Pharmacological Comparison of Transient and Persistent \[^{3}H\]Dopamine Release from Mouse Striatal Synaptosomes and Response to Chronic \(\text{L-Nicotine Treatment}^{1}\)

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ABSTRACT

\(\text{L-Nicotine stimulates a biphasic release of} \ [^{3}H] \text{dopamine from mouse striatal synaptosomes which does not persist after agonist is removed. Approximately 80\% of the initial release is transient and disappears with a half-time of less than 1 min; the other 20\% persists for several minutes (t_{1/2}, 5–10 min). Both the transient and persistent phases were investigated by 10-min exposures to agonists with an in vitro perfusion technique. A series of nicotinic agonists and antagonists were used to determine the pharmacological relationship of the two phases. Parameters measured included EC}_{50} \text{ and V}_{max} \text{ values and de-sensitization rates for both phases for agonists, } K_i \text{ values for antagonists and } K_i \text{ values for low concentrations of agonists. The results are consistent with both phases being mediated by a single type of receptor. In addition, the effects of chronic nicotine treatment on transient and persistent } [^{3}H] \text{DA release were measured. For both phases, release was decreased approximately 15\% by chronic infusion of 4.0 mg/kg/hr L-nicotine. Correlation of the results with inactivation of a portion of the receptors rather than a reversible desensitization is discussed.}

Nicotine-evoked \([^{3}H]DA \text{ release from striatal synaptosomes is mediated by a receptor exhibiting nicotinic pharmacology (Rapier et al., 1988, 1990; Grady et al., 1992, 1994; Rowell and Hillebrand, 1994; El-Bizri and Clarke, 1994; Lippiello et al., 1995) and is largely dependent upon calcium influx through voltage-gated calcium channels (Solliakov et al., 1995; Turner et al., 1993). NIC-stimulated } [^{3}H] \text{DA release from rodent striatal synaptosomes is biphasic with one component being a transient response with micromolar affinity for L-NIC and substantial initial release, and the other, a persistent response of low nanomolar affinity for L-NIC but low initial release (Grady et al., 1994). The persistent response does not require previous transient response; i.e., it is not a residual or secondary effect of high concentrations of L-NIC (Rowell, 1995; Grady et al., 1994). The persistent release is dependent on external calcium and is blocked by DH\(_{5}\)E, which indicates that it is a nicotinic response requiring calcium influx (Rowell, 1995). It is well established that nAChRs exist in multiple forms with somewhat different pharmacology depending on the subunit composition (McGehee and Role, 1995; Sargent, 1993). Clear evidence of different pharmacology for agonists and antagonists has been demonstrated for different combinations of subtypes expressed in Xenopus oocytes (Luetje and Patrick, 1991; Luetje et al., 1993; Cachelin and Jaggi, 1991; Gross et al., 1991; Cachelin and Rust, 1994; Gerzanich et al., 1995; McGeehe and Role, 1995). Electrophysiological measurements of agonist and antagonist potency for nAChRs in rat medial habenula, interpeduncular nucleus, superior cervical ganglion and hippocampus have shown that signals can be differentiated on the basis of pharmacology (Mulle and Changeux, 1990; Mulle et al., 1991; Coverton et al., 1994; Alkondon and Albuquerque, 1993, 1995). These potency variations are thought to reflect differences in subtype composition. Functional responses of nAChRs measured by ion flux and neurotransmitter release from brain slices, cell lines and synaptosomes also differ pharmacologically (Wonnacott et al., 1995; Lukas, 1993; Wong et al., 1995; Sacaan et al., 1995, 1996; Gopalakrishnan et al., 1996; Clarke and Reuben, 1996). For a subtype to form functional presynaptic receptors on dopaminergic terminals in striatum, the message should be detectable in substantia nigra. Message for alpha-3, alpha-4, alpha-5, beta-2 and beta-3 has been shown to exist in sub-

ABBREVIATIONS: ACh, acetylcholine; ATR, atropine; ATX, anatoxin-a; CARB, carbachol; CYT, cytisine; DA, dopamine; DEC, decamethonium; DFP, diisopropyl fluorophosphate; DH\(_{5}\)E, dihydro-\(\beta\)-erythroidine; EPI, (+)-epibatidine; HEPES, N-[2-hydroxyethyl]-piperazine-N’-[2-ethanesulfonate]; HEX, hexamethonium; MEC, mecamylamine; MeCARB, methylcarbachol; MLA, methyllycaconitine; nAChR, neuronal nicotinic acetylcholine receptor; nBTX, neuronal bungarotoxin; NIC, nicotine; dTC, \(\alpha\)-tubocurarine; TMA, tetramethylammonium.
stantia nigra of mice (Marks et al., 1992; Marks, M. J., unpublished results); therefore, it is possible that the transient and persistent phases of [3H]DA release from mouse striatal synaptosomes are the result of stimulation of two different subtypes of nAChR. In this case, the persistent and transient responses may express different pharmacologies. Alternatively, the two responses could be mediated by a single receptor which exists in two states. Such a two-state desensitizing model was originally proposed by Katz and Thesleff (1957) (see also Ochoa et al., 1989; Changeux, 1990), and subsequently this two-state model was shown to adequately explain the binding properties of L-[3H]NIC to rat brain membrane (Lippiello et al., 1987). Support for the single-receptor hypothesis comes from the slow onset of the persistent response (Rowell, 1995), and the ability of low concentrations of L-NIC to antagonize the transient response evoked by higher concentrations of the agonist (Grady et al., 1994; Lippiello et al., 1995). To determine whether it is likely that the two phases of [3H]DA release are the result of stimulation of two different receptor populations or kinetic manifestations of one receptor subtype, the pharmacology of the transient and persistent phases of agonist-induced [3H]DA release were compared.

