioid receptors (data not shown). This relatively weak activity was not examined further. (±)-Pseudophrynaminol at 10 μM had marginal effects on bind-

TABLE 1. Inhibition of nicotinic agonist-elicited sodium-22 influx in cultured cells and weak inhibition of agonist-binding to rat brain nicotinic receptors

	PC12 cells	TE671 cells	Brain membranes
	IC_{50} (μM)		K_i (μM)
(±)-Pseudophrynaminol	0.3 ± 0.06	0.3 ± 0.04	35 ± 9
(−)-Physostigmine	23 ± 1.0	67 ± 9	>100
(−)-Eseroline	35 ± 5	130 ± 10	89 ± 11

Assays were as described in Materials and Methods. The K_i values are for inhibition of binding of [^{3}H]nicotine to brain receptors, and the IC_{50} values are for inhibition of the sodium-22 influx elicited by 2 mM carbamylcholine in cultured cells. Values are mean ± SEM (N = 3).

ing of ligands to a variety of other central sites including β-adrenergic, dopaminergic, GABAergic, glutamatergic, muscarinic, and serotonergic receptors and dizocilpine and phencyclidine sites on *N*-methyl-D-aspartic acid (NMDA)-receptors, and dihydropyridine sites on L-type calcium channels (data not shown). At the high concentration of 100 μM, (±)-pseudophrynaminol caused only a 40% inhibition of the sodium-22 influx elicited by 1 μM batrachotoxin through sodium channels in guinea pig synaptoneurosomes (data not shown). (±)-Pseudophrynaminol did not cause significant inhibition of human red blood cell acetylcholinesterase at 30 μM, while being a weak inhibitor (IC_{50} 19 ± 3 μM) of human plasma butyrylcholinesterase (data not shown). In parallel experiments, (−)-physostigmine was, as expected, a potent inhibitor with an IC_{50} value of 28 ± 2 nM for acetylcholinesterase and an IC_{50} value of 16 ± 3 nM for butyrylcholinesterase. (−)-Eseroline, the descarbamyl analog of physostigmine, was nearly inactive, with only marginal effects on the cholinesterases at 30 μM.

DISCUSSION

A variety of noncompetitive blockers of nicotinic receptor-channels has been reported [14, 15]. However, little is known of the selectivity of such blockers for subtypes of nicotinic receptors. Several of the noncompetitive blockers are alkaloids, isolated from extracts of frog skins. These include the histrionicotoxins [16], gephyrotoxins [17], decahydroquinolines [18] and indolizidines [19]. Recently, spiropyrrolizidine oximes were added to this list of frog skin alkaloids that are potent blockers of nicotinic receptor-channels [20]. One unique class of amphibian alkaloids are the pseudophrynamines [1, 2], whose indolic structures are reminiscent of the plant alkaloids eseroline and physostigmine. The amount of pseudophrynamines, isolated from skins of Australian myobatrachid frogs, was insufficient for pharmacological evaluation. The synthesis of two of the many pseudophrynamines, detected in frog skin extracts, has been reported [21, 22], but no pharmacological studies were reported. Pseudophrynaminol had been synthesized in five steps from tryptamine in an overall yield of 2.3% [21]. Pseudophrynamine A has been synthesized in nine steps from tryptamine in an overall yield of 10% [22]. Pseudophrynaminol was an intermediate in the latter synthesis [22]. We have now developed a simple route to the synthesis of pseudophrynaminol by a one-step reaction of isoprene oxide with *N*-methyltryptamine in a yield of 56%.

The synthetic (±)-pseudophrynaminol proved to be a potent noncompetitive blocker of nicotinic receptor-channels (Fig. 3). It was equipotent with dihydroisohistrionicotoxin, itself one of the most potent such blockers [19]. (±)-Pseudophrynaminol was nonselective with respect to ganglionic and neuromuscular subtypes of nicotinic receptor-subtypes. Functional activity of (±)-pseudophrynaminol at the central neuronal ($\alpha_4\beta_2$) nicotinic subtype could not be assessed in parallel experiments, since cultured cell lines expressing that subtype are not known. A transfected cell line with $\alpha_4\beta_2$ receptors has been reported recently [23]. (±)-Pseudophrynaminol, (−)-physostigmine, and (−)-

