Pharmacologic Characterization of a Nicotine-Discriminative Stimulus in Rhesus Monkeys

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ABSTRACT

This study examined mechanisms by which nicotine (1.78 mg/kg base s.c.) produces discriminative stimulus effects in rhesus monkeys. In addition to nicotine, various test compounds were studied including other nicotinic acetylcholine receptor agonists (varenicline and cytisine), antagonists [mecamylamine and the $\alpha 4\beta 2$ receptor-selective antagonist dihydro- β -erythroidine (DH β E)], a nicotinic acetylcholine receptor antagonist/indirect-acting catecholamine agonist (bupropion), and non-nicotinics (cocaine and midazolam). Nicotine, varenicline, and cytisine dose-dependently increased drug-lever responding; the ED₅₀ values were 0.47, 0.53, and 39 mg/kg, respectively. Bupropion and cocaine produced 100% nicotine-lever responding in a subset of monkeys, whereas mecamylamine, DH β E, and midazolam produced predominantly vehicle-lever responding. The training dose of nicotine resulted in 1128 ng/ml cotinine in saliva. Mecamylamine antagonized the discriminative stimulus effects of nicotine and varenicline,

whereas DH β E was much less effective. Nicotine and varenicline had synergistic discriminative stimulus effects. In monkeys responding predominantly on the vehicle lever after a test compound (bupropion, cocaine, and midazolam), that test compound blocked the nicotine-discriminative stimulus, perhaps reflecting a perceptual-masking phenomenon. These results show that nicotine, varenicline, and cytisine produce discriminative stimulus effects through mecamylamine-sensitive receptors (i.e., nicotinic acetylcholine) in primates, whereas the involvement of DH β E-sensitive receptors (i.e., $\alpha 4\beta 2$) is unclear. The current nicotine-discrimination assay did not detect a difference in agonist efficacy between nicotine, varenicline, and cytisine, but did show evidence of involvement of dopamine. The control that nicotine has over choice behavior can be disrupted by non-nicotinic compounds, suggesting that non-nicotinics could be exploited to decrease the control that tobacco has over behavior.

Introduction

Cigarette smoking is a leading cause of respiratory disease, cardiovascular disease, cancer, and premature death. Various chemicals inhaled in cigarette smoke are responsible for the deleterious effects on health, whereas nicotine is the chemical in tobacco that drives cigarette smoking and other tobacco use. Nicotine binds to nicotinic acetylcholine receptors (Dale, 1914) located on ion channels permeable to sodium, potassium, and calcium; five protein subunits are differentially assembled from 12 known types (nine α and three β subunits) to yield various nicotinic acetylcholine receptor subtypes in brain (Gotti et al., 2006). Nicotinic acetylcholine receptors are widely distributed in the brain, are located predominantly on presynaptic nerve terminals, and regulate neurotransmitter release. Receptors associated with behavioral effects include homomeric $\alpha 7$ receptors that mediate the

effects of nicotine on cognition (Wallace and Porter, 2011) and heteromeric $\alpha 4\beta 2$ receptors that mediate nicotine abuse and dependence liability (Gotti et al., 2010). Establishing the contribution of various nicotinic acetylcholine receptor subtypes to behavioral effects will facilitate the development of novel therapeutics for tobacco dependence and other indications (cognitive deficits).

Nicotine replacement (transdermal patch, chewing gum, or inhaled spray) is the most common pharmacotherapy for tobacco dependence. As the name implies, nicotine replacement substitutes for and decreases the urge to use tobacco. Smoking cessation drugs also include orally administered nicotinic acetylcholine receptor agonists such as varenicline (Chantix, Pfizer, New York, NY) and cytisine (Tabex, Sopharma, Sofia, Bulgaria). Varenicline and cytisine were reported to have lower agonist efficacy than nicotine as evidenced by electrophysiological responses in vitro (Coe et al., 2005; Rollema et al., 2010). According to receptor theory, when the maximum effect of a low-efficacy agonist is less than that of a high-efficacy agonist and a common receptor type mediates the effects of both, the low-efficacy agonist antagonizes the effect of the high-efficacy agonist to the level

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ABBREVIATIONS: DH β E, dihydro- β -erythroidine; FR5, fixed ratio 5.

of effect produced by the low-efficacy agonist alone. Although antagonism of nicotine by varenicline *in vivo* has been proposed, the evidence for this is not unanimous. Bupropion is an antidepressant as well as a smoking cessation aid (Zyban, GlaxoSmithKline, Uxbridge, Middlesex, UK), and the mechanism responsible for the latter might involve both indirect-acting catecholamine agonism and nicotinic acetylcholine receptor antagonism (Slemmer et al., 2000).