Methods

Materials. The following compounds were products of Sigma Chemical Co., St. Louis, MO: acetylcholine iodide (ACh), cytosine (CYT), (+)-nicotine(+)di-p-toluoyltartrate (n-NIC), carbachol iodide (CARB), tetramethylammonium iodide (TMA), atropine sulfate (ATR), hexamethonium bromide (HEX), decamethonium bromide (DEC), d-tubocurarine chloride (dTC), sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, potassium dihydrogen phosphate, D(+)-glucose, ascorbic acid, pargyline and disopropyl fluorophosphate (DFP). Nicotine hydrogen (+) tartrate (l-NIC) was obtained from BDH Chemicals Ltd., Poole, England. Anatoxin-a hydrochloride (ATX), β-bungarotoxin (β-BTX), (+)-epibatidine-l-tartrate (EPI), methylcarbamylcholine chloride (MeCARB), dihydro-β-erythroidine hydrobromide (DHEB) and methyllycaconitine citrate (MLA) were products of Research Biochemicals International, Natick, MA. Sucrose and N,N,N,N′,N′-tetraethylenepiperazine-N′-2-ethanesulfonate hemisodium salt (HEPES) were obtained from Boehringer-Mannheim, Indianapolis, IN. Mecamylamine (MEC) was a gift from Merck Sharp and Dohme Research Laboratory, Rahway, N.J. Econo-Safe scintillation cocktail was purchased from Research Products International Corp., Mt. Prospect, IL. [7,8-3H]Dopamine (40–60 Ci/mmol) and [N-methyl-3H]nicotine (75 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL.

Animals. Mice of the C57BL/6J/hbg strain obtained from the breeding colony at the Institute for Behavioral Genetics (Boulder, CO), were maintained on a 12-hr light/12-hr dark cycle (lights on from 7 A.M. to 7 P.M.), and had free access to food and water. Females between the ages of 60 and 90 days were used. All animal procedures were in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the local animal care committee.

For chronic treatment experiments, mice were anesthetized by injection of pentobarbital (50 mg/kg) and chloral hydrate (100 mg/kg) and a cannula of silastic tubing was implanted in the right jugular vein by the method of Barr et al. (1979). Mice were transferred to individual treatment cages (15 × 15 × 30 cm) after 3 to 5 hr recovery time. The cannulas were connected to polyethylene tubing attached to glass syringes mounted on an Infusion Pump (Harvard Instruments, South Natick, MA), and continuous infusion with sterile saline was started. After 2 days of saline infusion half the mice were infused with 2 mg/kg/hr L-NIC for a day and then 4 mg/kg/hr L-NIC for 9 to 10 days. The rest of the mice were infused with saline for the whole treatment period. Infusion was discontinued 15 hr before synaptosome preparation. This time period allowed for total metabolism and elimination of nicotine (Petersen et al., 1984).

Synaptosome preparation. A crude P2 synaptosomal pellet was prepared by homogenization of the striatal tissue from one or two mice in 1 ml of 0.32 M sucrose buffered with 5 mM HEPES (pH 7.5) at 4°C with 16 strokes by hand in a glass-Teflon homogenizer. The homogenate was diluted to 3 ml with the HEPES-buffered sucrose and centrifuged at 3000 × g for 10 min. The supernatant was then centrifuged at 12,000 × g for 20 min. The resulting P2 pellet was resuspended in perfusion buffer (128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 7H2O, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbate, 0.01 mM pargyline).

L-[3H]Nicotine binding. A portion of the P2 synaptosomal preparation from one mouse included in the chronic treatment study was frozen (−20°C) for assay after completion of the study. Particulate fractions were prepared from P2 synaptosomal preparations combined from two animals. Assays were conducted as described previously (Marks et al., 1993a) with 20.8 ± 1.3 nM L-[3H]NIC purified by the method of Romm et al. (1990), with a 30 min incubation at 22°C. Nonspecific binding was determined in the presence of 10 μM L-NIC.

[3H]Dopamine uptake. Synaptosomes from one mouse suspended in 0.8 ml perfusion buffer were incubated at 37°C for 10 min. [3H]DA was added (4 μCi) for an approximate final concentration of 0.1 μM and the suspension was incubated for an additional 5 min. Aliquots (0.08–0.09 ml) were collected with mild suction onto 7-mm-diameter type A/E glass-fiber filters cut from larger sheets (Gelman Sciences, Ann Arbor, MI) and washed once with 0.5 ml perfusion buffer. These filters were then transferred to the perfusion apparatus. For experiments with ACh as agonist, the synaptosomes were treated with 100 μM DFP during the uptake procedure.

