chloroperoxybenzoic acid (53 mg, 0.31 mmol) in methanol (2 mL)/dichloromethane (10 mL) was stirred at room temperature for 16 h and then evaporated to dryness. The residue was purified by preparative TLC (1:9 methanol/dichloromethane) to give 40 mg (43%) of **17**. An analytical sample was obtained by recrystallization from ethanol: mp 215–217 °C; UV λ_{max} 283 nm (ϵ 11 300), 260 (15 200), 255 (15 300) (methanol); ^{1}H NMR (300 MHz, Me_2SO-d_6) δ 7.99 (s, 1 H, H-8), 5.43, 5.53 (AB, J = 13 Hz, 2 H, H-1′), 4.28–4.52 (m, 4 H, H-3′, H-5′), 3.62 (p, J = 6 Hz, 1 H, H-4′), 2.20 (s, 3 H, NHAc), 2.06 (s, 6 H, OAc). Anal. ($C_{15}H_{19}N_5O_7S$) C, H, N, S.

9-[(1,3-Dihydroxy-2-propylsulfinyl)methyl]guanine (15). A suspension of **2** (120 mg, 0.44 mmol) in 30% H_2O_2 (4 mL)/H_2O (4 mL) was stirred at room temperature for 48 h and then evaporated to dryness. The residue was recrystallized twice from water to give 30 mg (27%) of **15**: mp 209–210 °C; UV λ_{max} sh 274 nm (ϵ 9300) and 256 (12 600) (0.1 N HCl), 262 (11 200) (0.1 N NaOH); ^{1}H NMR (300 MHz, Me_2SO-d_6) δ 7.66 (s, 1 H, H-8), 6.60 (s, br, 2 H, NH_2), 5.29, 5.40 (AB, J = 13 Hz, 2 H, H-1′), 5.21 (s, br, 1 H, OH), 5.07 (s, br, 1 H, OH), 3.68–3.93 (m, 4 H, H-3′, H-5′), 3.00 (m, 1 H, H-4′). Anal. ($C_9H_{13}N_5O_4S{\cdot}0.5H_2O$) C, H, N, S.

9-[(1,3-Dihydroxy-2-propylsulfonyl)methyl]guanine (16). Repeated recrystallizations from water of the mother liquors of **15** gave 13 mg (10%) of **16**: mp 262–263 °C; UV λ_{max} 265 nm (ϵ 11 400) (0.1 N NaOH); ^{1}H NMR (300 MHz, Me_2SO-d_6) δ 7.70 (s, 1 H, H-8), 6.55 (s, br, 2 H, NH_2), 5.50 (s, 2 H, H-1′), 5.22 (s, br, 2 H, OH), 3.87 (m, 4 H, H-3′, H-5′), 3.44 (p, J = 6 Hz, 1 H, H-4′). Anal. ($C_9H_{13}N_5O_5S$) C, H, N, S.

Plaque Assays. Experiments were conducted with Vero (for HSV-1 and HSV-2 infections) and MRC-5cells (for HCMV infections) that were treated with the nucleoside analogue as described previously.[6] Inhibitory doses ID_{50} are defined as doses causing a 50% reduction in plaque numbers compared to untreated controls.

Animal Studies. Swiss–Webster female mice (Simonsen Laboratories, Gilroy, CA), weighing approximately 20 g each, were infected intraperitoneally with 5×10^4 PFU of HSV-2 (strain G). This challenge was approximately equivalent to 10 50% lethal doses. DHPG and **2** were administered subcutaneously once a day for 4 days starting 24-h post-infection. Deaths were recorded for 21 days after infection.

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Structure–Activity Relationships of Some Pyridine, Piperidine, and Pyrrolidine Analogues for Enhancing and Inhibiting the Binding of (±)-[^{3}H]Nicotine to the Rat Brain P_2 Preparation†

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Previous studies have shown that (±)-[^{3}H]nicotine binds to multiple sites in the rat brain P_2 preparation. Using a series of pyridine, piperidine and pyrrolidine analogues, the present studies identified drugs with specificity for a separate up-regulatory site that increases the density of nicotine binding at another site. Of these compounds, (±)-2-methylpiperidine was the most specific. Some compounds inhibited without enhancing (±)-[^{3}H]nicotine binding, but none bound with the very high affinity exhibited by nicotine and none could be classified as specific in inhibiting binding at a specific site. Structural changes in the 1- and 2-positions of pyridine and piperidine appear to be important for conferring specificity for the up-regulatory site whereas 3-position changes may be important for binding specificity.

Nicotine can induce diverse pharmacological actions in the central nervous system including analgesia,[1-3] euphoria,[4] ganglionic stimulation, or depression, leading to many effects such as changes in blood pressure and cardiac rhythmicity,[5-7] effects that can also be produced by intracerebroventricular administration.[1,3,8,9] Both behavioral arousal and depression have been observed[1,3,10,11] as well as alterations in conditioned responses,[12,13] alterations in myoneural junction transmission,[14,15] changes in respiration,[1,16] facilitation of memory, and reduction of aggression,[17,18] nausea, vomiting, and hormonal changes.[6]

On the basis of studies employing classic cholinergic agonists and antagonists the data suggest that nicotine exerts some of its pharmacological effects through different receptors (or subsets of receptors). Some of the actions of nicotine are clearly cholinergic since they are blocked by cholinergic antagonists.[2,9,10,19-32] Some other central actions of nicotine, however, appear to be mediated through noncholinergic mechanisms.[33-36] Different effects on behavior and the EEG have been observed with different doses of nicotine injected into the lateral ventricle of the conscious dog, thus lending further support to the hypothesis that nicotine has multiple modes of action.[3,11]

† Supported by the Tobacco and Health Research Institute of the University of Kentucky.

