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# RECEPTOR BINDING CHARACTERISTICS OF A $^3$H-LABELED AZETIDINE ANALOGUE OF NICOTINE*

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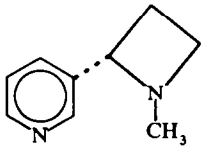
**Abstract**—A new radioligand, (±)-[$^3$H]-1-methyl-2-(3-pyridyl)-azetidine, which is an analogue of nicotine, has been used to investigate the binding characteristics of the nicotinic receptor in rat brain membranes. By Scatchard analysis, the azetidine analogue yielded a curvilinear plot with $K_d$ values of $7 \times 10^{-11}$ and $1.7 \times 10^{-9}$ M and $B_{max}$ values of $0.3 \times 10^{-14}$ and $2.5 \times 10^{-14}$ mol/mg protein respectively. Thermodynamic analyses yielded negative free enthalpy values for both sites, a decrease in the $B_{max}$ of only the lower affinity site, and no effect on either $K_d$. The psychotropic potency (prostration in rats following intraventricular injection) of the azetidine analogue was about 5-fold greater than (−)-nicotine, being among the greatest of any known nicotine analogues tested to date. Since only the higher affinity $K_d$ differed from that of (−)-nicotine, 3-fold greater, the psychotropic potency appears to be correlated with the higher affinity site. Insofar as [$^3$H]methylcarbamylcholine, a nicotinic ligand resembling acetylcholine, exhibits a linear Scatchard with a $K_d$ of $1 \times 10^{-9}$ M, the higher affinity site appears to be characterisic of nicotine analogues.

As part of an effort to explore the chemical and functional nature of the nicotinic receptor(s) in rat brain, various radioligands have been used, including (−)-[$^3$H]nicotine [1–4] and (+)-[$^3$H]nicotine [2, 5], [$^3$H]acetycholine [6] and [$^3$H]methylcarbamylcholine [7]. In the course of examining the structure–activity relationships of various nicotine analogues, it was observed that the (*R*,*S*)-3-pyridyl-1-methyl-2-(3-pyridyl)-azetidine (MPA)




exhibited a significantly greater receptor affinity and behavioral potency in rats than nicotine. The present study was undertaken to investigate the receptor binding characteristics of [$^3$H]MPA.

## METHODS

*Preparation of* [$^3$H]*MPA.* [$^3$H]MPA was prepared by New England Nuclear by methylation of (±)-1-methyl-2-(3-pyridyl)-norazetidine, using the procedure of Seeman *et al.* [8]. The final product, [$^3$H]MPA, was purified by HPLC and had a radioactive sp. act. of 80 Ci/mmol. Both the unlabeled MPA and the norazetidine analogue were gifts of the Philip Morris Research Center.

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*Preparation of* [$^3$H]*MPA and* [$^3$H]*nicotine binding.* The procedure for preparation of rat brain membranes and for measuring specific [$^3$H]MPA and (−)-[$^3$H]nicotine binding is described elsewhere [1]. Membranes were obtained from whole rat brain after homogenization in 30 vol. of 0.05 M $NaPO_4$, pH 7.0, and were centrifuged at 50,000 *g* for 30 min. To a 2-ml polypropylene tube was added 3 mg membrane protein along with various concentrations of either [$^3$H]MPA or (−)-[$^3$H]nicotine (New England Nuclear, sp. act. = 75 Ci/mmol) with or without various concentrations of unlabeled MPA, nicotine analogues, carbamate esters, and other agents, in a final volume of 1.2 ml of 0.05 M $NaPO_4$, pH 7.0. The relationship of pH to the binding of the two ligands was determined with 0.05 M $NaPO_4$ buffer. All assays were performed in triplicate. After incubating in an ice bath for 30 min, the tubes were centrifuged in an Eppendorf centrifuge for 2 min and the pellet was washed twice by filling the tubes with buffer and aspirating. The bottom of the tubes was then cut off (animal nail clipper), and the contents were counted by liquid scintillation. In a typical experiment, the total cpm obtained with $1 \times 10^{-9}$ M [$^3$H]MPA was almost 4000 cpm of which 1600 cpm represented specific binding.