Perfusion and release. The perfusion apparatus has been described previously (Grady et al., 1992). For these experiments, all conducted at room temperature, synaptosomes on 7-mm filters were placed on top of 13-mm-type A/E glass-fiber filters (Gelman Sciences, Ann Arbor MI) and perfused with buffer at a rate of 0.6 ml/min for 10 min before fraction collection was started. Fractions were collected for 18 or 30 sec depending on the experiment. Released radioactivity was primarily [3H]DA (Rapier et al., 1988).

Data analysis. Each sample was plotted as cpm vs. fraction number or time. An example of such data is shown in figure 1. Basal release was calculated as a single exponential decay, f = aev−kt, with fractions collected before and after agonist exposure (first 5 and last 2 fractions of fig. 1A). Curves were fit using the nonlinear least squares algorithm in SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA). The calculated basal at time t was subtracted from total cpm at time t for each fraction and the resulting cpm released above baseline were normalized to base line at time t to give units of release per fraction (total cpm – baseline)/line]. For short agonist exposures (not illustrated here), units of release for fractions with cpm above baseline during the agonist exposure were summed for total release. For longer exposures of agonist, [3H]DA release is biphasic (Grady et al., 1994), so data as units per fraction were fit to a double-exponential equation:

\[f = v_T e^{-d_T t} + v_P e^{-d_P t}\]

with use of SigmaPlot 5.0 where f is measured release; vT and vP are maximum release for the first (transient) and second (persistent) phases, respectively; dT and dP are desensitization rates for the first and second phases; and t is the time of agonist exposure. Figure 1B illustrates this curve fit for four experiments where synaptosomes were exposed to 10 μM L-NIC for 10 min. For purposes of the course fit, the first fraction is taken as the peak of response, and the time of each fraction is taken as the midpoint of the interval of collection. For experiments in which data for several concentrations of an
agonist were collected under conditions of long (5–10 min) exposure, the following method was used to determine activation constants and maximum initial release for each process. The parameters \( v_T \) and \( v_P \) were calculated as described above for various concentrations of an agonist. These data were then fit by SigmaPlot 5.0 to the Hill equation:

\[
f = \frac{(V_m \cdot A^n)/(K_m^n + A^n)}{f_0}
\]

where \( f = v_T or v_P \), \( A = \) agonist concentration, \( K_m = \) agonist concentration at half-maximal release (referred to as \( K_T \) and \( K_P \) for transient and persistent release, respectively), \( V_m = \) maximal release for each (referred to as \( V_T \) and \( V_P \), respectively) and \( n = \) the Hill coefficient (referred to as \( n \) for transient release and \( n' \) for persistent release). All parameters are reported \( \pm \) S.E.M.

An alternative method was used which enabled simultaneous calculation of maximum initial release, activation constants and desensitization rates for both phases. Units released per fraction were calculated as described above. Means for several experiments for 12 to 15 time points were determined for six to nine concentrations of an agonist. These data were then fit by SigmaPlot 5.0 to the Hill equation with double-exponential decay where the Hill coefficients were set to 1:

\[
f = \frac{(A^* \cdot V_T)/(A^* + K_T^*)e^{-(A^* \cdot D_T)\frac{t}{V_T}}}{1 + ((A^* \cdot V_P)/(A^* + K_P^*)e^{-(A^* \cdot D_P)\frac{t}{V_P}}}\]

where \( A \) is the agonist concentration, \( V_T \) and \( V_P \) are the maximum release values for the two processes, \( K_T \) and \( K_P \) are the activation constants for the two processes, \( D_T \) and \( D_P \) are the desensitization rates for the two processes, \( n \) and \( n' \) are the Hill coefficients and \( t \) is time in minutes of agonist exposure at the midpoint of each fraction. The results of this calculation are illustrated in figure 2 with \( L\text{-NIC} \) as agonist.

To determine \( K_i \) values for nicotinic antagonists for the two phases of agonist-stimulated \(^3\text{H}\)DA release, data were collected by use of 1 \( \mu \text{M} \) \( L\text{-NIC} \) as agonist with an exposure time of 10 min. The concentration of antagonist was varied on different filters within each experiment and synaptosomes were exposed to antagonist for 12 min before and during the \( L\text{-NIC} \) exposure. Base lines were calculated, and units of release per fraction were determined as above. These data were fit to the double-exponential decay equation to determine maximum release for the two processes (\( v_T \) and \( v_P \)) in the presence of various concentrations of inhibitor. These parameters were compared with controls from the same experiments to determine % control response. To determine \( K_i \) values for the two processes, the % control data were fit by use of SigmaPlot 5.0 to the equations for competitive inhibition:

\[
\frac{f}{f_0} = \frac{(N \cdot V)/(K_a(1 + I/K_i)) + N}{N + 1}
\]

or noncompetitive inhibition:

\[
\frac{f}{f_0} = \frac{(N \cdot V)/(K_a(1 + I/K_i)) + N}{N + 1}
\]

where \( N = \) \( L\text{-NIC} \) concentration (1 \( \mu \text{M} \)), I = antagonist concentration \( V = V_{\text{max}} \) for \( L\text{-NIC} \) calculated from agonist parameters setting 1 \( \mu \text{M} \) \( L\text{-NIC} \) response to 100 (171 and 108.5 for transient and persistent phases, respectively), \( K_a \) = activation constant for \( L\text{-NIC} \) for each process \( (K_T = 0.71 \text{ and } K_P = 0.085, \text{ respectively}) \) and \( K_i \) = the inhibition constant for the antagonist. The parameters used for \( L\text{-NIC} \) were calculated by the simultaneous fit procedure (see fig. 2).
Results

A preliminary characterization of the effects of nicotinic antagonists and external calcium and cadmium on both transient and persistent phases was conducted by a long exposure to L-NIC. The results presented in figure 3, A and B, show that both transient and persistent responses are dependent on external calcium and are almost totally inhibited by 200 \( \mu \)M cadmium. Both responses are inhibited by the nicotinic antagonists DH\( \beta \)E (fig. 3C) and MEC (fig. 3D), and neither are inhibited by aBTX (fig. 3E) or the muscarinic antagonist, ATR (fig. 3F).