Drug discrimination has played a prominent role in establishing the *in vivo* pharmacology of nicotinic acetylcholine receptor ligands in monkeys (Takada et al., 1988) and especially rats. In rats trained to discriminate nicotine from saline, both varenicline and cytisine shared discriminative stimulus effects with nicotine (Smith and Stolerman, 2009 for review). In one study (LeSage et al., 2009), the maximum effect of varenicline and cytisine was less than nicotine, and both attenuated the discriminative stimulus effects of nicotine. Bupropion substituted for the discriminative stimulus effects of nicotine in rats (Wiley et al., 2002; Wilkinson et al., 2010). However, bupropion did not substitute for the discriminative stimulus effects of nicotine in one study, nor did it attenuate the effects of nicotine in that study (Shoaib et al., 2003). Collectively, these studies suggest that effective smoking cessation therapies to some extent mimic the effects of nicotine.

The current study examined receptor mechanisms underlying the discriminative stimulus effects of nicotine in rhesus monkeys. This was accomplished by testing varenicline and cytisine as well as nicotinic antagonists alone and, for all but cytisine, in combination with nicotine. Antagonists included bupropion, the prototypic noncompetitive antagonist mecamylamine (Varanda et al., 1985), and the competitive $\alpha 4\beta 2$ nicotinic acetylcholine receptor-selective antagonist DH β E (Williams and Robinson, 1984). Isobolographic analysis was used to examine whether the combined effects of nicotine and varenicline were additive or not (greater than additive or synergistic; Tallarida, 2000). Non-nicotinic compounds (cocaine and midazolam) were tested alone and in combination with nicotine to examine the extent to which attenuation of discriminative stimulus effects was selective for nicotinic ligands. Cotinine, a primary metabolite of nicotine, was measured as a basis for comparison to cigarette smoking in humans.

Materials and Methods

Subjects. Three male and two female rhesus monkeys (*Macaca mulatta*) were experimentally and pharmacologically naive before the experiments described here. The monkeys were housed individually in stainless-steel cages on a 14-h light/10-h dark schedule (lights on at 6:00 AM). They were maintained at 95% free-feeding weight (range 6–10.5 kg) with a diet consisting of primate chow (High Protein Monkey Diet; Harlan Teklad, Madison, WI), fresh fruit, and peanuts; water was continuously available in the home cage. Monkeys were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Texas Health Science Center and the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, 2011).

Apparatus. Monkeys were seated in chairs (model R001; Primate Products, Miami, FL) and placed in ventilated, sound-attenuating chambers equipped with two levers and two lights, one positioned above each lever. Their feet were placed in shoes containing brass

electrodes to which a brief electric stimulus (3 mA, 250 ms) could be delivered from an a.c. generator (Coulbourn Instruments, Allentown, PA). Banana-flavored food pellets (300 mg; BioServ, Frenchtown, NJ) could be delivered from a dispenser (ENV-203-300; MED Associates, St. Albans, VT). An interface (MED Associates) connected the chambers to a computer, which controlled and recorded lever responses with Med-PC software (MED Associates).

Discrimination Training. Experimental sessions were conducted once daily, 7 days a week. Monkeys were trained initially to respond under a schedule of continuous reinforcement for food delivery on both levers, i.e., a single response on a lever resulted in delivery of a food pellet. The response requirement on a lever was systematically increased to a fixed ratio 5 (FR5). Thereafter, responding was maintained under an FR5 schedule of stimulus-shock termination. Illumination of the lights signaled that an electric stimulus was scheduled for delivery in 10 s; however, five consecutive responses on a lever extinguished the lights, prevented delivery of the electric stimulus, and postponed the schedule for 30 s. The 20-min experimental session was divided by an initial 10-min timeout; responses had no programmed consequence during the timeout. The timeout was followed by 10 min of responding under the FR5 schedule of stimulus-shock termination. The schedule of stimulus-shock termination ended after 10 min or the delivery of four electric stimuli, whichever occurred first.

Discrimination training was initially conducted in two monkeys at a dose of 0.1 mg/kg s.c. of nicotine base. The dose was increased in one-quarter log unit increments to 1 mg/kg. Training at 1 mg/kg did not result in acquisition, and the dose was increased to 1.78 mg/kg, at which point there was reliable discrimination of nicotine (see *Results* for additional details). For the other three monkeys, 1.78 mg/kg was used from the outset of discrimination training. For all five monkeys, the correct lever was determined by saline or nicotine administered subcutaneously in the first minute of the session. The nicotine- and saline-associated levers were right and left, respectively, for three monkeys; the assignment was reversed for the other two monkeys. Lever assignments remained the same for an individual throughout the study. A response on the incorrect lever reset the response requirement on the correct lever. Before the test criteria were satisfied, the pattern of training predominantly alternated between 2 consecutive nicotine-training days followed by 2 consecutive saline-training days; every 2 weeks on average, however, the training sequence alternated daily (saline, nicotine, and saline) for 3 to 4 days nonsystematically.

