**ABSTRACT**

Structural simplification of N-n-alkylnicotinium analogs, antagonists at neuronal nicotinic acetylcholine receptors (nAChRs), was achieved by removal of the N-methylpyrrolidino moiety affording N-n-alkylyridinium analogs with carbon chain lengths of C1 to C20. N-n-Alkylpyridinium analog inhibition of [3H]nicotine and [3H]methyllycaconitine binding to rat brain membranes assessed interaction with α4β2 and α7 nAChRs, respectively, whereas inhibition of nicotine-evoked [3H] overflow from [3H]dopamine ([3H]DA)-preloaded rat striatal slices assessed antagonist action at nAChR subtypes mediating nicotine-evoked DA release. No inhibition of [3H]nicotine and [3H]methyllycaconitine binding was observed, although N-n-alkylpyridinium analogs had low affinity for [3H]nicotine binding sites, i.e., 1 to 3 orders of magnitude lower than that of the respective N-n-alkylnicotinium analogs. These results indicate that the N-methylpyrrolidino moiety in the N-n-alkylnicotinium analogs is a structural requirement for potent inhibition of α4β2* nAChRs. Importantly, N-n-alkylnicotinium analogs with n-alkyl chains < C10 did not inhibit nicotine-evoked [3H]DA overflow, whereas analogs with n-alkyl chains ranging from C10 to C20 potently and completely inhibited nicotine-evoked [3H]DA overflow (IC50 = 0.12–0.49 μM), with the exceptions of N-n-pentadecylpyridinium bromide (C15) and N-n-eicosylpyridinium bromide (C20), which exhibited maximal inhibition of ~50%. The mechanism of inhibition of a representative analog of this structural series, N-n-dodecylpyridinium iodide, was determined by Schild analysis. Linear Schild regression with slope not different from unity indicated competitive antagonism at nAChRs mediating nicotine-evoked [3H]DA overflow and a Ks value of 0.17 μM. Thus, the simplified N-n-alkylnicotinium analogs are potent, selective, and competitive antagonists of nAChRs mediating nicotine-evoked [3H]DA overflow, indicating that the N-methylpyrrolidino moiety is not a structural requirement for interaction with nAChR subtypes mediating nicotine-evoked DA release.

Nicotine (Fig. 1, structure 1) activates neuronal nicotinic acetylcholine receptors (nAChRs), which are members of a ligand-gated ion channel family, consisting of transmembrane pentameric proteins with diverse composition (Le Noble et al., 2002). Twelve genes encode α2-α10 and β2-β4 nAChR subunits, and in situ hybridization reveals their discrete, but overlapping, central nervous system distribution (Wada et al., 1989; Dineley-Miller and Patrick, 1992). Individual neurons elaborate multiple nAChR subtypes, and more than two different subunits can assemble forming functional nAChRs (Forsayeth and Kobrin, 1997; Zoli et al., 2002), greatly increasing the complexity of nAChR pharmacology. Thus, nAChR subtype diversity originates from differences in amino acid sequences of subunit proteins and from multiple combinations of subunit assemblies forming functional nAChRs. The exact subunit composition, stoichiometry, and arrangement of native nAChRs remain to be elucidated (Lukas et al., 1999).

Although predominance does not necessarily reflect functional importance, the α4β2* subtype, probed by high-affinity [3H]nicotine binding predominates in brain. Greater than 90% of [3H]nicotine binding sites are immunoprecipitated with anti-β2 antibody (Whiting and Lindstrom, 1987; Flores et al., 1992), and β2-knockout mice do not exhibit high-
affinity $^{[3]H}$nicotine binding (Zoli et al., 1998). Homomeric α7 nAChRs, probed by $^{[3]H}$methyllycaconitine ($^{[3]H}$MLA; Fig. 1, structure 4) binding (Davies et al., 1999), are also abundant in brain (Wada et al., 1989; Flores et al., 1992).

nAChRs are preferentially located presynaptically and modulate neurotransmitter release (McGehee and Role, 1995; Wonnacott, 1997). nAChRs are located on the soma and terminals of substantia nigra dopamine (DA) neurons (Wonnacott, 1997), and nicotine evokes DA release in striatum (Teng et al., 1997). Subtype assignment of native nAChRs mediating nicotine-evoked DA release is based on several experimental approaches, including inhibition of agonist-induced response by subtype-selective nAChR antagonists, which are defined by inhibitory activity in cell systems expressing nAChR subunits of known composition, by results from studies using nAChR-subunit knockout mice, and by in situ hybridization and single cell polymerase chain reaction of mRNA in nigral neurons. Results from these current experimental approaches have generated considerable controversy regarding the composition of nAChR subtypes mediating nicotine-evoked DA release, as well as unraveling the complexity of the effect of nicotine on this response. Additionally, these subtype-selective antagonists may find utility as therapeutic agents in the treatment of neurological diseases associated with cholinergic modulation of dopaminergic neurotransmission.

Our previous studies show that structural modification of the nicotine molecule affords a series of $N$-$n$-alkylnicotinium analogs (Fig. 1, structure 2), exhibiting high affinity and selectivity as competitive antagonists at nAChRs. $N$-$n$-alkylpyridinium analogs (3, a–n), methyllycaconitine (4), and α-conotoxin-MII (5). R, alkyl substituent; X, halide anion.

![Chemical structures of nicotine (1), N-$n$-alkynicotinium analogs NONI (2a) and NDNI (2b), N-$n$-alklypyridinium analogs (3, a–n), methyllycaconitine (4), and α-conotoxin-MII (5). R, alkyl substituent; X, halide anion.](image-url)
NDNI are relatively selective and potent nAChR antagonists, but they act at different nAChR subtypes.

The purpose of the present study was to begin to elucidate the structural requirements for this class of N-n-alkyl nicotinum nAChR antagonists. The initial strategy was structural simplification by removal of the N-methylpyrrolidino moiety to afford N-n-alkylpyridinium analogs (Fig. 1, structure 3), which were assessed for their activity at αβ2* and α7* nAChR subtypes, and at nAChR subtypes mediating nicotine-evoked DA release.

