Pergamon

Biochemical Pharmacology, Vol. 47, No. 11, pp. 1965–1967, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0118-5

5-ISOTHIOCYANONICOTINE: A HIGH-AFFINITY IRREVERSIBLE LIGAND FOR BRAIN NICOTINIC RECEPTORS

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(*Received* 28 *October* 1993; *accepted* 2 *February* 1994)

Abstract—A newly synthesized affinity ligand, (*R,S*)-5-isothiocyanonicotine (ISCN-N) was found to inhibit irreversibly the binding of [^{3}H]methylcarbamylcholine (a specific nicotinic receptor ligand) to brain membranes. Plots of percent inhibition versus ligand concentration yielded an IC_{50} of 7×10^{-8} M for SCN-N and K_i values of 6×10^{-9} and 2×10^{-9} M for (*R,S*)-5-aminonicotine and (*S*)-nicotine, respectively. The IC_{50} value for irreversible inhibition of [^{3}H]methylcarbamylcholine by SCN-N was 2×10^{-7} M. The affinity ligand irreversibly inhibited brain nicotinic receptors *in vivo* in a dose-dependent manner, the inhibition being 49% at a dose of 20 μmol/kg. Behavioral studies in mice revealed that SCN-N had less than one-fifth the potency of nicotine in producing muscle weakness and seizures, whereas 5-aminonicotine was without significant behavioral effects at doses up to 20 μmol/kg.

Key words: 5-isothiocyanonicotine; nicotinic receptors; irreversible binding; brain membranes; 5-aminonicotine; [^{3}H]MCC binding

Various irreversible affinity ligands have been developed for the characterization and purification of nicotinic [1–3] and muscarinic cholinergic receptors [4–6]; however, no alkylating affinity ligands of nicotine with high affinity have been described. Isothiocyanate derivatives as alkylating agents have been utilized in the characterization of a number of neuronal receptors [5, 7, 8]. In the present paper, we describe the synthesis, receptor binding characteristics, and behavioral effects of SCN-N§, which has an affinity in the nanomolar range and irreversibly inhibits brain nicotinic receptors.

MATERIALS AND METHODS

Chemical synthesis. (*R,S*)-5-Aminonicotine was prepared by the method of Rondahl [9]. Purification by bulb-to-bulb distillation (130° in oil bath/0.7 mm Hg), followed by recrystallization from benzene/petroleum ether, yielded white crystals: m.p. 72–73°. MS: prominent peaks at *m/e* (rel. int. %): 177 (M^+, 34), 176 (16), 148 (26), 134 (7), 121 (14), 85 (7), 84 (100), 42 (19).

SCN-N. The procedure for the synthesis of SCN-N is based on the method of Sharma [10]. To a solution of 0.084 mmol (*R,S*)-5-aminonicotine in 0.5 mL methylene chloride at room temperature was added 80 μL of triethylamine followed by 110 μL of 1 M thiophosgene in methylene chloride. After the resulting black solution was stirred at room temperature for 10 min, 2 mL of anhydrous diethyl ether was added to yield a black precipitate. The material was filtered through silica gel, and then the filtrate was concentrated, redissolved in ether, and again passed through silica gel and concentrated to yield 13 mg (70%) of a yellowish oily product. MS: prominent peak at *m/e* (rel. int. %): 219 (M^+, 36), 218 (19), 190 (26), 176 (7), 84 (100), 82 (6). NMR: (C_6D_6), δ 8.32 (s 1H), 8.01 (d, J 3 Hz, 1H), 7.01 (d, J 2 Hz, 1H), 1.91–1.76 (m, 1H), 1.79 (s, 3H), 1.65–1.49 (m, 2H), 1.33–1.16 (m, 2H). IR: 2960, 2940, 2770, 2060, 1590, 1040, 1020, 1000, 880, 700 cm^{-1}.