The first test was conducted when for five consecutive or six of seven training sessions at least 80% of the total responses occurred on the correct lever and fewer than five responses occurred on the incorrect lever before completion of the first fixed ratio on the correct lever. After the test criteria were satisfied, sessions were divided into consecutive 20-min cycles, each consisting of a 10-min timeout followed by a 10-min schedule of stimulus-shock termination. Saline training was conducted by administering saline at the beginning of the first cycle followed by saline or sham (dull pressure applied to the midscapular region of the back) at the beginning of one to five additional cycles for a total of six cycles. Nicotine training was conducted by administering nicotine (1.78 mg/kg base) at the beginning of a cycle preceded by zero to five saline-training cycles.

Discrimination Testing. After the first test, further tests were conducted when performance for consecutive training sessions, including every cycle for both saline and nicotine training sessions, satisfied the test criteria. Test sessions consisted of one to six cycles and otherwise were identical to training sessions except that five consecutive responses on either lever postponed the schedule of stimulus-shock termination and animals received saline or a dose of test compound during a cycle.

To examine the effects of nicotine, varenicline, and cytisine over time, a dose was administered in the first cycle of a six-cycle test; saline or sham were administered nonsystematically in subsequent cycles. If the duration of effect was longer than 2 h (the maximum

duration of a six-cycle test), then a second test was conducted by administering a dose 100 min before a separate six-cycle test. To determine the onset of action of nicotine, the training dose (1.78 mg/kg base) was administered at various times during the timeout including 1, 3, and 5 min before the period of stimulus-shock termination. Bupropion, cocaine, mecamylamine, and DH β E were studied by administering a dose at the beginning of the first cycle followed by a dose of nicotine in a second cycle. Mecamylamine and DH β E were administered in the first cycle followed by varenicline in the second cycle. Midazolam, which has a relatively short duration of action, was administered alone or in combination with a dose of nicotine in a single test cycle. Nicotine and varenicline were combined in a constant, one-to-one proportion of their respective ED₅₀ values (Tallarida, 2000). They were administered in separate injections (one immediately after the other) at doses from one-eighth their respective ED₅₀ values (one-eighth the ED₅₀ value of nicotine in combination with one-eighth the ED₅₀ value of varenicline), with the dose combination increasing in one-quarter log unit increments up to 1½ the respective ED₅₀ values. Each dose combination was studied in separate tests comprising a single cycle. Otherwise, dose-response functions included ineffective doses up to doses that produced responding on the nicotine lever, decreased response rate to less than 20% of control, or attenuated nicotine-lever responding produced by another drug. The exception was DH β E, which was studied in all monkeys up to 3.2 mg/kg. A larger dose (5.6 mg/kg) of DH β E resulted in the death of one monkey, precluding further studies with this and larger doses.

Collection and Analysis of Cotinine. Saliva was collected by inserting a sterile, 6-inch, cotton-tipped applicator into the mouth while monkeys were seated in chairs. The applicator was inserted between the gums and lips toward the bottom of each side of the mouth. The cotton tip was separated from the applicator and placed into a 2-ml microcentrifuge tube containing a filter (Grace Davison Discovery Science, Deerfield, IL). The tube was placed into a centrifuge for 5 min at 10,000g. Cotinine was assessed with the High Sensitivity Salivary Cotinine Quantitative Enzyme Immunoassay kit (Salimetrics LLC, State College, PA) by using a 96-well plate reader with UV detection (Spectramax 384; Molecular Devices; Sunnyvale, CA). The cross-reactivity of the assay indicated that the antibody supplied with the kit recognized 100% cotinine, 55% 3-hydroxycotinine, and 1.4% nicotine. The samples were run in duplicate. Analytical results were adjusted to account for dilution of the samples. The lower limit of quantification was 1 ng/ml, intra-assay and interassay precision were less than or equal to 5%, and the final results were reported in nanograms per milliliters.

Drugs. Drugs were administered subcutaneously in the mid-scapular region of the back in volumes of 0.03 to 0.3 ml/kg; doses (milligrams per kilograms) were expressed as the weight of the forms listed below, with the exception of nicotine, which was expressed as the weight of the base. Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO), bupropion hydrochloride, cocaine hydrochloride, mecamylamine hydrochloride, and varenicline dihydrochloride (Research Triangle Institute, Research Triangle Park, NC), cytosine (Atomole Scientific, Hubei, China), and dihydro- β -erythroidine hydrobromide (Tocris Bioscience, Ellisville, MO) were dissolved in physiological saline. Midazolam hydrochloride (5 mg/ml in physiologic saline; Bedford Laboratories, Bedford, OH) was diluted in physiologic saline as needed.