Materials and Methods

Chemicals. N-n-Alkylpyridinium analogs were synthesized via direct N-alkylation of pyridine using the method of Ayers et al. (2002). Structures of these N-n-alkylpyridinium analogs are shown in Fig. 1. Iodide salts of analogs with n-alkyl chain lengths varying from C1 to C12 were prepared, whereas C15 and C20 analogs were prepared as bromide salts: N-methylpyridinium iodide (C1, NMPI; Fig. 1, structure 3a), N-etylpyridinium iodide (C2, NEPI; structure 3b), N-n-propylpyridinium iodide (C3, NPrPI; structure 3c), N-na-butylpyridinium iodide (C4, NBuPI; structure 3d), N-n-pentylpyridinium iodide (C5, NPPPI; structure 3e), N-n-hexylpyridinium iodide (C6, NHxPI; structure 3f), N-n-heptylpyridinium iodide (C7, NHPI; structure 3g), N-n-octylpyridinium iodide (C8, NOPI; structure 3h), N-n-nonylpyridinium iodide (C9, NNPI; structure 3i), N-n-decylpyridinium iodide (C10, NDP; structure 3j), N-n-undecylpyridinium iodide (C11, UNUPI; structure 3k), N-n-dodecylpyridinium iodide (C12, NDDPI; structure 3l), N-n-pentadecylpyridinium bromide (C15, NPD; structure 3m), and N-n-eicosylpyridinium bromide (C20, NEDPB; structure 3n). Structures of compounds were confirmed by H1 and 13C nuclear magnetic resonance and fast atom bombardment mass spectrometry. Analogs were also characterized by elemental analysis and afforded values that did not deviate by ±0.4% of theoretical values.

[3H]Nicotine (S−)-(−)-methyl-[3H]nicotine; specific activity, 81.5 Ci/mmol) and [3H]DA (3,4-ethyl-2-[methyl-3H]nicotine; specific activity, 27.1 Ci/mmol) were purchased from PerkinElmer Life Sciences (Crockers, MA). [3H]Methylxylonic acid ([1α,4α,S],8,14α,16β-20-ethyl-1,6,14,16-tetramethoxy-4-[[2-[3-[3H]-methyl-2,5-dioxo-1-pyrrolidinyl]benzoyl]oxy]methyl]cinnamate; 7,8-diol; [3H]MLA; specific activity, 25.4 Ci/mmol) and cold MLA were purchased from Tocris Cookson Ltd. (Bristol, UK). S−)-(−)-nicotine di-d-tartrate and nornicotine maleate were purchased from Regis (Meadowbrook, IL). Pargyline HCl, α-glucose, HEPES, Tris-(hydroxymethyl)aminomethane hydrochloride (Trizma HCl), Tris-(hydroxymethyl)aminomethane (Trizma base), polyethyleneimine, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). TS-2 tissue solubilizer was purchased from Research Products International (Mount Prospect, IL). Other chemical components used in the preparation of the binding and release assay buffers were purchased from Fisher Scientific Co. (Pittsburgh, PA). Chemicals and solvents used in the synthetic procedures were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Subjects. Male Sprague-Dawley rats (220–250 g) were obtained from Harlan (Indianapolis, IN) and were maintained on a 12-h light/dark cycle with two rats per cage and free access to food and water in the Division of Laboratory Animal Resources (College of Pharmacy, University of Kentucky, Lexington, KY). Experimental protocols involving the animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

[3H]Nicotine Competition Binding Assay. The procedures for the binding assay were a modification of a previously described method (Wilkins et al., 2003). Striatum was dissected, frozen and stored at −70°C. Striata from two rats were pooled and homogenized with a poltron homogenizer (setting 40; Tekmar, Cincinnati, OH), in 10 volumes of ice-cold 20 mM Krebs-HEPES buffer, pH 7.5, containing 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, and 1.2 mM MgSO4. Homogenate was incubated at 37°C for 5 min and then cooled to 4°C, and subsequently centrifuged (25,000g for 20 min at 4°C). The pellet was resuspended in 10 volumes of ice-cold Milli-Q water, incubated at 37°C for 5 min, and centrifuged (25,000g for 20 min at 4°C). The previous incubation and centrifugation steps were repeated twice using 10 volumes of ice-cold 10% Krebs-HEPES buffer. The final pellet was stored in 10% Krebs-HEPES buffer at −70°C until assay. Upon assay, the final pellet was resuspended in 2.0 ml of ice-cold Milli-Q water to obtain a final protein concentration of 150 to 200 μg/100 μl of membrane suspension, determined using the Bradford dye-binding method (Bradford, 1976) using bovine gamma globulin as the standard.

Competition binding experiments were performed at 4°C in a final volume of 200 μl of incubation buffer, containing 20 mM Krebs-HEPES buffer and 2 μM Tris buffer (pH 7.5). Reactions were initiated by addition of 100 μl of membrane suspension to duplicate tubes containing one of nine concentrations of analog (1.0 nM–1 mM, final concentration) and one concentration of [3H]nicotine (3 nM, final concentration), which was based on the observed Kd value (1.3 nM) for [3H]nicotine from preliminary experiments. Nonspecific binding was determined in the presence of 10 μM nicotine. The reaction was terminated after a 90-min incubation period by dilution of the samples with 3 ml of ice-cold 20 mM Krebs-HEPES buffer followed by immediate filtration through glass filters (grade 32; Schleicher & Schuell Keene, NH) presoaked in 0.5% polyethyleneimine using a Brandel harvester (Biomedical Research and Development Laboratory Inc., Gaithersburg, MD). Filters were rinsed three times with 3 ml of ice-cold 20 mM Krebs-HEPES buffer, transferred to vials, and scintillation cocktail (4 ml added). Radioactivity was determined using a Tri-Carb 2100 TR liquid scintillation analyzer (PerkinElmer Life Sciences).

[3H]MLA Binding Assay. The [3H]MLA binding assay was performed using previously described methods (Xu et al., 2002; Wilkins et al., 2003). Briefly, whole rat brain (minus cortex, striatum, and cerebellum) was homogenized in 20 volumes of ice-cold hypotonic buffer containing 2 mM HEPES, 14.4 mM NaCl, 0.15 mM KCl, 0.2 mM CaCl2, and 0.1 mM MgSO4 (pH 7.5). Homogenate was incubated at 37°C for 10 min and centrifuged (25,000g for 15 min at 4°C). Pellet was washed three times by resuspension in 20 volumes of the same buffer and centrifuged using the above-mentioned parameters. Final pellet was resuspended in the incubation buffer to yield ~150 μg protein/100 μl membrane suspension. Protein concentration was determined as described above.