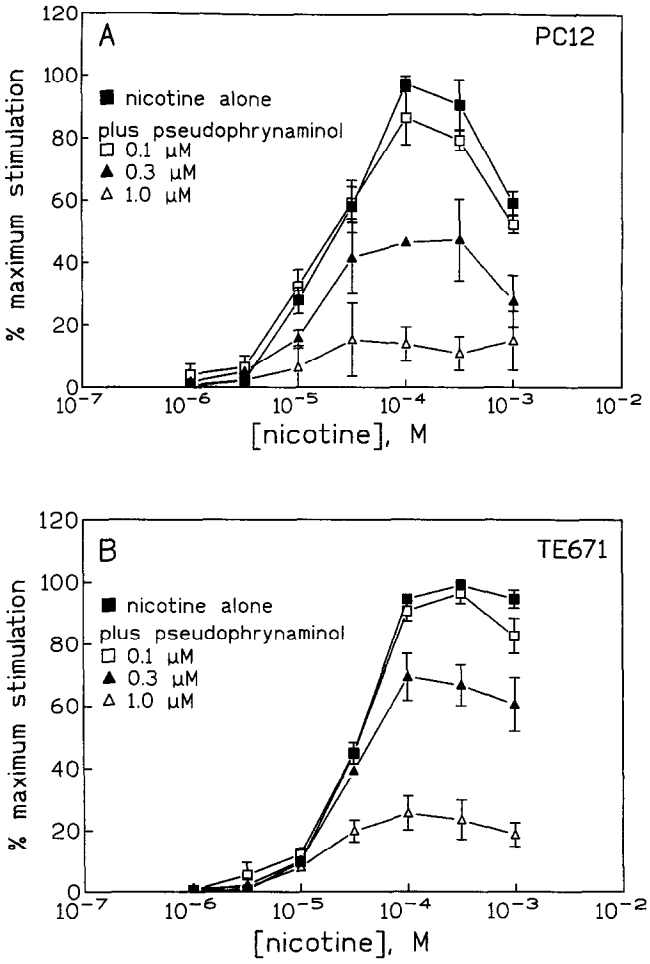


FIG. 3. Noncompetitive inhibition by (±)-pseudophrynaminol of nicotine-elicited sodium-22 influx in (A) PC12 cells and (B) TE671 cells. Influx assays were as described in Materials and Methods with increasing concentrations of (−)-nicotine alone or with 0.1, 0.3, or 1.0 μM pseudophrynaminol. Each value is reported as a percentage of maximum stimulation obtained with (−)-nicotine and is the mean ± SEM (N = 3). See legend of Fig. 2 for control values.

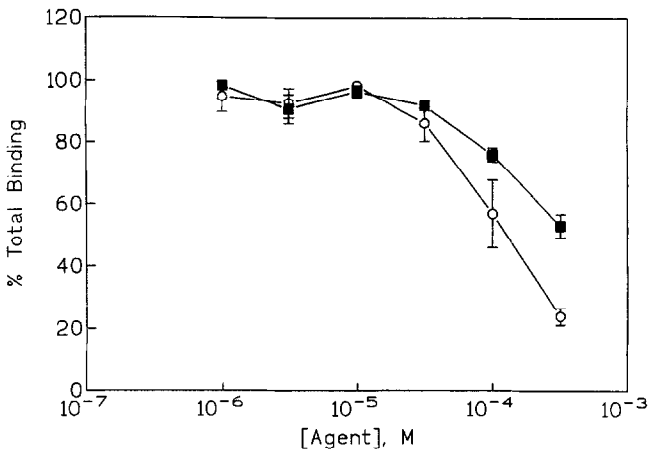


FIG. 4. Inhibition of [^{3}H]nicotine binding to rat cerebral cortical membranes. Binding assays were as described in Materials and Methods with the following alkaloids: (±)-pseudophrynaminol (○); and (−)-eseroline (■). Each value is reported as a percentage of specific binding of [^{3}H]nicotine in the absence of competing ligand and is the mean ± SEM (N = 3).

eseroline were relatively weak inhibitors versus agonist binding to central nicotinic binding sites (Fig. 4), which is consonant with activity as noncompetitive rather than competitive blockers of nicotinic receptor-chennels. (±)-Pseudophrynaminol was orders of magnitude more potent than either (−)-physostigmine or (−)-eseroline as a noncompetitive blocker of nicotinic receptors-channels of the ganglionic (PC12 cells) and neuromuscular (TE671 cells) nicotinic receptors (Fig. 2, Table 1). Neither (±)-pseudophrynaminol nor (−)-physostigmine stimulated sodium-22 influx in PC12 or TE671 cells when tested alone (data not shown).

(±)-Pseudophrynaminol had only weak effects on a variety of central receptors, and was a very weak inhibitor of voltage-dependent sodium channels (see Results). (±)-Pseudophrynaminol had no inhibitory effect on acetylcholinesterase, and was a very weak inhibitor of butyrylcholinesterase, not an unexpected finding since (−)-eseroline is inactive as a cholinesterase inhibitor (see Results) and, thus, a carbamate moiety is required for potent inhibition of cholinesterases, as is well known for such compounds [24].

In summary, (±)-pseudophrynaminol represents a further class of alkaloids with very potent activity as a noncompetitive blocker of nicotinic receptors. It, unlike many other classes of noncompetitive nicotinic blockers [16, 25, 26], has little effect on voltage-dependent sodium channels, and, unlike physostigmine, has no effect on acetylcholinesterase. Thus, (±)-pseudophrynaminol represents a new and specific tool for intervention with nicotinic receptor function.

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