Data Analyses. Discrimination and response rate data were expressed as the mean of individual values from three to five rhesus monkeys. Discrimination data were calculated as a percentage of responses on the nicotine lever out of total responses on the saline and nicotine levers. Response rate data were calculated as the number of responses per second excluding responses during timeouts. Discrimination data for an individual subject were not included for analyses when response rate was less than 20% of the control for that subject; the control was defined as the mean rate of five previous saline training sessions in which the test criteria were satisfied.

However, response rate data were always included in the group average.

The potency of a drug to produce nicotine-lever responding, either alone or in combination with another drug, was calculated by simultaneously fitting straight lines to individual dose-effect data by using Prism version 5.0 for Windows (GraphPad Software, Inc., San Diego, CA) with linear regression. Straight lines were fitted to the linear portion of dose-effect curves, defined by doses producing 20 to 80% nicotine-lever responding, including not more than one dose producing less than 20% nicotine-lever responding and not more than one dose producing more than 80% nicotine-lever responding. Other doses were excluded from the analyses. The slopes of dose-effect curves were compared with an *F*-ratio test using Prism. If the slopes were not significantly different, then a common, best-fitting slope was used for further analyses (Kenakin, 1997). Doses corresponding to the 50% level of the effect (ED₅₀), potency ratios, and their 95% confidence limits were calculated by parallel line analyses of data from individual subjects (Tallarida, 2000). Potencies were considered significantly different when the 95% confidence limits of the potency ratio did not include 1. Linear regression was used to examine the slope of the function between dose and response rate; a significant difference from 0 provided evidence for a significant effect of drug on response rate (*p* < 0.05).

To examine the combined effects of nicotine and varenicline, the experimentally derived individual dose-response curves for nicotine and varenicline were used to construct a theoretical additive dose-response curve (Tallarida, 2000). The slope and intercept of the theoretical additive function was compared with the slope and intercept of the experimentally derived function for the combination (¼ ED₅₀ of nicotine + ¼ ED₅₀ of varenicline, ½ ED₅₀ of nicotine + ½ ED₅₀ of varenicline, etc.) by using Prism. If a single line can be fitted to the experimental and theoretical functions (there is no significant difference in slope or intercept), then this is consistent with the additive effects of nicotine and varenicline. However, a significant difference in slope or intercept indicates that a single line is not sufficient to describe the two functions. For example, if the experimentally derived function is positioned to the left of the additive function, then the drugs have synergistic effects.

Results

Acquisition of the Nicotine Discrimination and Nicotine Dose Response. The initial training dose in two monkeys was 0.1 mg/kg nicotine base; after 15 sessions (including both nicotine and saline training sessions), the training dose was increased ¼ log unit; the training dose was increased ¼ log unit every 15 sessions such that the training dose was 1 mg/kg after 60 sessions. One monkey satisfied the criteria for testing after 33 training sessions at 1 mg/kg; however, the second monkey had not satisfied the criteria for testing after 270 training sessions (including both nicotine and saline training sessions) at 1 mg/kg. After increasing the dose to 1.78 mg/kg, the test criteria were satisfied after 12 sessions in the monkey that had not satisfied the criteria at 1 mg/kg and after five sessions (i.e., the minimum number of sessions required) in the monkey that had. Three other monkeys were trained from the onset with 1.78 mg/kg base weight of nicotine, and the test criteria were satisfied after 109, 169, and 222 sessions (including both nicotine and saline training sessions) in each monkey.

Nicotine dose-dependently increased the percentage of responses on the nicotine lever, whereas saline produced no responses on the nicotine lever (Fig. 1A). The ED₅₀ value (95% confidence limits) of nicotine to produce discriminative stimulus effects was 0.47 (0.35–0.64) mg/kg. The discrimina-

Fig. 1. Discriminative stimulus effects of nicotine, varenicline, and cytisine in rhesus monkeys discriminating nicotine base (1.78 mg/kg). Abscissae, saline or dose in milligrams per kilograms of body weight administered subcutaneously. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever.

tive stimulus effects of nicotine (1.78 mg/kg) had a rapid onset; monkeys responded 93% on the nicotine lever after 1 min. Responding on the nicotine lever was 51% at 30 to 40 min and decreased thereafter (Fig. 2A). A larger dose (3.2 mg/kg) of nicotine slightly extended the duration of action of nicotine. Nicotine did not significantly modify response rate as a function of dose (Fig. 1B) or time (Fig. 2D).