(1) Kamerling, S. G.; Wettstein, J. W.; Sloan, J. W.; Su, T.-P.; Martin, W. R.; *Pharmacol. Biochem. Behav.* **1982**, *17*, 733.
(2) Tripathi, H. L.; Martin, B. R.; Aceto, M. D. *J. Pharmacol. Exp. Ther.* **1982**, *221*, 91.
(3) Martin, W. R.; Kaplan, E.; Sloan, J. W. *Fed. Proc.* **1984**, *43*, 547.
(4) Henningfield, J. E.; Katsumasa, M.; Jasinski, D. R. *Pharmacol. Biochem. Behav.* **1983**, *19*, 887.
(5) Langley, J. N.; Dickinson, W. L. *Proc. R. Soc. London* **1889**, *46*, 423.
(6) Jaffe, J. H. In "The Pharmacological Basis of Therapeutics"; Gilman, A. G., Goodman, L. S., Gilman, A., Eds.; Macmillan: New York, 1980.
(7) Taylor, P. In "The Pharmacological Basis of Therapeutics"; Gilman, A. G., Goodman, L. S., Gilman, A., Eds.; Macmillan: New York, 1980.
(8) Lang, N. J.; Rush, M. L. *Br. J. Pharmacol.* **1973**, *47*, 196.
(9) Wu, K. M.; Martin, W. R. *J. Pharmacol. Exp. Ther.* **1983**, *227* (2), 302.
(10) Domino, E. F. *Ann. N.Y. Acad. Sci. U.S.A.* **1967**, *142*, 216.
(11) Haranath, P. S.; Venkatakrishna-Bhatt, H. *Br. J. Pharmacol.* **1977**, *59*, 231.
(12) Domino, E. F. In "Tobacco Alkaloids and Related Compounds"; von Euler, U. S., Ed.; Macmillan: New York, 1965.

0022-2623/85/1828-1245$01.50/0

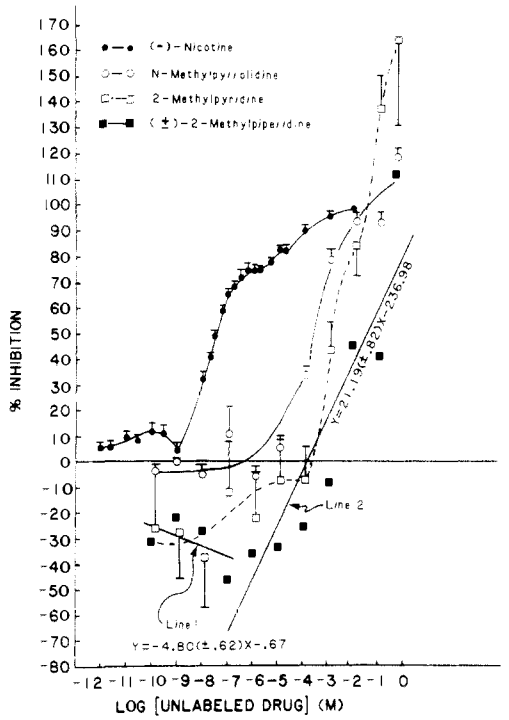


Figure 1. Inhibition of (±)-[^{3}H]nicotine binding by graded concentrations of (−)-nicotine, *N*-methylpyrrolidine, 2-methylpyridine, and (±)-2-methylpiperidine according to procedures described in the Experimental Section. The inhibition produced by each drug is shown as a percentage of the maximum displacement achieved by 10^{-2} M (−)-nicotine. Each point is the mean of four experiments. All points except those for (±)-2-methylpiperidine are shown with their standard error. Values that fall below "0" inhibition represent an enhancement of saturable (±)-[^{3}H]nicotine binding. The curve for (±)-2-methylpiperidine was quadratic in nature. The least-squares fits for line 1 (points 1–4) and line 2 (4–11) are shown with the equation for the fit. The slope for each line (± its 95% confidence limits) was calculated by regressing the percent inhibition against the log of the femtomolar concentration of (±)-2-methylpiperidine.

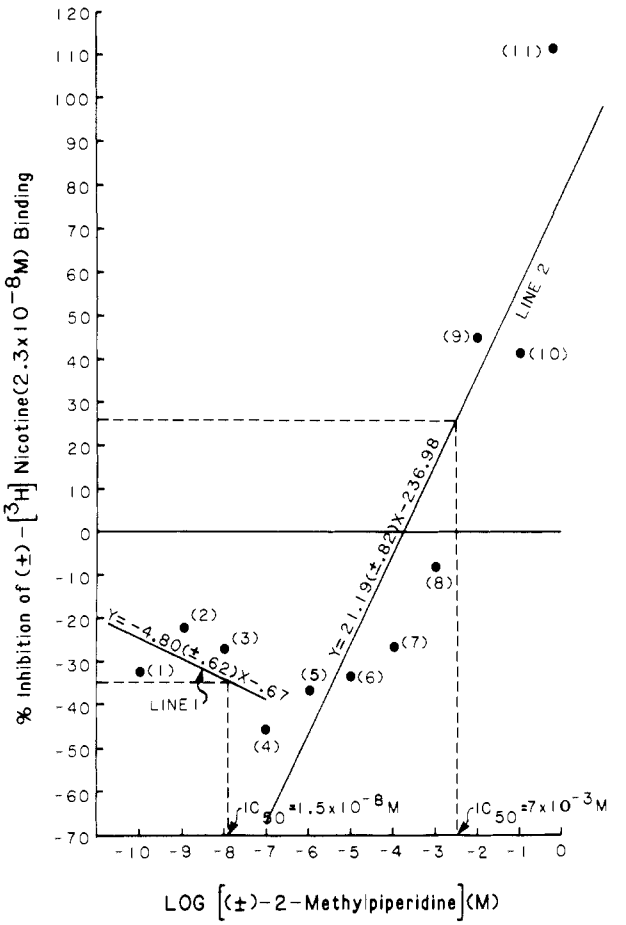
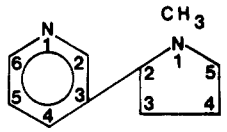


Figure 2. Inhibition of (±)-[^{3}H]nicotine binding by graded concentrations (±)-2-methylpiperidine. Each point is the mean of four experiments. The curve has a significant quadratic component. A significant regression on dose was produced by lines 1 and 2, which are shown with their IC_{50} values, slopes ± the 95% confidence limits, and their intercepts calculated as described in the legend for Figure 1.