In experiments with synaptosomes as a model, depletion of neurotransmitter and of labeled tracer versus endogenous compound are always questions. To assess the extent of these possible problems, synaptosomes were exposed for 1 min to 10 \( \mu \)M L-NIC at various time intervals after uptake was complete. Figure 4 (inset graph) shows that, although \([^{3}H]DA\) is being released continually during the perfusion, the base line normalizing procedure adequately corrects for any decrease in specific activity over the course of 40 to 50 min, in the absence of prior agonist exposure. The possibility exists that the two phases of release are representative of two pools of DA-containing vesicles with differing specific activity. If two such pools exist, the pool with higher specific activity would be depleted by the transient release, leaving the pool with lower specific activity to be released as the persistent phase. This appears to be a change in the amount of DA released when actually it is only a change in specific activity. In this case, the transient release would not be recoverable without uptake of additional \([^{3}H]DA\). Figure 4 presents a series of experiments in which a 5-min exposure to 10 \( \mu \)M L-NIC was followed by a second 5-min exposure with varying intervals between the two exposures. The two phases both recover, although in 20 min the transient phase has recovered to 55%, whereas the persistent phase has recovered to 80% of the original value by use of the base-line normalizing calculation. Therefore, it is likely that the two phases of release are dependent upon some aspects of function of the nicotinic receptor or the release mechanism, rather than any effect of depletion.

To gain information on whether transient and persistent \([^{3}H]DA\) release may be mediated by different types of nicotinic receptor, the effects of antagonist concentration on initial rates for transient (\(v_T\)) and persistent (\(v_P\)) responses were determined. Synaptosomes were exposed to various concentrations of antagonist for 12 min before and during exposure to 1 \( \mu \)M L-NIC for 10 min. Data from two to six experiments per concentration were fit to the double-exponential decay equation to determine \(v_T\) and \(v_P\). Based on previously published data on inhibition of \([^{3}H]DA\) release at 50 \( \mu \)M L-NIC (Grady et al., 1992) and inhibition of L-[3H]NIC binding and \(^{86}\)Rb\(^+\) efflux (Marks et al., 1993b) dTC and DH\( \beta \)E appear to be competitive antagonists. HEX, DEC and MEC likely have some competitive component. MLA is probably competitive in this system (Marks, M. J., unpublished results). Inhibition constants were calculated assuming competitive inhibition for dTC, DH\( \beta \)E and MLA, and noncompetitive inhibition for HEX and DEC. These results are presented in figure 5. A similar experiment was conducted with the noncompetitive antagonist MEC. Because this compound is an open-channel blocker, there is generally some response before the onset of antagonist action. The curve-fit adds this response to the transient portion of the release, which leads to an artifactually high \(K_i\) value, so this approach with MEC was abandoned. Regression analysis (see fig. 5F) for comparison of \(K_i\)
values for inhibition of transient versus persistent response for five antagonists gave a correlation coefficient of 0.98 (P < .005) with a slope of 0.89 and an intercept of −0.30. This correlation indicates that the effects of these antagonists on the transient and persistent phases of [3H]DA release were similar and could be mediated by the same receptor.

The effects of prolonged exposure to nine nicotinic agonists were also investigated. Dose-response curves for each agonist were determined by 10-min exposure of aliquots of synaptosomes to various concentrations of agonist. The results obtained at each concentration were fit to the double-exponential decay equation as described under “Methods.” These curves for the transient phase release (v_T) and the persistent phase (v_P) for the nine agonists are presented in figure 6. All nine nicotinic agonists activated both persistent and transient responses in a concentration-dependent, saturable manner. Because of the tendency for decreased response at high agonist concentrations (e.g., 100 μM L-NIC), data were fit to the Hill equation setting V_{max} to the highest actual release obtained. Parameters ± S.E.M. for the curve fits are given in table 1. Comparison of parameters v_T and v_P indicate that several of these compounds are partial agonists. Those that have the lowest efficacy for transient release (CYT and D-NIC) are also partial agonists for persistent release; however, the difference between the most and least efficacious agonist is greater for transient release (about 5-fold) than persistent release (about 2-fold). The ratio of activation concentrations for transient and persistent release (K_T/K_P) varied about 6-fold with L-NIC and D-NIC having a noticeably higher ratio and CYT and ACh a lower ratio than the other compounds. None of the Hill coefficients calculated were significantly higher than 1; most tended to be lower than 1, perhaps because of the curve-broadening effect of using averaged data points or the uncertainty of V_{max} values.