Binding assays were performed in duplicate, in a final volume of 250 μl of incubation buffer, containing 20 mM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, and 0.05% bovine serum albumin (pH 7.5). Binding assays were initiated by the addition of 100 μl of membrane suspension (~150 μg of protein) to samples containing one of seven concentrations of analog (1.0 nM–1.0 mM, final concentration) and [3H]MLA (2.5 nM, final concentration), and incubated for 2 h at room temperature. Nonspecific binding was determined in the presence of 10 μM MLA. Binding assays were terminated and radioactivity determined as described previously for the [3H]nicotine binding assay.

[3H]DA Overflow Assay. [3H]DA overflow from superfused rat striatal slices was determined using previously published methods (Dwooskin and Zahniser, 1986; Teng et al., 1997; Wilkins et al., 2002). Briefly, coronal striatal slices (500 μm in thickness, 6–8 mg) were incubated in Krebs' buffer (containing 1118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 1.3 mM CaCl2, 1.0 mM NaH2PO4, 11.1 mM glucose, 25 mM NaHCO3, 0.11 mM l-ascorbic acid, and 0.004 mM EDTA, pH 7.4, saturated with 95% O2, 5% CO2) in a metabolic shaker for 30 min at 34°C. Then, slices (6–8 slices/3 ml) were incubated with fresh buffer containing [3H]DA (0.1 μM, final concentration) for an additional 30 min. After rinsing in fresh buffer, each slice was transferred to a superfusion chamber and superfused at a flow rate of 1 ml/min with...
Krebs' buffer saturated with 95% O2, 5% CO2. Superfusion buffer contained 10 nM norfenfmine (a DA uptake inhibitor) and 10 μM paragline (a nonoamine oxidase inhibitor). After 60 min of superfusion, three 5-min samples (5 ml/sample) were collected to determine basal [3H]outflow. After collection of the third basal sample, each striatal slice from an individual rat was superfused for 60 min in either the absence or presence of one of six concentrations of analog (1 nM–0.1 mM) to determine analog-induced intrinsic activity (i.e., ability of analog to evoke [3H]overflow). Each slice was exposed to only one concentration of analog, which remained in the buffer throughout the experiment. After 60 min of superfusion in the absence or presence of analog, two 5-min samples were collected to assess basal [3H]outflow. Subsequently, nicotine (10 μM) was added to the buffer, and 5-min superfusate samples were collected for a period of 60 min to determine analog-induced inhibition of nicotine-evoked [3H]DA overflow. A control striatal slice in each experiment was superfused for 60 min in the absence of analog, followed by superfusion with nicotine (10 μM) to determine nicotine-evoked total [3H]DA overflow. Furthermore, each striatal slice was exposed to only one concentration of analog. Thus, a repeated measures design was used to determine analog-induced intrinsic activity and analog-induced inhibition of nicotine-evoked [3H]DA overflow using striatal slices from a single rat; the effect of each analog was determined using a group of five to six rats. At the end of the experiment, slices were solubilized in 1.0 ml of TS-2 tissue solubilizer, and the pH and volume of the solubilized samples were adjusted to those of the superfusate samples. Radioactivity was determined by liquid scintillation spectroscopy.

Another series of experiments determined the ability of mecamylamine (10 μM) to inhibit the intrinsic activity produced by a representative analog of this structural series, NDDPI, at a concentration (1 nM) to inhibit the intrinsic activity in the above-mentioned experiments. Experiments were performed as described above except that after collection of the third basal sample, each striatal slice from an individual rat was superfused for 60 min in either the absence or presence of mecamylamine, which remained in the buffer throughout the experiment. After 60 min of superfusion in the absence or presence of mecamylamine, NDDPI (10 μM) was added to the buffer, and 5-min superfusate samples were collected for a period of 60 min to determine mecamylamine-induced inhibition of NDDPI-evoked [3H]DA overflow. A control striatal slice in each experiment was superfused for 60 min in the absence of mecamylamine, followed by superfusion with NDDPI (10 μM) to determine NDDPI-evoked total [3H]DA overflow. Another control slice was superfused for the entire period in the absence of either mecamylamine or NDDPI. Thus, a repeated measures design was used, and the effect of NDDPI was determined using a group of six rats.

The mechanism of inhibition of NDDPI was determined by Schild analysis. In each experiment, the concentration response for nicotine (1 nM–100 μM) was determined in the absence and presence of a single concentration of NDDPI using striatal slices from a single rat. NDDPI inhibition of the effect of nicotine was determined at three concentrations of NDDPI (0.1, 0.3, or 0.6 μM), based on the previously determined IC50 value for NDDPI-induced inhibition of nicotine (10 μM)-evoked [3H]overflow. Slices were superfused for 60 min with buffer containing pargylene and nomifensine. Subsequently, slices were superfused in the absence or presence of a single concentration of NDDPI, which remained in the buffer throughout the experiment. After 60 min of superfusion in the absence or presence of NDDPI, one of six concentrations of nicotine (1 nM–100 μM) was added to the buffer and superfusion continued for an additional 60 min. Each slice from a single rat was exposed to only one concentration of nicotine and one concentration of NDDPI. These experiments used a repeated measures design, such that the concentration response for nicotine was determined using striatum from a single rat; and NDDPI concentration was a between-group factor. Tissue and superfusate samples were processed as described previously.

To assess the selectivity of NDDPI-induced inhibition of the effect of nicotine, the ability of NDDPI (1 nM–1 μM) to inhibit electrical field stimulation-evoked [3H]overflow was determined. Striatal slices were preloaded with [3H]DA, transferred to superfusion chambers, and superfused as described previously. After 60 min of superfusion, three 5-min samples were collected to determine basal [3H]overflow, and subsequently, slices were superfused for 60 min in the absence or presence of NDDPI, which remained in the buffer until the end of the experiment. Subsequently, electrical field stimulation was applied and consisted of a train of unipolar, rectangular pulses (1 Hz; 2-ms duration for 2 min; 120 pulses; model SD9 stimulator; Grass Instruments, Quincy, MA). The number of pulses was chosen to provide [3H]overflow equivalent to that evoked by superfusion with 10 μM nicotine. Superfusate samples were collected for an additional 60-min period. Each slice was exposed to only one concentration of NDDPI. One striatal slice in each experiment was superfused in the absence of NDDPI and stimulated with 120-pulse electrical field stimulation, serving as the control.