Discriminative Stimulus Effects of Varenicline and Cytisine. Varenicline and cytisine dose-dependently increased responding on the nicotine lever, although the maximum effect of cytisine was somewhat less than that of varenicline (Fig. 1A). The ED_{50} values (95% confidence limits) of varenicline and cytisine to produce discriminative stimulus effects were 0.53 (0.31–0.91) and 39 (24–64), respectively. In general, the time course for varenicline and cytisine was similar to that of nicotine (Fig. 2, B and C, respectively). Like nicotine, increasing the dose of varenicline from 1 to 1.78 mg/kg extended the duration of action. The duration of action of cytisine also was dose-dependent. Neither varenicline nor cytisine significantly modified response rate as a function of dose (Fig. 1B) or time (Fig. 2, E and F, respectively).

Saliva Cotinine. Nicotine dose-dependently increased saliva cotinine concentrations (Fig. 3). Two doses of nicotine produced peak cotinine at 120 min (339 and 1128 ng/ml for 1 and 1.78 mg/kg, respectively). At 480 min, cotinine was still detected in amounts larger than half the peak observed at 120 min, indicating that the half-life of salivary cotinine was longer than 480 min. The area under the curve 480-min values for time versus cotinine concentration (bioavailability) were 112,836 ng $\cdot$ min/ml for 1 mg/kg nicotine and 385,135 ng $\cdot$ min/ml for 1.78 mg/kg nicotine.

The Combined Effects of Mecamylamine or DH β E with Nicotinic Acetylcholine Receptor Agonists.

Mecamylamine (0.32 and 1 mg/kg) produced a maximum of 2% responding on the nicotine lever (Fig. 4A). The slopes of the dose-response curves for nicotine alone and in combination with mecamylamine (0.32 and 1 mg/kg) were not significantly different from each other. A relatively small dose (0.32 mg/kg) of mecamylamine produced a small, but not statistically significant, shift in the nicotine dose-effect curve, whereas a larger dose (1 mg/kg) produced a significant rightward shift in the nicotine dose-response curve (Fig. 4A). The potency ratios (95% confidence limits) for the nicotine dose-response curves in the presence of 0.32 and 1 mg/kg mecamylamine relative to control were 1.8 (0.7–6.8) and 3.2 (1.3–8.0) mg/kg, respectively. When combined with varenicline, mecamylamine (0.32 mg/kg) produced a 2.6-fold rightward shift (95% confidence limits were 1.8–3.7) in the varenicline dose-response curve (Fig. 4B). The slope of the varenicline dose-response function determined in the presence of the larger dose (1 mg/kg) of mecamylamine was significantly different from control (varenicline alone), and the potency ratio was not calculated. When cytisine (56 mg/kg) was combined with mecamylamine (1 mg/kg), control nicotine-lever responding (77%) was decreased to 4% ($\pm$ 1 S.E.M.). The rate of responding was not significantly altered by mecamylamine alone or in combination with nicotine (Fig. 4C), varenicline (Fig. 4D), or cytisine (data not shown).

DH β E (3.2 mg/kg) produced 25% responding on the nicotine lever (Fig. 5A); when combined with nicotine, there was a trend toward a rightward shift in the nicotine dose-response curve (Fig. 5A). However, the 95% confidence limit (1.0–4.1) of the potency ratio (1.9) included 1 and was therefore not statistically significant. Moreover, DH β E (3.2 mg/kg) did not significantly modify the dose-response curve for varenicline to produce discriminative stimulus effects (Fig. 5B); the potency ratio (95% confidence limit) was 1.3 (0.4–3.6). The combination of DH β E with either nicotine (0.32–1.78 mg/kg base) or varenicline (0.32–3.2 mg/kg) did not modify the rate of responding (Fig. 5, C and D, respectively). A larger dose (5.6 mg/kg) of DH β E in combination with nicotine (1.78 mg/kg) resulted in death in one monkey; studies with this and larger doses of DH β E were discontinued.

Discriminative Stimulus Effects of Nicotine in Combination with Varenicline. The combination of nicotine and varenicline in a constant proportion of their ED_{50} values ($\frac{1}{4} ED_{50} + \frac{1}{4} ED_{50}$ and $\frac{1}{2} ED_{50} + \frac{1}{2} ED_{50}$) yielded a function that was significantly different from the line of additivity ($F_{2,10} = 6.28$; $p < 0.05$). The slopes and intercepts of the two lines were significantly different, and the empirically derived function is left of the calculated line of additivity, primarily because of the effects of relatively small doses, consistent with a synergistic interaction (Fig. 6).