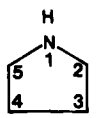
Recent in vitro binding studies using [^{3}H]nicotine as the radioligand have shown that nicotine binds to multiple sites in the rat brain.[37-48a] The complexities of nicotine binding as well as the binding characteristics of (−)- and

(13) Iwamoto, E. T.; Fudala, P.; Teoh, N. *Pharmacologist* **1984**, *26*, 161.
(14) Barlow, R. B.; Hamilton, J. T.; *Br. J. Pharmacol.* **1965**, *25*, 206.
(15) Wang, C. M.; Narahashi, T. *J. Pharmacol. Exp. Ther.* **1972**, *182*, 427.
(16) Rush, M. L.; Lang, W. J. *Eur. J. Pharmacol.* **1970**, *11*, 130.
(17) Larson, P. S.; Silvette, H. "Tobacco: Experimental and Clinical Studies", Suppl. III; Williams and Wilkins Co.: Baltimore, MD, 1975; pp 21–58.
(18) Jaffe, J. H.; Jarvik, M. E. In "Psychopharmacology: A Generation of Progress"; Lipton, M. A., DiMascio, A., Kollman, K. F., Eds.; Raven Press: New York, 1978.
(19) Eccles, J. C.; Fatt, P.; Koketsu, K. *J. Physiol.* **1954**, *126*, 524.
(20) Eccles, J. C.; Eccles, R. M.; Fatt, P. *J. Physiol.* **1956**, *137*, 154.
(21) Ginzel, K. H.; Eldred, E. Fourth International Congress on Pharmacology, Basel, 1969; Abstract, p 422.
(22) Ginzel, K. H.; Berry, N. C. *Pharmacologist* **1969**, *11* (2), 265.
(23) Ginzel, K. H.; Eldred, E.; Sasaki, Y. *Int. J. Neuropharmacol.* **1969**, *8*, 515.
(24) Ginzel, K. H.; Watanabe, S.; Eldred, E. *Neuropharmacology* **1970**, *9*, 369.
(25) Ginzel, K. H. *Fed. Proc.* **1970**, *29*, 779.
(26) Ginzel, K. H.; Eldred, E.; Watanabe, S.; Grover, F. *Neuropharmacology* **1971**, *10*, 77.
(27) Hudson, R. D. *Pharmacologist* **1971**, *13* (2), 254.
(28) Ginzel, K. H. *Neuropharmacology* **1973**, *12*, 149.
(29) Mansner, R.; Alhava, E.; Klinge, E. *Med. Biol.* **1974**, *52*, 390.
(30) Mansner, R.; Mattila, M. J. *Med. Biol.* **1975**, *53*, 169.
(31) Porsius, A. J.; VanZwieten, P. A. *Prog. Brain Res.* **1977**, 131.
(32) Andersson, K.; Fuxe, K.; Eneroth, P.; Gustafsson, J.-A.; Agnati, L. F. *Acta Physiol. Scand. Suppl.* **1980**, *479*, 27.
(33) Abood, L. G.; Lowy, K.; Tometsko, A.; Booth, H. *J. Neurosci. Res.* **1978**, *3*, 327.
(34) Abood, L. G.; Reynolds, D. T.; Bidlack, J. M. *Life Sci.* **1980**, *27*, 1307.
(35) Abood, L. G.; Reynolds, D. T.; Booth, H.; Bidlack, J. M. *Neurosci. Biobehav. Rev.* **1981**, *5*, 479.
(36) Andersson, K.; Fuxe, K.; Eneroth, P.; Agnati, L. F. *Acta Physiol. Scand.* **1984**, *120*, 489.
(37) Yoshida, K.; Imura, H. *Brain Res.* **1979**, *172*, 453.
(38) Romano, C.; Goldstein, A. *Science (Washington, D.C.)* **1980**, *No. 210*, 647.
(39) Vincek, W. C.; Martin, B. R.; Aceto, M. D.; Bowman, E. R. *J. Med. Chem.* **1980**, *23*, 960.
(40) Todd, G. D.; Sloan, J. W.; Martin, W. R. *Pharmacologist* **1982**, *24*, 222.
(41) Sloan, J. W.; Martin, W. R.; Todd, G. D. *Psychopharmacol. Bull.* **1983**, *19*, 402.
(42) Sloan, J. W.; Martin, W. R. *Pharmacologist* **1983**, *25*, 163.
(43) Abood, L. G.; Grassi, S.; Costanza, M. *FEBS Lett.* **1983**, *157*, 147.
(44) Costa, L. G.; Murphy, S. D. *J. Pharmacol. Exp. Ther.* **1983**, *226*, 392.
(45) Sloan, J. W.; Martin, W. R. *Fed. Proc.* **1984**, *43* (3), 547.
(46) Sloan, J. W.; Martin, W. R.; Hernandez, J.; Hook, R. *Pharmacologist* **1984**, *26*, 126.
(47) Sloan, J. W.; Todd, G. D.; Martin, W. R. *Pharmacol. Biochem. Behav.* **1984**, *20*, 899.



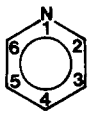
NICOTINE

1



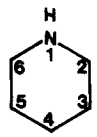
PYRROLIDINE

2



PYRIDINE

3



PIPERIDINE

4

(+)-nicotine to rat brain synaptosomal preparations have led Sloan and Martin to postulate five[47] or seven different binding sites.[48a] Subpopulations of binding sites were also identified by comparing the site densities of bound (+)- and (−)-nicotine.[48a] In order to facilitate the understanding of and to dissect out the physiologic and pharmacologic significance of these different binding sites, efforts are being made to identify specific ligands for each of these binding sites for further pharmacologic studies. This report is concerned with the binding characteristics of a series of substituted pyridines (**3**), piperidines (**4**), and pyrrolidines (**2**) that were selected for study because of their structural similarities to the pyridine or pyrrolidine moieties of nicotine (**1**) and related compounds.