Data Analysis. For the [3H]nicotine competition binding assay, the concentration (IC50) of N-n-alkylpyridinium analog that inhibited specific [3H]nicotine binding by 50% was determined by nonlinear regression fitting of the data to a one-site model. For each analog, the one-site model provided the best fit compared with the two-site model (F test). Pseudo Hill slopes were determined by nonlinear regression fit of the data to a sigmoidal dose–response equation (variable slope): % binding = bottom + (top - bottom)/(1 + 10(log IC50 - X))%, where X is the logarithm of inhibitor concentration, n is the pseudo Hill slope, and bottom is fixed to zero. The inhibition constant (Ki) for each analog was calculated from IC50 values using the Cheng-Prusoff equation [Ki = IC50(1 + ligand/Ki)]. One-way ANOVA followed by Tukey's post hoc test (p < 0.05) was used to determine significant differences among log Ki values. Linear regression was used to determine the relationship between log Ks values and alkyl chain length.

For the [3H]DA overflow assay, fractional release for each superfusion sample was calculated by dividing the [3H] present in each 5-min sample by the total [3H] present in the tissue at the time of sample collection; and these values were expressed as a percentage of basal [3H] overflow. Basal [3H] overflow was calculated from the average fractional release in the three 5-min samples just before addition of analog to the superfusion buffer. Fractional release data were analyzed by repeated measures two-way ANOVA with time and concentration as repeated measures factors. Total [3H]DA overflow was calculated as the sum of the increase in fractional release above basal [3H] overflow during superfusion with analog or nicotine. Analog-induced intrinsic activity was analyzed by one-way repeated measures ANOVA followed by Dunnett's post hoc test (p < 0.05). Analog-induced inhibition of nicotine-evoked [3H] overflow was expressed as a percentage of total [3H]DA overflow in the absence of analog (i.e., % control). Percentage of inhibition data were analyzed by one-way repeated measures ANOVA followed by Dunnett's post hoc test (p < 0.05). IC50 values were determined by nonlinear regression fit of the percentage of inhibition data to a sigmoid dose–response equation: response = bottom + (top - bottom)/(1 + 10logIC50 - X), where X is the logarithm of analog concentration.

The mechanism by which NDDPI inhibited nicotine-evoked [3H]DA overflow was determined using Schild analysis.Nicotine concentration-response curves in the absence and presence of ND- DPI were generated by nonlinear regression fit of the data to a sigmoidal dose–response equation (variable slope): response = bottom + (top - bottom)/(1 + 10(logIC50 - X)), where X is the logarithm of the nicotine concentration and n is the Hill slope. For each experiment, the dose ratio (dr) for each concentration of NDDPI was calculated as that producing an equivalent response in the absence and presence of NDDPI, such that total [3H]DA overflow was determined at a value of 1% of tissue tritium content. The log of the dose ratio − 1 was plotted as a function of log of the NDDPI concentration to provide the Schild regression. Data were fit by linear regression, the slope determined, and linearity assessed. The ability of NDDPI to
inhibit electrically evoked $^3$H overflow was analyzed by one-way repeated measures ANOVA, with NDDPI concentration as a within-subjects factor. All data analyses were performed using the commercially available programs GraphPad Prism (GraphPad Software, Inc., San Diego, CA) and SPSS standard version 9.0 (SPSS Science, Chicago, IL).

**Results**

* $N$-$n$-Alkylpyridinium-Induced Inhibition of [3H]Nicotine and [3H]MLA Binding to Rat Brain Membranes.*

$N$-$n$-Alkylpyridinium analogs with chain lengths ranging from C1 to C20 inhibited [3H]nicotine binding to rat striatal membranes (Fig. 2). With the exception of NEcPB (C20 analog), which inhibited binding by ~25%, this series of analogs completely inhibited [3H]nicotine binding at high concentrations (0.1–1.0 mM). The values for $K_i$ and pseudo Hill slope ($n_{1H}$) of the competition curves are presented in Table 1. Analogs with C4 to C11 chain length had the highest affinity ($K_i$ values from 9 to 20 μM) for the [3H]nicotine binding site relative to the other analogs in this series. However, these analogs had relatively low affinity for the [3H]nicotine binding site compared with nicotine ($K_i > 100$ μM) of the series for the [3H]nicotine binding site. A significant linear relationship was not found between $N$-$n$-alkylpyridinium affinity for the [3H]nicotine binding site and number of carbons in the $n$-alkyl chain ($r^2 = 0.324$). With respect to [3H]MLA binding, none of the analogs in the series inhibited [3H]MLA binding (data not shown), and thus, had no detectable affinity for this site.

* $N$-$n$-Alkylpyridinium-Induced Inhibition of Nicotine-Evoked [3H]DA Overflow from Superfused Striatal Slices.*

In an initial series of experiments, inhibition of nicotine (10 μM)-evoked [3H]DA overflow by the $N$-$n$-alkylpyridinium analogs (C1–C12) was determined at two concentrations (0.1 and 1.0 μM). Analogs with chain lengths from C1 to C9 did not inhibit nicotine-evoked [3H]DA overflow, and thus, were not evaluated further. However, the C10 (NDPI), C11 (NUPI), and C12 (NDDPI) analogs significantly inhibited nicotine-evoked [3H]DA overflow ($IC_{50} = 46.35$; $p < 0.0001$), such that a 1.0 μM concentration of analog inhibited nicotine-evoked [3H] overflow by ~70 to 90%. Subsequently, the complete concentration response (0.01–100 μM) for the C10 to C12 analogs was determined. To further evaluate the role of $n$-alkyl chain length on inhibition of nicotine-evoked [3H]DA overflow, the effect of C15 and C20 analogs was also determined. Intrinsic activity of the C10 to C20 $N$-$n$-alkylpyridinium analogs was assessed during the 60-min period of superfusion with each analog before addition of nicotine to the superfusion buffer (Table 2). Intrinsic activity was not observed for any of the analogs at concentrations ≤1.0 μM. NDPI (C10) and NEcPB (C20) also showed no intrinsic activity at 10 μM. However, NUPI (C11), NDDPI (C12), and NPDPB (C15) significantly increased [3H]DA overflow at 10 μM, whereas NDPI (C10) and NEcPB (C20) increased [3H]DA overflow at 100 μM (Table 2; repeated

$$\text{TABLE 1}$$

$K_i$ values and pseudo Hill slopes ($n_{1H}$) for $N$-$n$-alkylpyridinium analog inhibition of [3H]nicotine binding to rat striatal membranes.