Non-Nicotinic Compounds Alone and in Combination with Nicotine. Midazolam produced a maximum of 4% responding on the nicotine lever and significantly decreased response rate ($F_{1,13} = 12.83$; $p < 0.01$) to 0.57 responses per s at a dose of 1 mg/kg (Fig. 7). Cocaine and bupropion produced a greater percentage of responses on the nicotine lever compared with midazolam. In two monkeys, cocaine at doses more than 0.32 mg/kg produced a minimum of 99% responses

Fig. 2. Time course of discriminative stimulus effects after nicotine (A and D), varenicline (B and E), and cytisine (C and F). Abscissae, intervals of responding in minutes. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (A–C) and mean ($\pm$ S.E.M.) rate of responding expressed as responses per second (D–F).

Fig. 3. Time course of salivary cotinine after nicotine base (1 and 1.78 mg/kg). Abscissae, time in minutes. Ordinates, mean ($\pm$ S.E.M.) amount of cotinine expressed in nanograms per milliliters of saliva.

on the nicotine lever. In the other three monkeys, all doses of cocaine produced no more than 21% of responses on the nicotine lever. When expressed as an average, the maximum increase in nicotine-lever responding after cocaine (0.32 mg/kg) was 44% (Fig. 7A). One of the monkeys that responded

100% on the nicotine lever after cocaine also responded 100% on the nicotine lever after bupropion (3.2 and 10 mg/kg). The other four monkeys responded no more than 17% on the nicotine lever at all doses of bupropion, and the maximum group average was 23% at a dose of 10 mg/kg bupropion (Fig. 7A). Cocaine and bupropion did not significantly modify response rate up to the largest doses studied (Fig. 7B).

Bupropion (10 mg/kg) and cocaine (1 mg/kg) differentially modified the discriminative stimulus effects of nicotine depending on whether the test compounds alone produced relatively high or low levels of nicotine-lever responding. For monkeys responding 99 to 100% on the nicotine lever after bupropion ($n = 1$) or cocaine ($n = 2$), doses of nicotine less than 1 mg/kg also produced 100% nicotine-lever responding when combined with the test compounds. In monkeys responding predominantly on the vehicle lever after bupropion ($n = 4$) or cocaine ($n = 3$), however, the test drugs attenuated the nicotine discriminative stimulus (Fig. 8, A and B, respectively). Bupropion (10 mg/kg) produced a rightward and downward shift in the nicotine dose-response curve, as evidenced by a difference in slope relative to the nicotine control ($F_{1,25} = 9.03$; $p < 0.01$) and a maximum response of 25% up to 5.6 mg/kg nicotine. The magnitude of rightward shift in the nicotine

Fig. 4. Discriminative stimulus effects of nicotine and varenicline in combination with mecamylamine. Abscissae, saline or dose of nicotine base (A and C) or varenicline (B and D) in milligrams per kilograms of body weight administered subcutaneously. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (A and B) and mean ($\pm$ S.E.M.) rate of responding expressed as responses per second (C and D).

Fig. 5. Discriminative stimulus effects of nicotine and varenicline in combination with DHβE. Abscissae, saline or dose of nicotine base (A and C) or varenicline (B and D) in milligrams per kilograms of body weight administered subcutaneously. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (A and B) and mean ($\pm$ S.E.M.) rate of responding expressed as responses per second (C and D).

dose-response curve (increase in the nicotine ED₅₀ value) produced by cocaine (1 mg/kg) was 5.5-fold. Midazolam (0.1 mg/kg) also significantly attenuated the discriminative stimulus effects of nicotine 6.1-fold (Fig. 8C). Bupropion and cocaine, when combined with nicotine up to a dose of 5.6 mg/kg, did not significantly modify response rate (Fig. 8, D and E), whereas monkeys did not respond when 5.6

mg/kg nicotine was combined with 0.1 mg/kg midazolam (Fig. 8F).

Discussion

In rhesus monkeys discriminating nicotine (1.78 mg/kg base) from saline, both varenicline and cytosine substituted

Fig. 6. Discriminative stimulus effects of nicotine in combination with varenicline. Abscissae, dose of nicotine base or varenicline in milligrams per kilograms of body weight administered subcutaneously. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever.

Fig. 7. Discriminative stimulus effects of midazolam, cocaine, and bupropion in rhesus monkeys discriminating nicotine base (1.78 mg/kg). Abscissae, saline or dose of midazolam, cocaine, or bupropion in milligrams per kilograms of body weight administered subcutaneously. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (A) and mean ($\pm$ S.E.M.) rate of responding expressed as responses per second (B).

for the nicotine-discriminative stimulus. The effects of nicotine, varenicline, and cytisine were mediated by nicotinic acetylcholine receptors inasmuch as mecamylamine antagonized the discriminative stimulus effects of all three. DH β E was an ineffective nicotinic acetylcholine receptor antagonist. The combined effects of nicotine and varenicline were synergistic. Bupropion and cocaine substituted for the nicotine-discriminative stimulus in a subset of monkeys, but not in a majority, and bupropion and cocaine attenuated the nicotine-discriminative stimulus in the latter group of monkeys. Midazolam did not substitute for nicotine and attenuated the nicotine-discriminative stimulus.