[$^3$H]MPA binding was determined at various temperatures, ranging from 0° to 37°, using two concentrations of [$^3$H]MPA, $1 \times 10^{-9}$ and $1 \times 10^{-10}$ M. To eliminate the possibility that [$^3$H]MPA was degraded at 37°, the mixture was incubated at 37° for 30 min, followed by incubation at 0°. The results were the same as those obtained without preincubation.

*Measurement of the kinetics of* [$^3$H]*MPA binding.* The association and dissociation rate constants were determined as described elsewhere [5].

Table 1. Comparison of various constants of [$^3$H]MPA binding with (−)-[$^3$H]nicotine and [$^3$H]methylcarbamylcholine

| | | [$^3$H]MPA | (−)-[$^3$H]Nicotine | [$^3$H]MCC |
|---|---|---|---|---|
| Scatchard analysis | $K_d$ (M) | $7 \times 10^{-11}$<br>$1.7 \times 10^{-9}$ | $2 \times 10^{-10}$<br>$3 \times 10^{-9}$ | $1.1 \times 10^{-9}$ |
| | $B_{max}$ (mol/mg protein) | $0.3 \times 10^{-14}$<br>$2.5 \times 10^{-14}$ | $0.5 \times 10^{-14}$<br>$2.0 \times 10^{-14}$ | $3 \times 10^{-14}$ |
| Ratio of rate constants | $K_d$ (M) | $1.0 \times 10^{-9}$ | $1.2 \times 10^{-9}$ | $3.3 \times 10^{-9}$ |
| Hill coefficients | | 0.75 | 0.80* | 1.0 |

Data for (−)-[$^3$H]nicotine and [$^3$H]methylcarbamylcholine (MCC) were obtained from Refs. 5 and 7 respectively.
* Based on three experiments with a coefficient of variation of 7%.

*Kinetics of* [$^3$H]*MPA binding to membranes.* Briefly, the association rate constant of (−)-[$^3$H]MPA specific binding was measured by incubating rat brain membranes in an ice bath with $1 \times 10^{-9}$ M [$^3$H]MPA in the presence and absence of $1 \times 10^{-6}$ M unlabeled MPA. The appearance as a function of time of radioactivity specifically bound to membranes was measured by the centrifuge assay as described above. The apparent biomolecular rate constant was estimate from the initial linear portion of the association curve.

The dissociation rate constant was measured by incubating membranes to equilibrium (30 min at 0–4) with $1 \times 10^{-9}$ M [$^3$H]MPA in the presence and absence of $1 \times 10^{-6}$ M unlabeled MPA. After centrifuging and washing, the pellet was resuspended in buffer, incubated on ice for various times, and recentrifuged. The pellet was washed and counted. The data yielded a linear semi-log plot, and the dissociation rate constant was calculated as 0.693 $t_{\frac{1}{2}}$.

*Psychotropic evaluation of various agents.* The psychotropic action of the various agents was determined by administering various doses into the fourth ventricle through chronically implanted cannulae, as described elswhere [9]. A dose of 4 nmol of (−)-nicotine in 1 μl resulted in prostration of all four limbs, while 2 nmol ($IC_{50}$) produced prostration in the hind limbs and some weakness in the forelimbs.

## RESULTS

*Scatchard analyses of* [$^3$H]*MPA binding.* A Scatchard plot of [$^3$H]MPA in the presence of unlabeled MPA was curvilinear, yielding $K_d$ values of $7 \times 10^{-11}$ and $1.7 \times 10^{-9}$ M and $B_{max}$ values of $0.3 \times 10^{-14}$ and $2.5 \times 10^{-14}$ mol/mg protein, respectively, at a temperature of 4° (Table 1). At a temperature of 37°, the plot paralleled that at 4°, yielding $K_d$ values of $5 \times 10^{-11}$ and $1.5 \times 10^{-9}$ and $B_{max}$ values of $0.25 \times 10^{-14}$ and $3.8 \times 10^{-14}$ mol/mg protein respectively (Fig. 1). Replacement of unlabeled MPA with unlabeled (−)-nicotine also yielded a curvilinear Scatchard with comparable $K_d$ and $B_{max}$ values (data not shown). A Hill plot of the data [log $(B/B–B_{max}$ vs log $F)$] yielded a Hill coefficient of 0.8,