<table>
<thead>
<tr>
<th>Analog</th>
<th>Chain Length</th>
<th>$n_{1H}$</th>
<th>$K_i$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMPI</td>
<td>1</td>
<td>-0.95</td>
<td>20.1 (15.6–25.9)</td>
</tr>
<tr>
<td>NEPI</td>
<td>2</td>
<td>-0.91</td>
<td>28.7 (22.3–37.0)</td>
</tr>
<tr>
<td>NPPrPI</td>
<td>3</td>
<td>-1.00</td>
<td>42.3 (30.2–59.3)</td>
</tr>
<tr>
<td>NBuPi</td>
<td>4</td>
<td>-0.94</td>
<td>8.65 (7.57–12.3)</td>
</tr>
<tr>
<td>NPPI</td>
<td>5</td>
<td>-0.91</td>
<td>8.72 (5.68–13.4)</td>
</tr>
<tr>
<td>NHxPi</td>
<td>6</td>
<td>-0.98</td>
<td>9.07 (6.12–13.5)</td>
</tr>
<tr>
<td>NHPI</td>
<td>7</td>
<td>-0.90</td>
<td>15.0 (13.0–17.2)</td>
</tr>
<tr>
<td>NPOI</td>
<td>8</td>
<td>-1.01</td>
<td>20.1 (16.3–24.8)</td>
</tr>
<tr>
<td>NNPI</td>
<td>9</td>
<td>-1.06</td>
<td>18.0 (16.4–22.1)</td>
</tr>
<tr>
<td>NDDPI</td>
<td>10</td>
<td>-1.01</td>
<td>16.7 (13.6–20.4)</td>
</tr>
<tr>
<td>NDDPI</td>
<td>11</td>
<td>-1.07</td>
<td>17.0 (14.4–20.1)</td>
</tr>
<tr>
<td>NDDPI</td>
<td>12</td>
<td>-1.17</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NPDPB</td>
<td>15</td>
<td>-0.96</td>
<td>38.0 (28.2–51.3)</td>
</tr>
<tr>
<td>NEcPB</td>
<td>20</td>
<td>-1.12</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

CI, confidence interval.

![Fig. 2. Inhibition of specific [3H]nicotine binding to rat striatal membranes by $N$-$n$-alkylpyridinium analogs. Analog-induced inhibition is illustrated in three panels for clarity of presentation. Assays used a range of analog concentrations (0.1 μM–1 mM) to inhibit binding of 3 nM [3H]nicotine. Data are femtomoles per milligram of protein expressed as a percentage of control [3H]nicotine binding and represent the mean ± S.E.M. of 52 independent experiments (n = 4 independent experiments/analog). Control [3H]nicotine binding was 37.3 ± 0.54 fmol/mg of protein, range 34 to 45 fmol/mg of protein, in the absence of analog. Curves were generated by nonlinear regression. Numbers inside the parentheses indicate the length of the $n$-alkyl chain; analog abbreviations are provided in the text.](https://www.jpet.aspetjournals.org/content/1015/1/1015/F2.large.jpg)
measures one-way ANOVAs: NDNI (C10), F<sub>5,35</sub> = 27.19, p < 0.0001; NUPI (C11), F<sub>5,35</sub> = 55.53, p < 0.0001; NDDPI (C12), F<sub>5,35</sub> = 19.16, p < 0.0001; NPDPB (C15), F<sub>5,20</sub> = 36.784, p < 0.001; NEcPB (C20), F<sub>5,19</sub> = 4.830, p < 0.01). Thus, at high concentrations (10–100 μM), intrinsic activity was observed for the C10 to C20 N-n-alkylpyridinium analogs.

The ability of mecamylamine (10 μM) to inhibit NDDPI-induced intrinsic activity was determined. NDDPI (10 μM) evoked 20.9 ± 1.68 of total 3H overflow during the 60-min superfusion period. Inclusion of mecamylamine in the superfusion buffer for 60 min before addition of NDDPI to the buffer resulted in 17.2 ± 1.91 of total 3H overflow. At the concentration used, mecamylamine did not increase 3H overflow above that observed for control slices superfused with buffer only. Thus, mecamylamine did not inhibit intrinsic activity induced by the representative analog NDDPI.

The ability of the N-n-alkylpyridinium analogs to inhibit nicotine-evoked 3H overflow is illustrated in Fig. 3. High concentrations of analogs that produced intrinsic activity were not included in the analysis of inhibition of the effect of nicotine. For each analog, repeated measures one-way ANOVA revealed significant concentration-dependent inhibition of nicotine-evoked [3H]DA overflow: NDNI (C10), F<sub>5,35</sub> = 29.44, p < 0.0001; NUPI (C11), F<sub>4,29</sub> = 24.58, p < 0.0001; NDDPI (C12), F<sub>3,23</sub> = 11.07, p < 0.01; NPDPB (C15), F<sub>5,18</sub> = 24.31, p < 0.01; NEcPB (C20), F<sub>5,15</sub> = 9.29, p < 0.001. IC<sub>50</sub> values derived from the nonlinear sigmoid curve fits ranged from 0.12 to 0.49 μM (Table 3). NDNI (C10), NUPI (C11), and NDDPI (C12) completely inhibited nicotine-evoked [3H]DA overflow (Fig. 3). Interestingly, the longer chain analogs, NPDPB (C15) and NEcPB (C20), inhibited nicotine-evoked [3H]DA overflow by a maximum of 45 to 55%.

The time course of the NDDPI-induced inhibition of nicotine-evoked [3H]DA overflow is also shown in Fig. 3. Repeated measures two-way ANOVA revealed significant main effects of NDDPI concentration (F<sub>5</sub> = 36.616, p < 0.001) and time (F<sub>11,55</sub> = 80.497, p < 0.001), and a significant concentration × time interaction (F<sub>55,165</sub> = 4.215, p < 0.001). Fractional release evoked by nicotine peaked 10 min after its addition to the buffer and subsequently decreased toward basal levels, despite the presence of nicotine in the buffer throughout the remainder of the experiment. The time course illustrates the concentration-dependent inhibition, with low concentrations of NDDPI (0.01–1.0 μM) inhibiting the response to nicotine across the time course of exposure.