Results

Table I summarizes the binding characteristics of a series of pyridine, piperidine, and pyrrolidine analogues as determined by their ability to enhance and inhibit the binding of (±)-[^{3}H]nicotine to the rat brain P_2 fraction.

Enhancement of (±)-[^{3}H]Nicotine Binding. As can be seen in Tables I and II a large number of drugs have the ability to enhance (±)-[^{3}H]nicotine binding although the amount of enhancement varies markedly between drugs. Of the drugs listed in Table II, acetylcholine produced the greatest amount of enhanced binding. Of the pyridines, piperidines, and pyrrolidines investigated in the present studies, the 2-methyl congeners were the most efficacious in this regard (Tables I and II). Further, (±)-2-methylpiperidine was the most specific drug studied in that it enhanced binding in a dose-related way over a wide range of concentrations but had little ability to inhibit the binding of (±)-[^{3}H]nicotine (Figures 1 and 2). Although 2-amino- or 2-hydroxypyridine produced less enhancement than 2-methylpyridine, these congeners inhibited (±)-[^{3}H]nicotine binding by more than 100% and to a greater extent than either 2-methylpyridine or (±)-2-methylpiperidine. Similarly, an *N*-hydroxy substitution on piperidine enhanced (±)-[^{3}H]nicotine binding less than an *N*-methyl or -ethyl substitution but inhibited binding more than 100%. The N-substituted piperidines studied showed less specificity in enhancing binding than the 2-substituted pyridines and piperidines. All of the 3- and 4-substituted pyridines and piperidines produced less enhancement of binding than the 2-methyl-substituted compounds. The pyrrolidines produced less enhancement of binding that the pyridines and piperidines. Some compounds such as *N*-benzhydrylpiperidine, 3-methylpyridine, anabasine, and cotinine produced no enhancement of binding. One of these compounds, 3-methylpyridine, produced significantly more than 100% inhibition, however (Table I). These data taken together suggest that 2-position substitutions on pyridine and piperidine are more important than substitutions on other positions for increasing the efficacy and specificity for up-regulating nicotine binding in the rat brain P_2 preparation.

Inhibition of (±)-[^{3}H]Nicotine Binding. Table I shows that several piperidines, pyridines, and pyrrolidines interact with multiple binding sites of different K_D values; however, none of these drugs had K_D values as low as nicotine.

Discussion

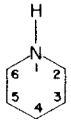
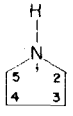
In our previous work[40-42,45,47] evidence was obtained that demonstrated that the binding of (±)-[^{3}H]nicotine to the rat brain P_2 preparation can be enhanced by a variety of drugs. In these studies it was found that there was a great disparity among drugs in their ability to produce enhanced nicotine binding. (+)-Nicotine produced a greater degree of enhanced binding, and the enhancement occurred at lower concentrations and over a wider range than (−)-nicotine. Acetylcholine produced the greatest amount of enhancement of any drug studied whereas compounds such as carbachol were markedly less effective and some such as (−)-cytisine produced no enhancement of (±)-[^{3}H]-nicotine binding.

In the present studies ligands were found to also vary greatly in their ability to enhance nicotine binding. Drugs were identified that showed specificity for the enhancement of (±)-[^{3}H]nicotine binding in that they enhanced binding over a wide range of concentrations but had little ability to inhibit binding. Other drugs were identified that inhibited but did not enhance binding. The most specific drug for producing enhanced binding was (±)-2-methylpiperidine, a compound that enhanced binding in very low concentrations whereas very high concentrations were required to inhibit nicotine binding. Other agents that exhibited specificity for enhancing binding were (±)-*N*-methyl-, (±)-*N*-hydroxy-, (±)-3-methyl-, (±)-3-hydroxy-, (±)-4-methyl-, (±)-4-hydroxy-, (±)-3-hydroxy-*N*-methyl-, and (±)-4-hydroxy-*N*-methylpiperidine; 2-ethyl-, 2-hydroxy-, 2-amino-, 3-amino-, 4-methyl-, and 2,6-dimethylpyridine; and pyridine.

It was originally thought that enhanced binding was attributable to positive cooperativity. The fact that some compounds were specific in enhancing binding argued against this hypothesis in that they enhanced binding in concentrations that did not inhibit binding. Further, many of the substituted piperidines and pyridines inhibited the binding of (±)-[^{3}H]nicotine more than 10^{-2} M (−)-nicotine (Table I). The magnitude of this increased displacement of radiolabeled nicotine in the presence of these cold ligands does not seem to be adequately explained by positive cooperativity for at least four reasons: (1) Up-regulation occurs at a site that is different from the binding site. (2) Calculations of the binding density for the best fit lines to the data indicate an increase in the number of binding sites for some compounds as can be seen in Table I. (3) Enhanced (±)-[^{3}H]nicotine binding occurs in concentrations where there is no evidence of inhibition of binding. (4) In recent kinetic studies where (−)-[^{3}H]nicotine (1.2×10^{-8} M) was incubated across time in the absence and presence of a single concentration of (±)-2-methylpiperidine (1×10^{-9} M), it was found that the association rates of (−)-[^{3}H]nicotine were not different under the two conditions. This suggests that positive cooperativity is not the explanation for the enhanced binding observed.

(48) (a) Sloan, J. W.; Martin, W. R.; Hernandez, J.; Hook, R. *Pharmacol., Biochem. Behav.*, in press. (b) Sloan, J. W.; Martin, W. R.; Hook, R.; Bostwick, M.; Howell, A.; Smith, W. T., in preparation.