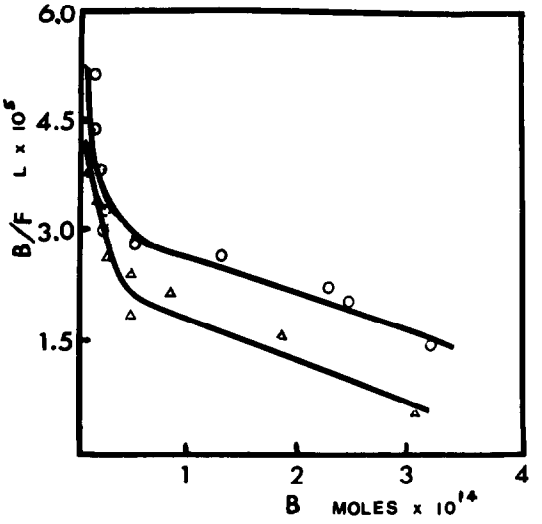


Fig. 1. Scatchard plot of [$^3$H]MPA binding to rat brain membranes. [$^3$H]MPA binding was determined with and without a 1000-fold excess of unlabeled MPA at 0° (○) and 37° (△). $B$ = bound MPA in mol/mg protein; $F$ = molar concentration of free ligand.

which is suggestive of a heterogeneity of sites (data not shown). The $K_d$ and $B_{max}$ values for [$^3$H]MPA were similar to those for the higher and lower affinity sites of [$^3$H]nicotine (Table 1). The binding constants were derived from linear regression analyses of the Scatchard plots of the data.

$K_d$ *calculated from rate constants for association and dissociation.* The rate constant for association for [$^3$H]MPA binding to rat brain membranes was determined to be $2.1 \times 10^6$ $M^{-1}$ $sec^{-1}$ and for the dissociation, $2.0 \times 10^{-3}$ $sec^{-1}$. The $K_d$, calculated from the ratios of the rate constants, was $1.0 \times 10^{-9}$ M, a value which is in close agreement with the lower affinity value obtained by Scatchard analysis (Table 1).

*Thermodynamics of* [$^3$H]*MPA binding.* An analysis of the binding of [$^3$H]MPA as a function of temperature revealed that the binding increased with decreasing temperature. Furthermore, the lower

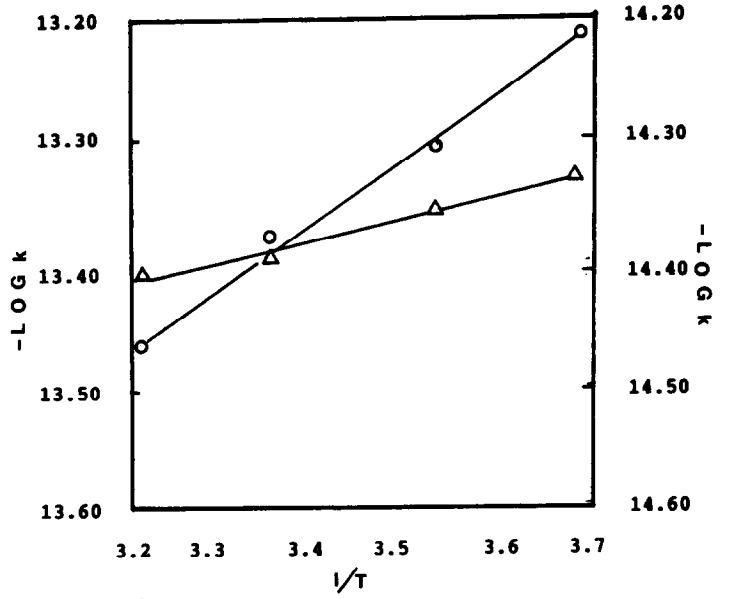


Fig. 2. van't Hoff plot of [³H]MPA binding. Binding was determined with either $1 \times 10^{-9}$ M (○) or $1 \times 10^{-10}$ M (△) [³H]MPA. The results are an average of three separate experiments with a coefficient of variation under 10%.