Calculating desensitization rates by an analogous procedure (fitting d_T and d_P to the Hill equation), proved less successful because of high errors in the desensitization parameters calculated from lower concentrations of agonists. To reduce these errors, simultaneous fit of data from all concentrations of an agonist was performed as described under “Data Analysis,” setting the Hill coefficients to 1. Parameters ± S.E.M. calculated by this method for all nine agonists are given in table 3. The maximum release parameters (V_T and V_P) calculated by the simultaneous fits (table 2) were all similar to those estimated previously (table 1). Values for potency of stimulating transient release (K_T) for all nine agonists, as well as values for potency of stimulating persistent release (K_P) for six of the agonists, were not significantly different from the previous calculation. Values of K_P for three agonists, D-NIC, CARB and TMA, were significantly higher by this curve-fitting procedure as indicated in table 2. Maximal desensitization rates for the transient response varied about 2-fold. The rates measured for ACh, TMA and CARB were significantly higher than rates of desensitization for the other agonists. Maximal desensitization rates for the persistent response varied less than 2-fold among the agonists, and none were significantly different by one-way analysis of variance.

One of the properties of a desensitizing nAChR is that response can be inhibited by low concentrations of agonist. This property has been established for L-NIC-induced [3H]DA release from mouse striatal synaptosomes (Grady et al., 1994). To determine inhibition constants for agonists as inhibitors of transient release, the following protocol was used. After uptake of [3H]DA, the synaptosomes were perfused with buffer containing varying concentrations of agonist for 15 to 20 min. This interval is approximately ten times the t_{1/2} for onset (Grady et al., 1994). Release was then evoked by exposure to 10 μM L-NIC for 30 sec. The short test stimulation time was chosen to minimize the persistent re-
sponse component. Not only is the ratio of transient to persistent response highest at early time points, the persistent phase has a somewhat slower onset (Rowell, 1995; Grady et al., 1994). The low concentration of agonist present before and during test exposure stimulated some persistent response which is included in the base line and therefore subtracted from the transient response. As a result, this method measures inhibition of the transient response only. Units of release for the L-NIC test exposure were calculated as described under “Data Analysis” and are presented as % control in figure 7. Figure 8 presents the correlation of low concentration inhibition of transient response (K_T) with activation of persistent response (K_P, from table 2). The correlation coefficient is 0.99 with an intercept of −0.79 and a slope of 0.95, which indicates a strong correlation between these parameters but not identity (slope and correlation coefficient are close to 1, but intercept is not 0).

To assess whether chronic L-NIC treatment changes the release profile, [3H]DA release was measured from synaptosomes prepared from mice chronically treated with L-NIC or saline (n = 11 per group) as described under “Methods.” Individual animals were assayed by a 10-min exposure to 10 μM L-NIC. Data were expressed as units of release per fraction as means of two to four filters per animal and were fit to a double-exponential equation (see “Data Analysis” and fig. 1). Figure 9 shows the data as mean ± S.E.M. and curve fits for NIC- and saline-treated mice. In addition, L-[^3H]NIC binding was measured on a portion of the P2 striatal synaptosomes prepared from each mouse. For this measurement, pooled tissue from two mice were assayed (n = 5 determinations per group) with 20 nM L-[^3H]NIC (a concentration close to saturation for binding). Parameters calculated are presented in table 3. The parameter ν_T was significantly different (P < .05) between groups. Although differences for V_T were not highly significant (P < .20), the decrease to 82% of saline control value indicated a decrease in function similar to that for V_T. Uptake and base-line release before exposure to L-NIC during the assay did not differ between groups; however, [3H]DA remaining in the synaptosomes at the end of the assay was higher for the L-NIC-treated group, probably reflecting the decreased release. Binding of L-[^3H]NIC for the L-NIC-treated group was not significantly higher than for the saline-treated group.

**Discussion**

Nicotinic agonist-induced release of [3H]DA from striatal synaptosomes consists of two distinct phases, one transient and the other persistent. Most of the [3H]DA release from rodent synaptosomes stimulated by micromolar concentrations of L-NIC is transient, is blocked by nictinic antagonists and requires external calcium (Grady et al., 1992; Soliakov et al., 1995; Rowell et al., 1987; El-Bizri and Clarke, 1994). The persistent response, remaining after the transient portion has desensitized, is also calcium-dependent and inhibited by DHβE (figs. 3 and 5; cf. Rowell, 1995). In addition, both responses are inhibited by MEC at approximately the same concentrations (fig. 3D), as expected for a noncompetitive antagonist, but not by atropine or α-BTX. The absolute dependence of both responses on external calcium and the total blockade of both transient and persistent release by 200 μM cadmium (fig. 5) indicate that both phases are mediated by voltage-sensitive calcium channels (Miller, 1990; Rathouz and Berg, 1994). External calcium is known to modify the response of some nAChRs; however, this effect of calcium is not absolute and in fact may be minimal at high agonist concentrations (Mulle et al., 1992b). Thus, although nAChRs can flux calcium (Mulle et al., 1992a; Vernino et al., 1994), it appears that insufficient calcium enters through the nAChR to directly promote release of [3H]DA.
The desensitization time course may be a function of the release mechanism. Desensitization rates were similar for all the compounds tested for persistent release. Values of desensitization rates for the transient phase ($D_t$) varied about 2-fold with three agonists, ACh, CARB and TMA, having significantly higher rates than the other compounds. Similar desensitization rates for $[^3H]$DA release from rat striatal synaptosomes stimulated by TMA and l-NIC have been reported (Lippiello et al., 1995). Experiments with other agents, such as 20 mM K$^+$ or 100 µM kainate, which promote $[^3H]$DA release via calcium-dependent processes but do not involve the nAChR, indicated similar rates of desensitization (Grady, S. R., unpublished results). However, a more direct measure of nAChR desensitization, $[^3Rb]^+$ flux experiments (Marks et al., 1996), has shown that neuronal nAChRs do desensitize but at slightly slower rates than measured by the $[^3H]$DA release assay. Although these assays may not measure the same receptor subtype, this result shows that neuronal nAChRs do desensitize as expected. The observation that the rate of desensitization of the transient phase of $[^3H]$DA release does not vary from agonist to agonist and closely resembles the desensitization rate seen after depolarization could mean that receptor desensitization is not the rate-limiting step for attenuation of release. For example, there is evidence for biphasic depletion of cytosolic calcium concentrations and existence of two calcium sensors (Kao and Schneider, 1986; Greengard et al., 1993; Geppert et al., 1994; Littleton and Bellen, 1995). However, in bovine chromaffin cells that exhibit a biphasic pattern of release for