Table I. Effects of a Series of Pyridines, Piperidines, and Pyrrolidines on the Binding of (±)-[^{3}H]Nicotine Using the Rat Brain P_2 Fraction[a]

	pyridine analogues				piperidine analogues				pyrrolidine analogues			
				III: max %			III: max %				III: max %	
substituent	binding site	I: K_D or [enhanced binding rg]	II: site density	enhancement	inhibn	I: K_D or [enhanced binding rg]	II: site density	enhancement	inhibn	I: K_D or [enhanced binding rg]	II: site density	enhancement	inhibn
none	1	[10^{-9}–10^{-11}]		16	154	[10^{-9}–10^{-6}]		22	58	[<10^{-10}–10^{-6}]		7	111
	2	3.3×10^{-3}	1.7×10^{-10}			1.2×10^{-7}	3.0×10^{-16}			2.5×10^{-5}	1.1×10^{-12}		
	3	8.6×10^{-2}	1.7×10^{-9}			6.9×10^{-5}	1.6×10^{-12}			3.3×10^{-4}	9.4×10^{-12}		
	4									4.1×10^{-2}	4.2×10^{-10}		
N-methyl	1					[<10^{-10}–10^{-5}]		24	173	[<10^{-10}–10^{-6}]		6	120
	2					1.2×10^{-3}	6.2×10^{-11}			5.2×10^{-5}	1.5×10^{-12}		
	3									5.9×10^{-4}	3.0×10^{-11}		
N-ethyl	1					[<10^{-10}–10^{-6}]		35	185				
	2					3.3×10^{-6}	7.6×10^{-12}						
	3					2.1×10^{-0}	2.4×10^{-7}						
N-hydroxy	1					[10^{-8}–10^{-4}]		9	287				
	2					6.0×10^{-3}	5.5×10^{-11}						
	3					5.7×10^{-2}	6.6×10^{-9}						
N-benzhydryl	1					3.9×10^{-3}	3.2×10^{-10}	...	130				
2-methyl	1	[<10^{-10}–10^{-4}]		38	166	[<10^{-10}–10^{-3}]		46	112				
	2	5.0×10^{-8}	1.6×10^{-16}			3.2×10^{-3}	1.1×10^{-10}						
	3	8.9×10^{-4}	2.8×10^{-11}										
	4	6.4×10^{-2}	2.4×10^{-9}										
2-ethyl	1	[<10^{-10}–10^{-6}]		14	126	[<10^{-10}–10^{-6}]		19	128				
	2	1.0×10^{-4}	6.2×10^{-12}			7.9×10^{-3}	5.0×10^{-10}						
2-propyl	1	[10^{-10}–10^{-5}]		20	136	[<10^{-10}–10^{-5}]		13	91				
	2	1.7×10^{-6}	6.6×10^{-15}			9.7×10^{-5}	1.2×10^{-12}						
	3	3.7×10^{-4}	3.2×10^{-11}			9.8×10^{-4}	5.4×10^{-11}						
2-hydroxy	1	[<10^{-10}–10^{-4}]		13	259								
	2	8.8×10^{-4}	5.9×10^{-12}										
	3	1.6×10^{-1}	2.3×10^{-8}										
2-amino	1	[<10^{-10}–10^{-7}]		20	378								
	2	2.0×10^{-4}	6.3×10^{-12}										
	3	3.4×10^{-2}	3.9×10^{-9}										
2-(3-pyridyl) (anabasine)	1					2.6×10^{-6}	1.4×10^{-13}	...	91				
	2					1.3×10^{-3}	2.6×10^{-11}						
3-methyl	1	1.8×10^{-3}	2.3×10^{-11}	...	121	[<10^{-10}–10^{-5}]		7	109				
	2					8.9×10^{-4}	5.1×10^{-11}						
3-ethyl	1	[<10^{-10}–10^{-6}]		20	109								
	2	9.5×10^{-6}	7.7×10^{-14}										
	3	2.8×10^{-3}	1.3×10^{-10}										
3-hydroxy	1					[<10^{-10}–10^{-4}]		13	85				
	2					8.2×10^{-4}	5.0×10^{-11}						
3-amino	1	[<10^{-10}–10^{-6}]		6	178								
	2	3.8×10^{-4}	8.3×10^{-12}										
	3	2.2×10^{-2}	2.8×10^{-9}										
(-)-1-methyl-2-(3-pyridyl)-pyrrolidine (nicotine)	1	2.2×10^{-11}	7.0×10^{-17}										
	2	[10^{-9}–10^{-8}]		>5	100								
	3	5.2×10^{-9}	1.2×10^{-15}										
	4	4.5×10^{-5}	8.0×10^{-13}										

(+)-nicotine	1	4.9×10^{-13}	8.0×10^{-18}	8	100					
	2	$[10^{-12}$–$10^{-9}]$								
	3	4.3×10^{-7}	3.1×10^{-15}							
	4	1.1×10^{-5}	2.9×10^{-13}							
	5	4.3×10^{-2}	3.0×10^{-10}							
1-methyl-2-(3-pyridyl)-2-pyrrolidinone (cotinine)	1	6.2×10^{-7}	7.7×10^{-15}							
	2	4.7×10^{-3}	3.7×10^{-10}							
4-methyl	1	$[<10^{-10}$–$10^{-4}]$		17	119	$[<10^{-10}$–$10^{-6}]$		9	126	
	2	2.2×10^{-3}	1.4×10^{-10}			3.9×10^{-3}	3.6×10^{-10}			
4-ethyl	1	$[<10^{-10}$–$10^{-5}]$		24	130					
	2	8.7×10^{-5}	1.5×10^{-12}							
	3	1.5×10^{-3}	4.2×10^{-11}							
4-hydroxy	1					$[10^{-10}$–$10^{-4}]$		9	132	
	2					3.0×10^{-3}	9.4×10^{-11}			
	3					3.2×10^{-1}	1.9×10^{-8}			
2,6-dimethyl	1	$[10^{-9}$–$10^{-4}]$		8	102					
	2	1.0×10^{-2}	8.1×10^{-10}							
3-hydroxy-*N*-methyl	1					$[<10^{-10}$–$10^{-4}]$		9	103	
	2					2.7×10^{-3}	1.7×10^{-10}			
	3					1.6×10^{-1}	4.1×10^{-9}			
4-hydroxy-*N*-methyl	1					$[<10^{-10}$–$10^{-4}]$		20	88	
	2					3.4×10^{-3}	2.2×10^{-10}			

[a] The values for each drug were obtained from the mean of four experiments with the exception of (±)-3-hydroxypiperidine ($n = 3$) and (±)-piperidine ($n = 6$). Column I shows the concentrations ranges for enhanced binding (in brackets) and the K_Ds for the inhibition of binding for the different receptor sites, all in molar units. Column II shows the site densities (in mol/mg of tissue) for the various binding sites. The maximum percent enhancement and inhibition of binding are indicated in column III.