[^{3}H]*MCC and* [^{3}H]*QNB binding.* The procedure for the measurement of the binding of [^{3}H]MCC, a specific ligand for the nicotinic cholinergic receptor, is described elsewhere [11]. [^{3}H]QNB binding was determined by the method of Yamamura and Snyder [12]. Membranes were obtained from cortical gray matter of fresh calf brains after homogenation in 0.05 M sodium phosphate buffer, pH 7.0, and centrifugation at 50,000 *g* for 30 min. The pellet was then resuspended in sodium phosphate buffer and stored at −70°. To a 2-mL polypropylene centrifuge tube was added 0.8 mL of 1.25 mg/mL membrane protein along with 0.1 mL of 1×10^{-8} M [^{3}H]MCC (80 Ci/mmol) or [^{3}H]QNB (45 Ci/mmol) with or without 10^{-5} M unlabeled MCC or QNB, respectively, in a final volume of 1.0 mL. After incubation in an ice bath for 60 min, the samples were filtered *in vacuo* on GF/B glass filters and washed twice with 3 mL of ice-cold buffer; radioactivity of the filters was determined by liquid scintillation. All assays were performed as three separate experiments run in triplicate, and the data are expressed as moles of specific binding per milligram of membrane protein. Specific [^{3}H]-naloxone (sp. act. 56 Ci/mmol) binding to brain

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§ Abbreviations: SCN-N, (*R,S*)-5-isothiocyanonicotine; MCC, *N*-methylcarbamylcholine; and QNB, 3-quinuclidinyl benzilate.

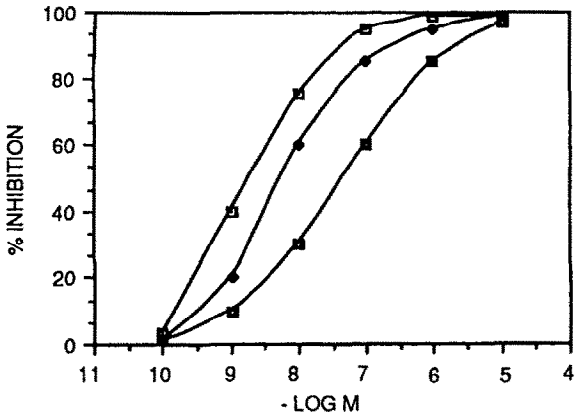

Fig. 1. Inhibition plots for nicotine, 5-aminonicotine, and SCN-N on [^{3}H]MCC binding in calf brain membranes. [^{3}H]MCC binding was determined in the presence of various concentrations of SCN-N (■), 5-aminonicotine (◆), and (*S*)-nicotine (□). Results are an average of three separated experiments run in triplicate; the variation from the mean was 7%. The control value was 12 fmol/mg membrane protein.

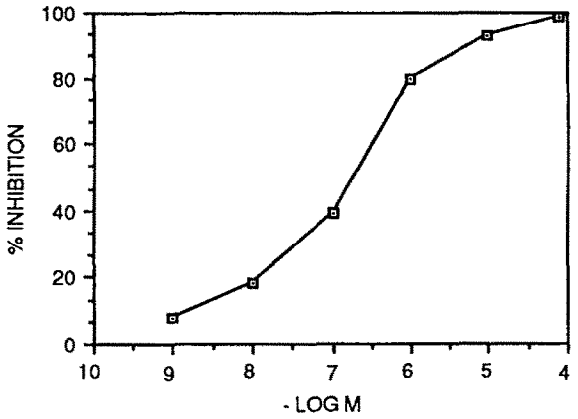

Fig. 2. Irreversible inhibition of [^{3}H]MCC binding *in vitro* versus the concentration of SCN-N. Results represent an average of three separate experiments run in triplicate, with the variation from the mean being less than 8%.

membranes was determined by the method of Pasternak and Snyder [13].

Measurement of irreversible binding to affinity ligands in vitro. After exposure of membranes containing 1 mg of membrane protein/mL in 0.05 M sodium phosphate, pH 7.0, to various concentrations of SCN-N for 30 or 60 min at 37°, they were centrifuged at 20,000 *g*. The pellet was washed three times after homogenizing (polytron) in 30 mL of ice-cold phosphate buffer and then was recentrifuged. The binding of [^{3}H]MCC and [^{3}H]QNB was measured as described above.

Behavioral studies and in vivo *receptor binding studies with SCN-N of 5-aminonicotine in mice.* Male Swiss Webster mice (25–35 g) were administered intraperitoneally 5, 10, or 20 μmol/kg of SCN-N or 5-aminonicotine and then were observed for respiratory changes, locomotor activity, weakness in the hindlimbs, tremors, and seizures. The mice were also tested for analgesia using the tail-flick test [14]. Five mice were used at each dose of SCN-N. The mice were then decapitated 30 min later, and the membranes were prepared from whole brain by centrifugation at 20,000 *g*; the pellet was washed twice with 20 mL of ice-cold phosphate buffer. Irreversible [^{3}H]MCC and [^{3}H]QNB binding was measured as described for the *in vitro* studies.