### Table 1

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$V_t$ (units)</th>
<th>$k_t$ (µM)</th>
<th>$n$</th>
<th>$V_p$ (units)</th>
<th>$k_p$ (µM)</th>
<th>$n'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NIC</td>
<td>4.28 ± 0.75</td>
<td>1.05 ± 0.74</td>
<td>0.73 ± 0.27</td>
<td>1.15 ± 0.12</td>
<td>0.13 ± 0.07</td>
<td>0.57 ± 0.16</td>
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<tr>
<td>ACh</td>
<td>3.59 ± 0.39</td>
<td>0.30 ± 0.13</td>
<td>0.80 ± 0.20</td>
<td>0.99 ± 0.07</td>
<td>0.16 ± 0.05</td>
<td>0.89 ± 0.19</td>
</tr>
<tr>
<td>CYT</td>
<td>1.11 ± 0.12</td>
<td>0.075 ± 0.044</td>
<td>0.61 ± 0.17</td>
<td>0.83 ± 0.10</td>
<td>0.054 ± 0.033</td>
<td>0.64 ± 0.21</td>
</tr>
<tr>
<td>d-NIC</td>
<td>1.20 ± 0.45</td>
<td>4.81 ± 6.28</td>
<td>0.82 ± 0.72</td>
<td>0.61 ± 0.02</td>
<td>0.52 ± 0.07</td>
<td>1.13 ± 0.15</td>
</tr>
<tr>
<td>CARB</td>
<td>4.30 ± 0.50</td>
<td>8.75 ± 3.21</td>
<td>0.99 ± 0.23</td>
<td>0.99 ± 0.05</td>
<td>2.10 ± 0.47</td>
<td>0.97 ± 0.18</td>
</tr>
<tr>
<td>EPI</td>
<td>2.51 ± 0.24</td>
<td>0.0027 ± 0.0009</td>
<td>0.78 ± 0.23</td>
<td>0.94 ± 0.28</td>
<td>0.00065 ± 0.00005</td>
<td>0.69 ± 0.45</td>
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<tr>
<td>MeCARB</td>
<td>2.36 ± 0.14</td>
<td>0.70 ± 0.19</td>
<td>0.86 ± 0.18</td>
<td>0.87 ± 0.11</td>
<td>0.12 ± 0.07</td>
<td>0.62 ± 0.20</td>
</tr>
<tr>
<td>ATX</td>
<td>3.40 ± 0.55</td>
<td>0.092 ± 0.063</td>
<td>0.72 ± 0.25</td>
<td>1.15 ± 0.10</td>
<td>0.024 ± 0.009</td>
<td>0.92 ± 0.28</td>
</tr>
<tr>
<td>TMA</td>
<td>5.34 ± 1.46</td>
<td>17.1 ± 11.9</td>
<td>1.15 ± 0.57</td>
<td>1.16 ± 0.11</td>
<td>3.62 ± 1.23</td>
<td>1.03 ± 0.28</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$V_t$ (units)</th>
<th>$k_t$ (µM)</th>
<th>$D_t$ (min$^{-1}$)</th>
<th>$V_p$ (units)</th>
<th>$k_p$ (µM)</th>
<th>$D_p$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NIC</td>
<td>3.72 ± 0.20</td>
<td>0.71 ± 0.11</td>
<td>1.86 ± 0.18</td>
<td>1.03 ± 0.07</td>
<td>0.085 ± 0.010</td>
<td>0.089 ± 0.011</td>
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<tr>
<td>ACh</td>
<td>3.31 ± 0.23</td>
<td>0.22 ± 0.03</td>
<td>2.95 ± 0.28</td>
<td>1.10 ± 0.04</td>
<td>0.28 ± 0.02</td>
<td>0.099 ± 0.008</td>
</tr>
<tr>
<td>CYT</td>
<td>0.89 ± 0.08</td>
<td>0.033 ± 0.010</td>
<td>1.56 ± 0.27</td>
<td>0.75 ± 0.05</td>
<td>0.024 ± 0.003</td>
<td>0.102 ± 0.011</td>
</tr>
<tr>
<td>d-NIC</td>
<td>1.21 ± 0.06</td>
<td>2.55 ± 0.32</td>
<td>1.45 ± 0.14</td>
<td>0.62 ± 0.04</td>
<td>1.83 ± 0.13</td>
<td>0.103 ± 0.010</td>
</tr>
<tr>
<td>CARB</td>
<td>4.25 ± 0.21</td>
<td>8.82 ± 0.99</td>
<td>2.60 ± 0.20</td>
<td>1.06 ± 0.06</td>
<td>5.08 ± 0.49</td>
<td>0.145 ± 0.011</td>
</tr>
<tr>
<td>EPI</td>
<td>2.40 ± 0.09</td>
<td>0.0030 ± 0.0004</td>
<td>1.71 ± 0.15</td>
<td>0.77 ± 0.06</td>
<td>0.00037 ± 0.00005</td>
<td>0.154 ± 0.015</td>
</tr>
<tr>
<td>MeCARB</td>
<td>2.22 ± 0.08</td>
<td>0.54 ± 0.07</td>
<td>1.65 ± 0.12</td>
<td>0.72 ± 0.04</td>
<td>0.14 ± 0.02</td>
<td>0.097 ± 0.010</td>
</tr>
<tr>
<td>ATX</td>
<td>3.09 ± 0.13</td>
<td>0.066 ± 0.009</td>
<td>1.28 ± 0.12</td>
<td>1.14 ± 0.10</td>
<td>0.020 ± 0.002</td>
<td>0.079 ± 0.013</td>
</tr>
<tr>
<td>TMA</td>
<td>5.70 ± 0.29</td>
<td>20.8 ± 2.4</td>
<td>2.65 ± 0.21</td>
<td>1.21 ± 0.07</td>
<td>6.84 ± 0.71*</td>
<td>0.135 ± 0.012</td>
</tr>
</tbody>
</table>