Table 2. Thermodynamic constants for [³H]MPA binding

| | $\Delta H$ (cal/mol) | $\Delta S$ (cal/mol·deg) | $\Delta G$ (cal/mol) |
|---|---|---|---|
| Lower affinity site | −3560 | 27 | −11,300 |
| Higher affinity site | −1495 | 41 | −12,500 |

The thermodynamic constants were obtained using $1 \times 10^{-9}$ M and $1 \times 10^{-10}$ M [³H]MPA to examine the lower and higher affinity sites respectively.

affinity site exhibited a greater change with temperature than did the higher affinity site. A Scatchard analysis of the binding at 0° and 37° revealed that the decrease in binding with increasing temperature was attributable to a decrease in the $B_{max}$ of the lower but not the higher affinity site, while the $K_d$ of either site was unaffected.

A van't Hoff plot of the binding determined at either a final concentration of $1 \times 10^{-10}$ or $1 \times 10^{-9}$ M [³H]MPA yielded a greater positive slope at the higher than at the lower concentration of [³H]MPA (Fig. 2). An enthalpy change ($\Delta H$) of −1495 and −3560 cal/mol was determined for the higher and lower affinity sites, respectively (Table 2), from the equation

$$\ln K = \frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

where $K$ = the affinity constant. A positive entropy ($\Delta S$) of 41 and 27 cal/mol·degree was calculated for the higher and lower affinity sites. From the Gibbs equation

$$-\Delta G = RT \ln K$$

assuming affinity constants of $1 \times 10^{10}$ and $1 \times 10^{9}$ M for the higher and lower affinity constant, $K$, respectively. The free enthalpy ($\Delta G$) at 0° was −12,500 cal/mol for the higher and −11,300 cal/mol for the lower affinity site (Table 2).

*Comparison of various agents in competition with* [³H]*MCC and* [³H]*nicotine.* A variety of nicotine and cholinergic agents were compared for their abilities to compete with [³H]MPA binding to rat brain membranes (Table 3). At a 1 nM concentration of (−)-[³H]MPA or (−)-[³H]nicotine, unlabeled (−)-nicotine had similar $IC_{50}$ values, whereas (+)-nicotine exhibited a 10-fold greater affinity with (−)-[³H]nicotine than [³H]MPA. With either radioligand, increasing the alkyl chain length on the pyrrolidine N resulted in a 3-fold decrease with the *N'*-ethyl, about a 100-fold decrease in affinity with the *N'*-isopropyl, and a 1000-fold decrease with the *N'*-butyl analogues of nicotine. The affinity of *N'*-nicotonium was about 3 orders of magnitude less than (−)-nicotine with both radioligands. Comparable affinities with both radioligands were observed with the various carbamate esters, methylcarbamylcholine having an affinity approaching that of (−)-nicotine. With both ligands, the $K_d$ value for acetylcholine was about $5 \times 10^{-5}$ M, whereas that of hexamethonium and atropine was greater than $1 \times 10^{-4}$ M. The relative $IC_{50}$ values were similar when $1 \times 10^{-10}$ M MPA was used instead of $1 \times 10^{-9}$ M.

*Psychotropic action of MPA and other nicotine*

Table 3. Comparison of $IC_{50}$ values for binding of various nicotinic agents using [$^3$H]MPA and (−)-[$^3$H]nicotine