The fact that some of these agents produced more inhibition than 10^{-2} M (−)-nicotine needs special comment. Under the conditions of these experiments 10^{-2} M (−)-nicotine inhibited the binding of (±)-[^{3}H]nicotine 38.8% ± 1.5% ($n = 108$). With receptor up-regulation, theoretically, the total amount of binding increases while the amount of nonspecific binding does not change. Thus, these pyridines, piperidines, and pyrrolidines that up-regulate the binding of [^{3}H]nicotine have more nicotine binding sites available where displacement can occur because they produce more up-regulation than (−)-nicotine.

Chemicals that inhibit binding more than 100% probably lack specificity and are inhibiting binding in concentrations that are also up-regulating the receptor. Further, when the possibility is considered that the radioligand, (±)-[^{3}H]nicotine, has already up-regulated these sites,[41,46-48] the ability of some of these substituted piperidines and pyridines to up-regulate is very large. Since up-regulation occurs within minutes in a brain fraction that probably has no, or limited, ability to synthesize binding sites de novo, the most likely explanation of this up-regulation is that pro-nicotine binding sites are converted to nicoting binding sites, probably as a consequence of drug–receptor induced allosteric changes.

With regard to the structural activity relationship it is necessary to limit the discussion to the ability of these compounds to produce up-regulation of binding and their ability to interact with binding sites. It is our belief that up-regulation is also mediated by a chemical interaction with another site or sites. Thus, when the term up-regulation is used, it will be referring to an up-regulatory site. At the present, data for the effects of N substitutions are available for piperidine only (Table I); however, several generalizations seem reasonable. Substitutions of alkyl groups on the nitrogen either enhanced or did not markedly alter up-regulation. Compounds with these substitutions exhibited a lesser affinity for binding sites, suggesting that these substitutions increase specificity for up-regulation. The substitution of the large benzhydryl group on the nitrogen abolished up-regulatory activity and decreased binding.

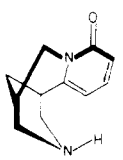
Substitutions on the 2-positions of both piperidine and pyridine enhanced up-regulation, but the effects on binding affinity were not consistent. Thus, a methyl substitution on the 2-position of piperidine markedly decreased the interaction with binding sites while the same substitution on pyridine enhanced binding site affinity. Of importance is the fact that 2-hydroxypyridine produced marked up-regulation. Substitution on the 3- and 4-positions, in general, produced lesser up-regulation than substitution on the 2-position of both pyridine and piperidine. Both substituted pyridines and piperidines can produce marked up-regulation, and in this regard the data that are available would suggest that they have greater activity than pyrrolidine. Both (+)- and (−)-nicotine have a lesser ability to produce up-regulation of (±)-[^{3}H]nicotine binding than some of the substituted pypridines and piperidines. This suggests that the up-regulatory site probably is less complicated than the nicotine binding sites.

It is also apparent that none of the drugs interact with the binding sites with as high an affinity as (+)- or (−)-nicotine. This would suggest that both the pyridine and pyrrolidine moieties are necessary for high-affinity interactions with binding sites. However, many of the substituted pyridines, piperidines, and pyrrolidines interact with several binding sites, indicating that several of the nicotine binding sites probably have more complex structures than the up-regulatory site. As yet, a method of estimating K_D values for the up-regulatory site has not

Table II. Efficacy of Some Pyridines, Piperidines, Pyrrolidines, and Other Compounds for Enhancing (±)-[^{3}H]Nicotine Binding

compd	max % enhanced binding	mean enhancement of binding: mean ± SE (*n*)	dose range, M
acetylcholine	73	DR[b]	10^{-9}–10^{-3}
(±)-2-methylpiperidine	46	DR	10^{-10}–10^{-7}
2-methylpyridine	38	20.7 ± 6.6 (28)[a]	10^{-10}–10^{-4}
(±)-*N*-ethylpiperidine	35	23.0 ± 3.8 (20) [a]	10^{-10}–10^{-6}
(−)-lobeline	34	18.2 ± 6.5 (12)[a]	5×10^{-9}–1.5×10^{-8}
(±)-*N*-methylpiperidine	24	10.0 ± 3.3 (24)[a]	10^{-10}–10^{-5}
(±)-atropine	24	14.9 ± 2.8 (64)[a]	10^{-12}–10^{-5}
4-ethylpyridine	24	DR	10^{-10}–10^{-5}
piperidine	22	10.3 ± 3.9 (24)[a]	10^{-9}–10^{-6}
2-propylpyridine	20	15.2 ± 3.2 (20)[a]	10^{-10}–10^{-5}
2-aminopyridine	20	12.4 ± 5.7 (16)[a]	10^{-10}–10^{-7}
3-ethylpyridine	20	5.4 ± 9.6 (20)	10^{-10}–10^{-6}
(±)-4-hydroxy-*N*-methylpiperidine	20	13.0 ± 6.0 (28)[a]	10^{-10}–10^{-4}
mecamylamine	20	16.4 ± 2.3 (60)[a]	2.5×10^{-9}–2.5×10^{-5}
4-methylpyridine	17	6.6 ± 2.3 (28)[a]	10^{-10}–10^{-4}
pyridine	16	12.7 ± 5.6 (24)[a]	10^{-9}–10^{-4}
carbachol	15	15.0 ± 3.8 (4)[a]	2.5×10^{-9}–7.5×10^{-8}
2-ethylpyridine	14	10.9 ± 5.6 (20)	10^{-10}–10^{-6}
(±)-2-propylpiperidine	13	8.6 ± 2.1 (24)[a]	10^{-10}–10^{-5}
(±)-3-hydroxypiperidine	13	8.6 ± 1.4 (21)[a]	10^{-10}–10^{-4}
2-hydroxypyridine	13	10.0 ± 2.5 (24)[a]	10^{-10}–10^{-4}
(±)-*N*-hydroxypiperidine	9	8.5 ± 2.3 (4)[a]	10^{-8}–10^{-4}
(±)-4-methylpiperidine	9	6.1 ± 0.8 (28)[a]	10^{-10}–10^{-4}
(±)-4-hydroxypiperidine	9	4.0 ± 1.6 (24)[a]	10^{-10}–10^{-4}
(±)-3-hydroxy-*N*-methylpiperidine	9	5.2 ± 1.0 (28)[a]	10^{-10}–10^{-4}
2,6-dimethylpyridine	9	3.1 ± 1.6 (24)	10^{-10}–10^{-4}
(+)-nicotine	8	4.7 ± 1.1 (16)	10^{-11}–10^{-8}
(±)-3-methylpiperidine	7	5.2 ± 1.5 (24)[a]	10^{-10}–10^{-5}
pyrrolidine	7	4.5 ± 2.1 (20)[a]	10^{-10}–10^{-6}
N-methylpyrrolidine	6	4.1 ± 1.6 (16)[a]	10^{-10}–10^{-6}
3-aminopyridine	6	2.4 ± 1.7 (20)	10^{-10}–10^{-6}
(−)-nicotine	<5		
(−)-anabasine	none		
choline	none		
3-methylpyridine	none		
(−)-cotinine	none		
N-benzhydrylpiperidine	none		
(−)-cytisine[c]	none		