*Asterisks denote significant differences from previously calculated parameters (table 1); *$P < .05$. 

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**Fig. 6.** Dose-response curves for nicotinic agonist stimulation of transient and persistent $[^3H]$DA release. Data points are means ± S.E.M. or range ($n = 2–6$ experiments) for $V_t$ (○) or $V_p$ (●) calculated by double-exponential fit of data expressed as units per fraction (see “Methods” for details). Lines drawn are curve fits by SigmaPlot 5.0 to the Hill equation setting $V_m$ to the highest actual release achieved and omitting any decreasing values at high concentrations of agonist from the curve fits.
catecholamines, evidence has been presented that replenishment of release granules is not the cause of the biphasic release pattern (McKay and Schneider, 1984). Several observations indicate that efficacy and potency parameters measured by the release assay are indicative of nAChR function. That persistent response is agonist dependent and can occur without prior transient release shows that the persistent phase is not simply the direct result of biphasic calcium depletion. That transient response can be modulated by treatment with low concentrations of agonists in the absence of significant release argues against these parameters being affected by calcium or vesicle depletion. In addition agonists show a range of transient efficacy which would be unlikely if these parameters were determined by calcium channel activity or some function of the release mechanism.

Data presented here support the concept that both transient and persistent components of l-NIC-evoked [3H]DA release are mediated by a single receptor. If the two responses are mediated by a single type of receptor, some predictions can be made assuming the kinetics follow the desensitization model of Katz and Thesleff (1957).
If RL is the only opening form of the receptor, the transient phase of \([\text{H}]\text{DA}\) release could be a measure of \(R \rightarrow RL\) (rate constant, \(k_1\)) which then desensitizes via \(RL \rightarrow R'L\) (rate constant, \(k_3\)). The persistent phase of release could be a measure of \(R'L \rightarrow RL\) (rate constant, \(k_2\)). This could occur if the receptor that mediates dopamine release has a higher ratio of \(k_2/k_3\) than previously studied subtypes of the neuronal nicotinic receptor. For example, if the ratio of \(k_2/k_3\) is about 1:1 as expected from binding studies (Grady et al., 1994), the measured \(V_{\text{max}}\) for a functional assay would be 50% of the theoretical \(V_{\text{max}}\). If the ratio of \(k_2/k_3\) is 10:1, persistent release would be 10% of theoretical \(V_{\text{max}}\) or approximately 20% of the measured \(V_{\text{max}}\) for transient release.