| Agent | Binding $IC_{50}$ [$^3$H]MPA (M) | Binding $IC_{50}$ [$^3$H]Nicotine (M) | Prostration $EC_{50}$ (nmol) |
|---|---|---|---|
| MPA | $1 \times 10^{-9}$ | $2 \times 10^{-9}$ | 0.4 |
| (−)-Nicotine | $5 \times 10^{-9}$ | $3 \times 10^{-9}$ | 2 |
| (+)-Nicotine | $1 \times 10^{-7}$ | $1 \times 10^{-8}$ | 40 |
| *N'*-Methylnicotonium | $2 \times 10^{-6}$ | $7 \times 10^{-6}$ | 100 |
| *N'*-Ethylnornicotine | $3 \times 10^{-8}$ | $3 \times 10^{-8}$ | 20 |
| *N'*-Isopropylnornicotine | $2 \times 10^{-7}$ | $6 \times 10^{-7}$ | 100 |
| *N'*-Butylnornicotine | $1 \times 10^{-6}$ | $1 \times 10^{-6}$ | >100 |
| Acetylcholine | $3 \times 10^{-5}$ | $6 \times 10^{-5}$ | >100 |
| Carbamylcholine | $3 \times 10^{-7}$ | $4 \times 10^{-7}$ | 100 |
| Methylcarbamylcholine | $1 \times 10^{-8}$ | $8 \times 10^{-9}$ | 20 |
| Hexamethonium | $>1 \times 10^{-4}$ | $>1 \times 10^{-4}$ | Inactive |
| Atropine | $>1 \times 10^{-4}$ | $>1 \times 10^{-4}$ | Inactive |
| γ-Bungarotoxin | $>1 \times 10^{-4}$ | $>1 \times 10^{-4}$ | Inactive |

To determine psychotropic potency, expressed as $ED_{50}$, at least six rats were used for every agent. $IC_{50}$ values were obtained from log plots of agent concentration. All *N'*-alkynornicotines were the (−)-isomer.

*agents*. MPA was about five times more potent than (−)-nicotine in producing prostration in rats following intraventricular administration (Table 3). The relative pharmacologic potency of MPA with respect to various nicotine analogues was comparable to that of nicotine.

The pH curve for the binding of [$^3$H]MPA was identical to that of (−)-[$^3$H]nicotine [7] with an optimum at pH 6.0–6.5 (data not shown).

## DISCUSSION

The present study has demonstrated that, among a number of radiolabeled nicotinic ligands used to investigate nicotinic receptors in rat brain, MPA, the azetidine analogue of nicotine, exhibits the highest affinity and psychotropic potency. Although some investigators [1, 2] have reported the existence of a curvilinear Scatchard plot for (−)-[$^3$H]nicotine, others have reported only a single, lower affinity site [3]. With the use of the more potent analogue, [$^3$H]MPA, the existence of the higher affinity site, with a $K_d$ value of less than $10^{-10}$ M, and the existence of a curvilinear Scatchard for nicotine have been reinforced. It has been demonstrated recently [7] that, using [$^3$H]methylcarbamylcholine, a nicotinic ligand resembling acetylcholine, a linear Scatchard was obtained, yielding only the lower affinity site seen with the nicotine ligands.

The distinct characters of the higher and lower affinity sites for MPA are borne out by the thermodynamic analyses. Although both sites had a negative enthalpy, that of the lower affinity site was over twice that of the higher affinity site. Correspondingly, the higher affinity site exhibited a greater positive entropy and free enthalpy. It was also found from a comparison of the Scatchard analysis of [$^3$H]MPA binding at 0° and 37° that the decreased binding at higher temperature was associated with a decrease in the $B_{max}$ of the lower affinity site with no effect on either $K_d$ or the $B_{max}$ of the higher affinity site.

A comparison of the psychotropic potencies revealed that MPA was about 5-fold greater than nicotine and, although the higher affinity $K_d$ value of MPA was 3-fold greater than that of nicotine, the lower $K_d$ values were similar. This finding would suggest that the psychotropic potency of the nicotine analogues correlates with the higher affinity, rather than with the lower affinity, site. The difficulty with this explanation, however, is that the concentration of nicotine and its analogues needed to produce prostration when injected into the fourth ventricle of the rat [9] or into the vestibular cerebellum [10]—a presumed site for the nicotine-induced prostration—is estimated to be in the range of $10^{-7}$–$10^{-8}$ M. One possible explanation is that the concentration of nicotine at neuroanatomical sites of action, such as the vestibular cerebellum [10], may be at least a magnitude less than that in the area of the fourth ventricle. Preliminary studies, in which the vestibular cerebellum and other neuroanatomical sites surrounding the fourth ventricle were excised and measured for radioactivity within 2 min after administering (−)-[$^3$H]nicotine into the fourth ventricle, have revealed that the concentration of bound nicotine ranges between $10^{-8}$ and $10^{-9}$ M.

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