Nicotine was among the first drug discriminations to be trained under operant conditioning procedures (Morrison and Stephenson, 1969) and has been used extensively since. Relatively novel parameters of the current study include species (rhesus monkey) and the type of reinforcer (stimulus-shock

termination) maintaining operant behavior. The current results, including substitution of varenicline and cytisine for the nicotine-discriminative stimulus and antagonism by mecamylamine, are consistent with previous findings with rats responding for food (Smith and Stolerman, 2009 for review). Training dose is one factor that can determine the pharmacologic profile of nicotine discriminations (Jutkiewicz et al., 2011); however, only one training dose (1.78 mg/kg base) was used here. This training dose seems large, as evidenced not only by emesis in a subset of monkeys, but also by cotinine levels comparable with those measured in daily, heavy cigarette smokers.

Antagonism of the discriminative stimulus effects of nicotine, varenicline, and cytisine by the nonselective antagonist mecamylamine demonstrates a nicotinic acetylcholine receptor mechanism. In contrast, the α 4 β 2 nicotinic acetylcholine receptor-selective antagonist DH β E did not significantly antagonize the discriminative stimulus effects of nicotine or varenicline. In addition to differences in selectivity for receptor subtypes, DH β E is a competitive antagonist, whereas mecamylamine is a noncompetitive antagonist (Williams and Robinson, 1984; Varanda et al., 1985). DH β E has been shown to antagonize the discriminative stimulus effects of nicotine in previous studies (Stolerman et al., 1997; Gommans et al., 2000; Shoaib et al., 2000; Struthers et al., 2009). However, antagonism of nicotine by DH β E is not unanimously reported when the training dose of nicotine is relatively large (Stolerman et al., 1997; Jutkiewicz et al., 2011). Nicotine has the highest binding affinity for α 4 β 2 receptors (Coe et al., 2005). Other subtypes of nicotinic acetylcholine receptor aside from α 4 β 2 are expected to mediate the effects of large doses of nicotine, thereby decreasing the role played by α 4 β 2 receptors and, in turn, the effectiveness of DH β E as a nicotine antagonist. Alternatively, given that DH β E was studied up to a lethal dose, it could be that DH β E has adverse effects in monkeys that preclude antagonism of the behavioral effects of nicotine.

The maximum effect of varenicline and cytisine can be lower than nicotine in vitro (Coe et al., 2005) and in vivo (Rollema et al., 2007, 2010; LeSage et al., 2009), which is attributed to differences in agonist efficacy at nicotinic acetylcholine receptors. The maximum effect of varenicline and cytisine in the current study was the same as that of nicotine, suggesting that each of the agonists has sufficiently high-agonist efficacy in rhesus monkeys. In a previous study, nicotine and varenicline were reported to be similarly effective at increasing the potency of cocaine to produce discriminative stimulus effects in rhesus monkeys (Gould et al., 2011). To the extent that the current training dose of nicotine is large, then any difference in efficacy seems to be associated

Fig. 8. Discriminative stimulus effects of nicotine alone and in combination with bupropion, cocaine, or midazolam. Saline or dose of nicotine base in milligrams per kilograms of body weight administered subcutaneously. Ordinates, mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (A–C) and mean ($\pm$ S.E.M.) rate of responding expressed as responses per second (D–F).

with a mechanism that is not important for discriminative stimulus effects. When two drugs produce the same maximum effect through the same pharmacologic (receptor) mechanism, the combined effects of the two drugs are expected to be additive. Here, the combined discriminative stimulus effects of nicotine and varenicline were synergistic, primarily at relatively small dose combinations. The underlying mechanism could be pharmacokinetic, e.g., a decrease in metabolism of one drug by the other. However, if the mechanism underlying the synergism is not pharmacokinetic, then nicotine and varenicline might have similar, although not identical, receptor mechanisms. The extent to which any synergistic effects with nicotine influence the effectiveness of varenicline as a smoking cessation aid remains to be established.