[a] Significant enhancement, $P < 0.5$ for dose range shown. [b] DR = significant dose-related enhancement. [c] Structure of (−)-cytisine:



been devised. Further, the specificity of ligands for the various binding sites that are up-regulated is unknown.

Up-regulation of binding sites in vitro in the presence of low ligand concentrations has been reported in the adrenergic system where it has been shown that in rat whole brain homogenates (−)-norepinephrine and phenylephrine markedly up-regulate the number of α receptors but do not alter the binding affinity of the specific α ligand [^{3}H]-2-[[[(2′,6′-dimethoxyphenoxy)ethyl]amino]methyl]-benzodioxan. These investigators postulated an unmasking of receptors but did not speculate about the mechanism.[49] Similar up-regulation of α receptors by epinephrine without a change in K_D has been observed in the rat vas deferens[50] and in the human platelet after exposure to α adrenergic agonists.[51]

Several studies have also shown that nicotine may produce up-regulation in vivo. Nicotine, in doses that did not contract the nictitating membrane of the chloralose anesthetized cat, potentiated submaximal responses to low rates of preganglionic stimulation.[52] In this regard, Schwartz and Kellar[53] demonstrated that, after repeated administration of nicotine for 10 days, the binding of [^{3}H]acetylcholine in the cortices of rats was increased, a result that was due to an increase in the density of [^{3}H]-acetylcholine binding sites. Further, Marks et al.[54] have shown that the chronic administration of nicotine to mice resulted in significant increases in (±)-[^{3}H]nicotine binding in cortex, midbrain, hindbrain, hippocampus, and hypothalamus. An increase in α-[^{125}I]bungarotoxin binding was seen in midbrain and hippocampus. These changes in B_{max} were not associated with changes in K_D. In contrast, no change was observed in L-[^{3}H]quinuclidinyl benzilate binding. Marks et al.[54] suggest that this finding can be explained by assuming that chronic exposure increases the inactive form of the receptor, resulting in a decrease of agonistic activity that results in receptor up-regulation.

(49) Hata, F.; Uchida, S.; Takeyasu, K.; Ishida, H.; Yoshida, H. *Jpn. J. Pharmacol.* **1980**, *30*, 570.
(50) Hata, F.; Takeyasu, K.; Uchida, S.; Yoshida, H. *Eur. J. Pharmacol.* **1980**, *67*, 193.
(51) Cooper, B.; Handin, R. I.; Young, L. H.; Alexander, R. W. *Nature* **1978**, *274*, 703.
(52) Armitage, A. K. In "Electrophysiological Effects of Nicotine"; Redmond, A., Izard, C. Eds.; Elsevier/North-Holland Biomedical Press: New York, 1979.
(53) Schwartz, R. D.; McGee, R., Jr.; Kellar, K. J. *Mol. Pharmacol.* **1982**, *22*, 56.
(54) Marks, M. J.; Burch, B.; Collins, A. C. *J. Pharmacol. Exp. Ther.* **1983**, *226*, 817.

Schwartz and Kellar[53] suggest similar basic mechanisms in which chronic nicotine either decreases the synaptic release of acetylcholine or alternatively yields a metabolite of nicotine that is acting as an antagonist. These mechanisms decrease acetylcholine's agonistic activity with a resulting up-regulation of ACh receptors.

The results reported in this paper offer yet another explanation of ACh nicotinic receptor up-regulation.