Mechanism of N-n-Alkylpyridinium Inhibition of Nicotine-Evoked [3H]DA Overflow. The competitive versus noncompetitive nature of inhibition of nicotine-evoked 

### Table 2

<table>
<thead>
<tr>
<th>Analog</th>
<th>n</th>
<th>Concentration</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>NDPI (10)</td>
<td>6</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NUPI (11)</td>
<td>6</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NDDPI (12)</td>
<td>6</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NPDPB (15)</td>
<td>5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NEcPB (20)</td>
<td>5</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are mean ± S.E.M. total [3H]DA overflow expressed as a percentage of tissue tritium.  
<sup>*</sup>P < 0.05, compared to control (absence of analog). 

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**Fig. 3.** Concentration-dependent inhibition of nicotine-evoked [3H]DA overflow by N-n-alkylpyridinium analogs. Superfusion buffer contained nomifensine (10 μM) and pargyline (10 μM). N-n-Alkylpyridinium analog was added to the buffer after 60 min of superfusion, and superfusion continued for 60 min with analog before addition of nicotine (10 μM) to the buffer. Slices were superfused with analog plus nicotine for an additional 60 min. In each experiment, one striatal slice was superfused with 10 μM nicotine in the absence of analog and served as the nicotine control. Top, illustrates the data for NDNI, NUPI, NDDPI, NPDPB, and NEcPB as mean ± S.E.M. total [3H]DA overflow expressed as a percentage of nicotine control. The response to nicotine (10 μM) under control conditions was 2.14 ± 0.19 of total [3H]DAoverflow (mean ± S.E.M.). Numbers in parentheses indicate the number of carbons in the n-alkyl chain for each analog. Curves were generated by nonlinear regression. Bottom, illustrates the time course of the inhibition of nicotine-evoked fractional release by the representative analog NDDPI. The vertical arrow indicates addition of nicotine to the buffer. Data are expressed as mean ± S.E.M. as a percentage of basal 3H outflow as a function of time of superfusion (minutes). Basal 3H outflow before nicotine exposure was 0.89 ± 0.04 fractional release. n = 5 to 6 rats/anallog.
N-n-Alkylpyridinium Analogs Inhibit nAChRs

TABLE 3
Concentration (IC_{50}) of N-n-alkylpyridinium analogs required to reduce nicotine-evoked [3H]DA overflow from superfused rat striatal slices by 50%

<table>
<thead>
<tr>
<th>Analog</th>
<th>IC_{50} (95% CI)</th>
<th>μM</th>
</tr>
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<tbody>
<tr>
<td>NDPI (10)</td>
<td>0.13 (0.02–0.87)</td>
<td></td>
</tr>
<tr>
<td>NUPI (11)</td>
<td>0.49 (0.01–16.9)</td>
<td></td>
</tr>
<tr>
<td>NDPI (12)</td>
<td>0.26 (0.02–4.23)</td>
<td></td>
</tr>
<tr>
<td>NDPPB (15)</td>
<td>0.22 (0.11–0.87)</td>
<td></td>
</tr>
<tr>
<td>NEnPB (20)</td>
<td>0.12 (0.01–2.38)</td>
<td></td>
</tr>
</tbody>
</table>

[3H]DA overflow was determined by Schid analysis for the representative analog NDDPI. The concentration-response for nicotine was determined in the absence and presence of three NDDPI concentrations (0.1, 0.3, and 0.6 μM; Fig. 4). Inclusion of 0.3 and 0.6 μM NDDPI in the buffer produced a rightward shift in the concentration-response curves for nicotine relative to the response curves obtained under the control condition (in the absence of NDDPI). A linear fit (r^2 = 0.9889) to the Schid-transformed data (Fig. 4, inset) revealed a slope not different from unity, and the regression did not deviate significantly from linearity, as determined by runs test. The log K_B value of –6.774 for NDDPI was derived from the x-intercept. The anti-log transform provided a K_B value of 0.17 μM, which was in good agreement with the IC_{50} value of 0.26 μM obtained by determining NDDPI-induced inhibition of 10 μM nicotine. Furthermore, NDDPI did not augment [3H]DA overflow evoked by low concentrations (1 nM–1 μM) of nicotine. Thus, the results from the Schid analysis indicate that NDDPI interacts in a competitive manner with nAChRs mediating nicotine-evoked [3H]DA overflow.

NDDPI Does Not Inhibit Field Stimulation-Evoked [3H]DA Overflow. [3H]DA-preloaded striatal slices were superfused for 60 min in the absence or presence of NDDPI (1 nM–1 μM), and were subsequently field stimulated with 120 electrical pulses (1-Hz stimulation for 2 min). Table 4 provides the results demonstrating that electrical field stimulation-evoked [3H] overflow was not inhibited by NDDPI. Electrical field stimulation resulted in total [3H] overflow of 2.24 ± 0.35% of [3H] tissue content. ANOVA revealed that the main effect of NDDPI concentration was not significant (F_{4,20} = 2.309; p > 0.05). Thus, NDDPI did not inhibit electrical stimulation-evoked [3H] overflow.

Discussion

Structural simplification of a series of N-n-alkylnicotinium antagonists was achieved by removal of the N-methylpyrrolidino moiety, which afforded N-n-alkylpyridinium analogs with carbon chain lengths ranging from C1 to C20. These N-n-alkylpyridinium analogs had generally greater water solubility than the respective N-n-alkylnicotinium analogs, which allowed for evaluation of longer chain compounds (C15 and C20) in the current study. N-n-Alkylpyridinium analogs had low affinity for [3H]nicotine binding sites, i.e., 1 to 3 orders of magnitude lower than those of the respective N-n-alkylnicotinium analogs (Wilkins et al., 2003; present study). Similar to the N-n-alkylnicotinium antagonists, the N-n-alkylpyridinium analogs exhibited no inhibition of [3H]MLA binding. These results indicate that the N-methylpyrrolidino moiety in the N-n-alkylnicotinium series is a structural requirement for potent inhibition of α4β2* nAChRs probed by high-affinity [3H]nicotine binding.