Cytisine was less potent than nicotine or varenicline and produced a maximum of 77% responding on the nicotine lever; however, unlike varenicline, the nicotine-like effects of cytisine were accompanied by other observable signs such as pale skin, increased vocalization, and impaired motor control. In rats discriminating nicotine, cytisine is approximately 10-fold less potent than nicotine (Chandler and Stolerman 1997). Here, cytisine was 83-fold less potent than nicotine. The lower maximum effects of cytisine, along with cytisine being much less potent than nicotine or varenicline in rhesus monkeys despite having similar binding affinity for nicotinic acetylcholine receptor subtypes (e.g., $\alpha 4\beta 2$ nicotinic acetylcholine receptors; Rollema et al., 2010), are consistent with limited penetration of cytisine through the blood-brain

barrier (Romano et al., 1981). Subcutaneous nicotine, varenicline, and cytisine had a relatively short duration of action. The short duration is not ideal for therapeutics, although it is overcome by altering the route and method of delivery (continuous transdermal administration of nicotine or the nicotine patch).

Bupropion and cocaine fully substituted for nicotine in a subset of monkeys, suggesting that dopamine agonism is a component of the mechanism underlying the discriminative stimulus effects of nicotine. The dopaminergic mechanism is consequent to nicotinic acetylcholine receptor stimulation inasmuch as nicotine does not bind to dopamine transporters or receptors. In a majority of monkeys, cocaine and bupropion did not substitute for and antagonized the discriminative stimulus effects of nicotine. Bupropion, in addition to being an indirect-acting dopamine agonist, is a nicotinic acetylcholine receptor antagonist (Slemmer et al., 2000), and the latter could be responsible for nicotine antagonism. However, non-nicotinic drugs (cocaine and midazolam) attenuated the nicotine-discriminative stimulus, so it seems that nicotinic acetylcholine receptor antagonism was not necessarily involved in the effects of bupropion. Alternatively, the underlying mechanism could be perceptual masking of the training stimulus (nicotine) by another drug stimulus (bupropion, cocaine, and midazolam) that is qualitatively different from the training stimulus (Gauvin and Young, 1989). Cocaine and midazolam have well established discriminative stimulus effects in animals including rhesus monkeys (de la Garza and Johanson, 1982; Lelas et al., 1999). The current study is not the

first to provide evidence for perceptual masking of a nicotine-discriminative stimulus by midazolam (Mariathasan and Stolerman, 1993), although it does expand the list to include indirect-acting dopamine agonists.

Saliva was collected by a novel technique, and saliva cotinine levels were measured to compare nicotine doses in the monkeys with those measured in heavy smokers. The levels of saliva cotinine, which are considered to be a good indicator of smoking in humans, measured at two doses of nicotine suggest that the monkeys received doses of nicotine similar to those produced by heavy cigarette smoking. In monkeys and humans, nicotine is converted to cotinine and subsequently 3-hydroxy cotinine, the principal metabolites of nicotine, by the action of the enzyme CYP2A6 in the liver (Nakajima et al., 1996; Schoedel et al., 2003; Dempsey et al., 2004). The peak saliva cotinine levels achieved in the monkeys in this study at doses of 1 and 1.78 mg/kg (339 and 1128 ng/ml, respectively) were slightly higher than saliva cotinine levels observed in humans who smoked between 5 and 25 cigarettes every 24 h and whose saliva cotinine levels ranged from 50 to 600 ng/ml (Swan et al., 1993). Plasma cotinine levels for humans who smoked approximately 20 cigarettes per day were also similar (Malaiyandi et al., 2006), because saliva and plasma cotinine levels are essentially identical (Jarvis et al., 2003). The procedure used to collect saliva samples from monkeys is noninvasive (does not require blood draws). This innovative procedure allows saliva samples to be collected as frequently as desired without a concern about the amount of sample to be collected as is the case with blood draws.

In summary, varenicline and cytosine shared discriminative stimulus effects with nicotine in rhesus monkeys, and these effects were antagonized by mecamlamine but not DH β E. These results question the extent to which α 4 β 2 receptors are involved in the discriminative stimulus effects of nicotine or suggest that DH β E is an ineffective nicotine antagonist in rhesus monkeys. The combined effects of nicotine and varenicline were synergistic. Bupropion and cocaine substituted for or attenuated the nicotine-discriminative stimulus depending on the monkey, whereas midazolam attenuated the nicotine-discriminative stimulus. Many different non-nicotinic compounds are either in use as smoking cessation aids or are being considered for that indication (Buchhalter et al., 2008; Polosa and Benowitz, 2011), and many of these have discriminative stimulus effects on their own. To the extent that perceptual masking of nicotine is involved in the effectiveness of smoking cessation aids, then a variety of drugs with discriminative stimulus effects could be exploited as treatments for tobacco dependence.

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Authorship Contributions

Participated in research design: Cunningham, Javors, and McMahon.

Conducted experiments: Cunningham and Javors.

Performed data analysis: Cunningham, Javors, and McMahon.

Wrote or contributed to the writing of the manuscript: Cunningham, Javors, and McMahon.

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