RESULTS AND DISCUSSION

Inhibition plots of SCN-N and 5-aminonicotine on [^{3}H]MCC binding of calf brain membranes in vitro. Plots of the inhibition of [^{3}H]MCC binding versus ligand concentration yielded an IC_{50} of 7×10^{-8} M for SCN-N and apparent K_i values of 2×10^{-9} and 6×10^{-9} M for nicotine and 5-aminonicotine, respectively (Fig. 1). The control value was 12 fmol/mg membrane protein. Apparent K_i values were determined from the inhibition curves by the method of Cheng and Prusoff [15]. No significant inhibition was observed in either [^{3}H]QNB for [^{3}H]naloxone binding in the presence of concentrations of SCN-N up to 10^{-5} M (data not shown).

Irreversible inhibition of [^{3}H]MCC binding by

Table 1. Effect of SCN-N on [^{3}H]MCC and [^{3}H]QNB binding *in vivo*

Dose of SCN-N (μmol/kg)	[^{3}H]MCC		[^{3}H]QNB	
	(fmol/mg)	% Inhibition	(pmol/mg)	% Inhibition
Control	12.2 ± 0.8*		1.9 ± 0.2*	
5	11.9 ± 0.7	3	2.0 ± 0.04	+5
10	9.0 ± 0.5†	26	1.8 ± 0.03	5
20	6.2 ± 0.3†	49	1.7 ± 0.02	9

Results are based on three separate mice for the control and experimental points, each brain run in triplicate.

* mean ± S.E.M.

† $P < 0.01$.

SCN-N in vitro. A plot of the irreversible inhibition of [^{3}H]MCC binding versus the concentration of SCN-N following a 60 min incubation revealed an inhibition of 20% at 1×10^{-8} M; this inhibition increased linearly to 50% at 2×10^{-7} M and became complete at 1×10^{-5} M (Fig. 2). The results were essentially the same when the incubation time was reduced to 30 min (data not shown). In control experiments where 5-aminonicotine was substituted for SCN-N, no significant inhibition was observed even at the higher concentrations (data not shown). When the concentration of membrane protein exposed to various concentrations of SCN-N was increased from 1 mg/mL incubation medium to 7 mg/mL, the degree of inhibition at a given concentration of SCN-N was found to be approximately 50% less (data not shown).

Irreversible inhibition of [^{3}H]*MCC binding by SCN-N* in vivo. Following the administration of SCN-N to mice, [^{3}H]MCC binding in membrane preparations of excised brains was inhibited 26% at a dose of 10 μmol/kg and 49% at a dose of 20 μmol/kg; whereas [^{3}H]QNB binding was not significantly different from the control (Table 1).

Behavioral effects of SCN-N and 5-aminonicotine in mice. At SCN-N doses of 10 and 20 μmol/kg, i.p., mice exhibited a marked decrease in locomotor activity, weakness in the hindlimbs, decreased respiratory rate, and ptosis in all mice tested at each dose. With the exception of a moderate decrease in locomotor activity and only slight muscle weakness, no other changes were noted in mice receiving a dose of 5 μmol/kg. At a dose of 20 μmol/kg, no analgetic effect was observed. Doses of 10 μmol/kg and greater resulted in seizures and lethality 30–60 min after the administration of SCN-N, death being due to respiratory failure. No significant behavioral effects were seen with 10 μmol/kg of 5-aminonicotine and only moderate hindlimb weakness and slight respiratory depression at 20 μmol/kg. Neither SCN-N nor 5-aminonicotine at doses up to 20 μmol/kg was able to antagonize or prevent the behavioral effects of nicotine.

It can be concluded that SCN-N is a potent specific affinity ligand for nicotinic cholinergic receptors, both *in vitro* and *in vivo*. From a comparison of the binding data for nicotine and SCN-N, the affinity of SCN-N for the nicotinic receptor was 1/35 that of nicotine. With respect to behavioral–pharmacologic effects, SCN-N appeared to be comparable to nicotine in its toxicity, but because of this toxicity, it was not possible to compare SCN-N with nicotine for such parameters as prostration, tremors, and seizures [6]. Since irreversible inhibition of [^{3}H]-MCC binding *in vivo* was observed following i.p. administration of SCN-N, it can be inferred that some of the intact ligand entered the brain. The failure to observe any significant behavioral effects with 5-aminonicotine except at high doses would suggest that the agent does not readily pass the blood–brain barrier.

Acknowledgements—This research was supported by NIH Grant DA 00464. N.L-M. was supported by NIDA Training Grant DA 07232.

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