This series of N-n-alkylpyridinium compounds was initially evaluated for intrinsic activity in the DA release assay (i.e., analog-induced [3H]DA overflow). As in the N-n-alkylnicotinium series, relatively high concentrations of analogs with n-alkyl chain lengths greater than or equal to C10 exhibited intrinsic activity in the DA release assay; however, in contrast to the N-n-alkylnicotinium analogs, intrinsic activity produced by the current series of analogs was not

![Fig. 4. NDDPI competitively inhibits nicotine-evoked [3H]DA overflow from superfused rat striatal slices. Superfusion buffer contained nomifensine (10 μM) and pargyline (10 μM) from the start of superfusion. After 60 min of superfusion, slices were superfused in the absence or presence of 0.1, 0.3, or 0.6 μM NDDPI for a 60-min period before the addition of nicotine (1.0 nM–100 μM) to the buffer, and superfusion continued for an additional 60 min. In each experiment examining the effect of a single concentration of NDDPI, a set of slices was superfused with 1 nM to 100 μM nicotine in the absence of NDDPI, serving as the nicotine control condition. Because the results from the nicotine control condition (i.e., concentration response for nicotine in the absence of NDDPI) were not significantly different among the series of experiments (F_{4,10} = 2.92, p > 0.05), these data were pooled for graphical presentation. An additional slice in each experiment was superfused in the absence of either drug and served as the buffer control, and is indicated as control. Data are expressed as mean ± S.E.M. total [3H]DA overflow during the 60-min period of exposure to nicotine in the absence and presence of NDDPI as a function of log nicotine concentration. Curves were generated by nonlinear regression fit of the data to a sigmoid dose-response equation. Schid regression plot of the data are presented in the inset; slope determined by linear regression. n = 3 to 5 rats/NDDPI concentration.](Image 44x242 to 295x418)

![TABLE 4](Image 11002)

NDDPI does not inhibit field electrical stimulation-evoked [3H]overflow from [3H]DA preloaded striatal slices. Superfusion buffer contained pargyline (10 μM) and nomifensine (10 μM). Slices were superfused with a range of NDDPI concentrations. Each slice was exposed to only one concentration of NDDPI and subsequently was stimulated with 120 electrical pulses (1 Hz) of electrical field stimulation. Data represent [3H]overflow during the 60-min period of superfusion following electrical field stimulation.

<table>
<thead>
<tr>
<th>NDDPI</th>
<th>120 Pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>[3H] overflow (49.0 ± 0.37)</td>
</tr>
<tr>
<td>Control (0)</td>
<td>2.24 ± 0.35*</td>
</tr>
<tr>
<td>0.001</td>
<td>1.47 ± 0.28</td>
</tr>
<tr>
<td>0.01</td>
<td>2.45 ± 0.39</td>
</tr>
<tr>
<td>0.1</td>
<td>2.23 ± 0.36</td>
</tr>
<tr>
<td>1.0</td>
<td>1.23 ± 0.38</td>
</tr>
</tbody>
</table>

* Data are mean ± S.E.M. of total [3H] overflow expressed as a percentage of tissue tritium. n = 6 rats.
related to chain length (Wilkins et al., 2002; present study). Mecamylamine, which inhibits all known nAChR subtypes, including the αβ2* subtype probed by [3H]nicotine binding, was used to evaluate whether the N-n-alkylpyridinium analogs produced intrinsic activity via stimulation of nAChRs. However, mecamylamine did not inhibit intrinsic activity induced by the representative analog NDPI. These results indicate that N-n-alkylpyridinium analog-induced intrinsic activity is not the result of stimulation of nAChRs (i.e., these analogs do not act as nAChR agonists). Thus, intrinsic activity induced by these compounds is likely the result of stimulation of non-nicotinic receptors.

The most significant finding in the current study is that N-n-alkylpyridinium analogs, with alkyl chains ranging from C10 to C20, potently and selectively inhibited nicotine-evoked [3H]DA overflow (IC_{50} = 0.12–0.49 μM). The representative N-n-alkylpyridinium analog NDPI inhibited nicotine-evoked [3H]DA overflow at low concentrations (0.1–1.0 μM), whereas NDPI inhibited [3H]nicotine binding at high concentrations of 100 μM. These results support the suggestion that different nAChRs mediate nicotine-evoked DA release and [3H]nicotine binding (see Introduction), and moreover, demonstrate the greater than 2 orders of magnitude selectivity of NDPI as an antagonist at nAChRs mediating nicotine-evoked DA release in striatum relative to αβ2* nAChRs. Furthermore, the IC_{50} value for NDPI to inhibit nicotine-evoked [3H]DA overflow was 0.26 μM, whereas significant intrinsic activity for NDPI was observed only at a 40-fold higher concentration of 10 μM. As such, NDPI exhibits 40-fold selectivity as an antagonist at nicotinic receptors mediating nicotine-evoked DA release relative to its action to intrinsically evoke DA release likely via a non-nicotinic receptor-mediated mechanism. With the exception of NPDPB, which exhibits similar selectivity to NDPI, the remaining analogs exhibit 330-fold selectivity for the nicotinic receptor mediating nicotine-evoked DA release (IC_{50} of ~0.3 μM), whereas intrinsic activity (stimulation of DA release) was observed at relatively higher concentrations (100 μM). Thus, the 40- to 330-fold separation between analog-induced inhibition of nicotine-evoked dopamine release and analog-induced intrinsic activity supports the contention that these compounds are relatively selective as antagonists at nicotinic receptors mediating nicotine-evoked dopamine release.