Experimental Section

Drugs and Chemicals. (±)-[^{3}H]Nicotine (71.2 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and the purity checked periodically (>97%) with three TLC solvent systems (methanol/ammonium hydroxide, 99:1, silica gel; chloroform/methanol/diethylamine, 80:15:1, silica gel; methanol/acetic acid, 99:1, alumina). The (±)-[^{3}H]nicotine stock solution was diluted to 2 μM with mercaptoacetic acid and stored at 4 °C.[38] Other drugs and chemicals and their sources were as follows: (-)-nicotine, (-)-cytisine, and (-)-anabasine, Research Plus (Bayonne, NJ); carbachol and (±)-atropine, Sigma Chemical Co. (St. Louis, MO); piperidine, ICN Pharmaceuticals, Inc. (Plainview, NY); (±)-*N*-methyl-, (±)-*N*-ethyl-, (±)-*N*-hydroxy-, (±)-2-methyl-, (±)-3-methyl-, (±)-4-methyl-, (±)-4-hydroxy-, (±)-3-hydroxy-*N*-methyl-, and (±)-4-hydroxy-*N*-methylpiperidine, 2-methyl-, 2-ethyl-, 2-hydroxy-, 2-amino-, 3-methyl-, 3-ethyl-, 3-amino-, 4-methyl-, and 4-ethylpyridine, and pyrrolidine, Chemical Dynamics Corp. (South Plainfield, NJ); pyridine, Burdick & Jackson Laboratories Inc. (Muskegon, MI); (±)-2-propylpiperidine (coniine), Pfaltz & Bauer, Inc. (Stamford, CT); (±)-3-hydroxypiperidine, 2,6-dimethylpyridine, and *N*-methylpyrrolidine, Fluka Chemical Corp. (Hauppange, NY); acetylcholine chloride and choline chloride, Calbiochem-Behring (San Diego, CA); 2-propylpyridine, Aldrich Chemical Co. (Milwaukee, WI). (+)-Nicotine was resolved by Dr. W. T. Smith and A. Howell (Chemistry Department, University of Kentucky). Other drugs were generous gifts from the following sources: mecamylamine hydrochloride and *N*-benzhydrylpiperidine, Dr. Clement Stone, Merck Sharp and Dohme (West Point, PA); (-)-cotinine hydrochloride, Dr. Jeffery Seeman, Philip Morris Research Lab (Richmond, VA). The purity of all nonlabeled drugs was also checked by the above three TLC solvent systems used for (±)-[^{3}H]nicotine.

Protein Determination. Protein was determined in 5 μL of the P_2 preparation by the method of Lowry et al.[55] with bovine serum albumin as the standard. The reading obtained with 5 μL of Hepes buffer was subtracted from each tissue sample since it produced significant interference.

Binding Studies. Binding was determined according to procedures previously described.[47] Briefly, a P_2 fraction was prepared from the whole brain of female Sprague–Dawley rats (200–300 g) at 4 °C. Eleven concentrations of the cold ligands ranging from 1×10^{-10} to 5×10^{-1} M were used to compete with the binding of 2.4×10^{-8} M (±)-[^{3}H]nicotine. The concentration of the radioligand was chosen in order to label both high- and low-affinity sites in a single experiment. This concentration is similar to that used by other investigators.[38,44,56] Binding was determined in triplicate for each drug concentration, and each experiment was repeated usually four times with different homogenate preparations. Each tube contained 0.5 mL of the P_2 suspension (~2 mg protein) and 0.25 mL of either Hepes (total binding) or the appropriate concentration of the competing drug followed immediately by 0.25 mL of (±)-[^{3}H]nicotine. Nonspecific binding was determined in the presence of 10^{-2} M (-)-nicotine. This concentration of nicotine was chosen because lower concentrations produced less inhibition and higher concentrations produced no greater inhibition of (±)-[^{3}H]nicotine (2.4×10^{-8} M) binding. The samples were incubated at 4 °C for exactly 1 h in a shaking ice bath, a time and condition under which the binding of 2.4×10^{-8} M (±)-[^{3}H]nicotine has been shown to be at equilibrium.[47] After incubation, each sample was diluted with 3.5 mL of ice-cold Hepes and filtered at a reduced pressure (460–510 mmHg) on a filter apparatus (Hoeffer Scientific Instruments, San Francisco, CA) and Whatman GF/C glass fiber filters previously soaked in poly-L-lysine, 0.1%. With this treatment [^{3}H]nicotine binding to the filters was less than 6%. The filters were washed four times with 3.5 mL of cold Hepes and after 20 s of suction were counted by liquid scintillation.

Data Analysis

Curve-Fitting Procedures. An iterative, nonlinear, computerized curve-fitting program, LIGAND,[57] was used to provide an objective measurement of binding parameters. This program, available in Basic language through Vanderbilt University [Biomedical Computing Technology Information Center (BCTIC) R-1302, Vanderbilt Medical Center, Nashville, TN 37232] uses the total ligand concentration (labeled and unlabeled) and provides a weighted least-squares estimate of association constants (K_A), binding capacity, and nonspecific binding. The results are presented as dissociation constants (K_D values) in molar units and site densities in mol/mg of tissue. Only three sites were identified by the LIGAND program. Other sites were identified with use of chemicals with different specificities. Thus, the up-regulatory site has been unequivocally identified by using (+)- and (-)-2-methylpiperidine.[48b] The K_D values obtained for (+)- and (-)-nicotine in competition studies employing (±)-[^{3}H]nicotine were obtained as previously described.[47] For these studies ANOVA I and ANOVA II, a nonlinear curve-fitting program that provides a nonweighted iterative least-mean-squares fit of multiple regression lines of bound/free against bound Scatchard data, was also used to estimate the binding parameters. Both the program and the details of the procedures used have been described.[47]

Enhancement of Binding. Two lines of evidence indicated that the binding of (±)-[^{3}H]nicotine was enhanced or up-regulated: (1) More (±)-[^{3}H]nicotine was bound in the presence of some concentrations of a variety of ligands than was bound in their absence. Figure 1 illustrates this phenomenon. The level of enhancement often varied between homogenates. Drugs that enhanced the binding of (±)-[^{3}H]nicotine produced this effect at several concentrations. In order to test the statistical significance of this enhancement, several statistical procedures were used. The data were analyzed by a two-way analysis of variance (ANOVA) that partitioned the variance into between homogenates and between doses. The between-dose variance was partitioned into linear and quadratic regression components and the significance of these components assessed. If the between-doses variance obtained by the two-way ANOVA was not statistically significant, then each concentration of the drug for each homogenate was considered to be an independent determination, the data were pooled, and the significant of the mean was determined by a t-test. (2) Some drugs inhibited the binding of (±)-[^{3}H]nicotine more than 100%. This inhibition of binding was compared to that produced by 10^{-2} (-)-nicotine by a paired t-test.

(55) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
(56) Marks, M. J.; Collins, C. *Mol. Pharmacol.* **1982**, *22*, 554.
(57) Munson, P. J.; Rodbard, D. *Anal. Biochem.* **1980**, *107*, 220.