The N-n-alkylpyridinium analogs completely inhibited the effect of nicotine in the DA release assay, with the exceptions of NECPB (C20 analog) and NPDPB (C15 analog), both compounds exhibiting maximal inhibition of only ~50%. High concentrations (10–100 μM) of NPDPB produced significant amounts of intrinsic activity, which may have contributed to the observed incomplete inhibition of the effect of nicotine. However, only the high concentration (100 μM) of NECPB elicited a small, but significant, amount of intrinsic activity. At lower concentrations (10 μM), NECPB induced maximal inhibition and no intrinsic activity was observed. Thus, maximal incomplete inhibition was observed at a concentration of NECPB eliciting no intrinsic activity. The Conus snail neurotoxin, α-conotoxin-MII (Fig. 1, structure 5), also has been shown to inhibit ~50% of the response to nicotine in the DA release assay (Kulak et al., 1997; Kaiser et al., 1998; Kaiser and Wonnacott, 2000). Whether the latter observations indicate that NECPB and α-conotoxin-MII act at the same nAChR subtype remains to be determined. α-Conotoxin-MII inhibits acetylcholine electrophysiological responses in Xenopus oocytes expressing α8/α6 and αβ/α4 nAChRs containing either β2 or β4 subunits (Luefte et al., 1990; Cartier et al., 1996; Kuryatov et al., 2000). Also, α-conotoxin-MII binds with high affinity to immunopurified αβ2* nAChRs (Zoli et al., 2002), implicating nAChR subtypes containing these two subunits as mediating this response. Furthermore, [3H]α-conotoxin-MII binding was eliminated in α6-knockout mice, but not in α3-knockout mice, suggesting an interaction with native α6-containing nAChRs (Champtiaux et al., 2002; Whiteaker et al., 2002). Additionally, studies using β2-knockout mice also implicate β2-containing nAChRs in nicotine-evoked DA release (Picciotto et al., 1998; Grady et al., 2002). The fact that α-conotoxin-MII and NECPB inhibited only 50% of nicotine-evoked DA release indicates that more than one subtype of nAChR is involved (Kulak et al., 1997; Kaiser et al., 1998; present study). Because nigral neurons express α3/α7 and β2/β4 mRNAs (Deneris et al., 1989; Wada et al., 1989; Le Novère et al., 1996; Charpentier et al., 1998; Arroyo-Jimenez et al., 1999; Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002), it is quite possible that a diversity of nAChR subunit combinations and subtypes are involved in this response. Evidence is emerging that suggests that different DA neurons in the nigra can be categorized based upon the expression of particular nAChR subtypes with varying compositions of nAChR subunits (Azam et al., 2002). Although speculative at the current time, it may be that the expression of nAChR subtypes by dopaminergic neurons is a dynamic process, which is regulated in part by the history of the organism, including environmental exposure and drug experience. With respect to drug discovery, NECPB is a small molecule, but seems to produce the same functional effect (i.e., ~50% maximal inhibition) as the relatively larger neuroactive peptide, α-conotoxin-MII. The discovery of NECPB sets the precedence for the development of small, nonpeptide molecules that are selective antagonists for a subset of nAChR subtypes mediating nicotine-evoked DA release.

In terms of structure-activity relationships, analogs in the current N-n-alkylpyridinium series required longer n-alkyl substituents (i.e., C10–C20) compared with those in the respective N-n-alkylnicotinium series (C8–C12) to obtain a comparable affinity (IC_{50} = 0.1–0.5 μM) for nAChRs mediating nicotine-evoked DA release from striatum. Furthermore, in the N-n-alkylnicotinium series, n-alkyl chain length was linearly related to nAChR affinity, whereas in the N-n-alkylpyridinium series, no such linear relationship was observed. In this respect, C10 to C20 analogs were equipotent in inhibiting nicotine-evoked DA release, whereas analogs with alkyl chain lengths of less than C10 did not inhibit nicotine-evoked [3H]DA overflow. Interestingly, the C10 analog NDPI was a potent inhibitor (IC_{50} = 0.13 μM) of nicotine-evoked DA release (present study). This is in marked contrast to the C10 analog NDNI, which had no inhibitory activity in the nicotine-evoked DA release assay (Wilkins et al., 2002). In the latter study, NDNI was proposed to exhibit a unique conformation to explain its unusual pharmacological profile; this unique conformation is obviously not maintained in the NDPI structure, after removal of the N-methylpyrrolidino moiety.

Although it seems that the nature of the cationic head group and the size of the n-alkyl substituent are both impor-
tant in establishing affinity for nAChR subtypes mediating nicotine-evoked DA release, the lack of a linear relationship in the C10 to C20 analogs of the N-n-alkylypyridinium series indicates that antagonist potency is insensitive to chain length within this 10-carbon range. Thus, the inhibitory effect of these C10 to C20 N-n-alkylypyridinium analogs may be more generally related to a common physicochemical property of these molecules rather than to their differences in chemical structure.

The mechanism of inhibition of a representative analog in this structural series, NDDPI, was determined by Schuld analysis. Nicotine concentration-response curves were obtained in the absence and presence of three concentrations of NDDPI. NDDPI produced a rightward shift in the concentration-response curve for nicotine, and a linear Schuld regression with slope not different from unity, providing a $K_I$ value of 0.17 $\mu M$. These results are indicative of competitive and potent antagonism at nAChRs mediating nicotine-evoked DA release. The $K_I$ value from the Schuld analysis was in good agreement with the $IC_{50}$ value obtained from the inhibition analysis with the single concentration of nicotine (10 $\mu M$). Furthermore, the observation that low concentrations of NDDPI (0.01–0.6 $\mu M$) did not augment DA release evoked by the very low concentrations (1 nM–1 $\mu M$) of nicotine provides additional support for the contention that NDDPI is not acting as an agonist at nAChRs mediating nicotine-evoked DA release. Moreover, the findings from the Schuld analysis indicate that NDDPI is a competitive antagonist at nAChRs mediating nicotine-evoked DA release. Thus, the simplified N-n-alkylypyridinium analogs are potent, selective, and competitive antagonists of nAChRs mediating nicotine-evoked DA release, indicating that the N-methylpyridylidino moiety is not a structural requirement for interaction with these nAChR subtypes. Competitive inhibition most likely results from the pyridinium moiety of NDDPI, which may interact competitively with the agonist-binding region on the nAChR protein that normally accommodates the protonated N-methylpyridylidino moiety of the nicotine molecule. In this respect, it is important to note that NDDPI did not inhibit depolarization-induced release evoked by electrical field stimulation, which provides additional supporting evidence that these analogs selectively interact with the agonist-binding site on nAChRs mediating nicotine-evoked DA release.

Evidence indicates that high micromolar concentrations (30–100 $\mu M$) of nicotine activate $\alpha7^*$ homeric nAChRs to evoke DA release through an indirect mechanism of action, i.e., nicotine-induced stimulation of $\alpha7^*$ nAChRs releases glutamate from glutamatergic terminals, which then indirectly releases DA from dopaminergic terminals (Kaiser and Wonnacott, 2000). Furthermore, glutamate receptor antagonists have been reported to inhibit ~20 to 50% of nicotine-evoked DA release from striatal slices, but not from striatal synaptosomes (Wonnacott et al., 2000), indicating that local circuitry within the striatal slice is sufficient to detect this indirect effect of nicotine on DA release. The concentration of nicotine (10 $\mu M$) used in the present study to evoke DA release may not have been sufficient to activate this indirect glutamate-mediated mechanism of DA release. Moreover, the observation that N-n-alkylypyridinium analogs have no affinity for the $[3H]$MLA binding site excludes the involvement of homeric $\alpha7^*$ nAChRs as a possible target for these antagonists.

In summary, structural simplification of N-n-alkylnicotin- 

In summary, structural simplification of N-n-alkylnicotin- 
inium antagonist molecules (e.g., NONI and NDNI) by re- 

In summary, structural simplification of N-n-alkylnicotin- 

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