It has been shown for nAChR from Torpedo that \(K_i\) values for competitive antagonists are similar for the desensitized form, measured by competition binding assays, and the active form, measured by \(^{22}\text{Na}^+\) efflux (Popot et al., 1976). The \(K_i\) values for competitive antagonists measured for \([\text{H}]\text{nor}-\text{epinephrine release and L-}[\text{H}]\text{NIC binding in bovine adrenal chromaffin cells are also similar} (Higgins and Berg, 1988). Therefore, if the transient and persistent phases of \([\text{H}]\text{DA}\) release are mediated by agonist binding to different forms of the same receptor, it is likely that \(K_i\) values for inhibition by competitive antagonists as well as noncompetitive antagonists would be similar. If, however, the two phases of \([\text{H}]\text{DA}\) release are mediated by different receptor subtypes, \(K_i\) would likely be different (Alkondon and Albuquerque, 1993; Mulle et al., 1991; Cachelin and Rust, 1994, 1995; Clarke and Ruben, 1996). The results (fig. 5) for five antagonists show that the \(K_i\) values are similar for the two phases of release. Regression analysis yielded a good correlation \((r = 0.98; \text{slope} = 0.89; \text{intercept} = -0.30)\) between \(K_i\) values for the five antagonists for the two phases of release. The slope and intercept deviate somewhat from expected (1 and 0), which possibly reflects an error in the assumption that DEC and possibly HEX are purely noncompetitive inhibitors. Inhibition by these antagonists, however, is similar enough to conclude that the two processes may be mediated by the same receptor.

Further support for the same receptor hypothesis is derived from a comparison of agonist-induced activation of the persistent phase and agonist-induced desensitization of the transient phase. If the transient and persistent responses are manifestations of the kinetics of a single receptor, as opposed to responses of two separate types of release, the concentrations required to evoke persistent response \((K_p)\) should be correlated with inhibition of the transient response \((K_\text{IA})\), which is produced by preexposure to low concentrations of agonist. Such a correlation is expected because both processes require agonist interaction with the desensitized form of the receptor (the \(R' \rightarrow R'L\) step). High correlation between the activation constants for persistent release \((K_p)\) and the inhibition by low agonist concentrations of the transient response to L-NIC \((K_\text{IA})\) was obtained (fig. 6). Whereas \(K_p\) and \(K_\text{IA}L\) are highly correlated, \(K_p\) is always larger than \(K_\text{IA}L\) (ratio, 6.1 ± 0.7; range, 2.9–8.8). Calculations based on the Katz and Thesleff model described by Marks et al. (1996) have shown that \(K_\text{IA}L\) values for inhibition of function are predicted to be higher than \(K_iL\) values for inhibition of binding. Similar calculations with ratios of \(k_2/k_4\) in the range of 10:1 and other constants set at values determined in experiments reported here and previously (Grady et al., 1994) indicate that \(K_p\) is expected to be larger than \(K_\text{IA}L\).

A range of efficacy was seen for the nine agonists tested in this study. For the transient release process, initial release \((V_r)\) varied about 5-fold with CYT giving the lowest response and TMA having the highest transient release. Persistent release \((V_p)\) varied much less (about 2-fold), with n-NIC having the lowest and TMA having the highest efficacy. In agreement with these data, CYT has been shown to have lower efficacy than L-NIC for release of \([\text{H}]\text{DA}\) from rat synaptosomes (El Bizri and Clarke, 1994; Wonnacott et al., 1995). An initial characterization (Grady et al., 1992) indicated that efficacy was similar for various agonists including L-NIC, CYT, n-NIC, ACh and CARB. These early experiments with the perfusion system were conducted with Percoll-purified synaptosomes and without current improved line washing procedures, which resulted in considerably lower agonist-stimulated release values than are now achieved. A greater proportion of the response measured for this initial characterization was possibly of the persistent phase which varies little in efficacy.

Marks et al. (1993a) showed that chronic treatment of mice with 4 mg/kg/hr L-NIC produces a functional down-regulation of striatal \([\text{H}]\text{DA}\) release as measured by a 1-min exposure to L-NIC. This treatment protocol was repeated in the experiment reported here, which used a 10-min L-NIC exposure for the \([\text{H}]\text{DA}\) assay and a 15-hr withdrawal from chronic treatment, a length of time sufficient to clear L-NIC from the bloodstream of mice (Petersen et al., 1984). A small but statistically insignificant increase in L-\([\text{H}]\text{NIC binding in striatum was seen. Chronic exposure to L-NIC produces a well-documented up-regulation of L-NIC binding sites in brain. Regional differences are seen in the amount of up-
regulation measured after chronic treatment, with striatum showing minimal up-regulation (Marks et al., 1993a). The results reported here are in agreement with this previous study. Results of functional assays (fig. 8) show that both phases of [3H]DA release are decreased by 16 to 18% (P < .05 for the transient phase; P < .20 for the persistent phase). This result is similar to previously reported down-regulation of [3H]DA release (Marks et al., 1993a) where release was measured with a 1-min exposure to L-NIC. The data reported here indicate that the down-regulation of response by chronic treatment affects both transient and persistent [3H]DA release similarly. Therefore, if both responses are mediated by one receptor, functional down-regulation is not produced by locking the receptor in a desensitized state, because that possibility would have resulted in greatly reduced transient phase release with increased or no change in persistent response. Instead, some receptors may be functionally inactive or partially active as measured by the [3H]DA assay, but still able to bind L-[3H]NIC. This interpretation is consistent with the results and conclusions of Peng et al. (1994) who investigated functional responses of α4β2 receptors expressed in oocytes and M10 cells chronically treated with L-NIC.

Assuming that a single type of nAChR is mediating both phases of [3H]DA release, the question of subtype assignment arises. Multiple subunits likely coexist in mouse striata as messenger RNA for alpha-3, alpha-4, alpha-5, beta-2 and beta-3 has been detected by in situ hybridization in the substantia nigra of mice (Marks et al., 1992; Marks, M. J., unpublished data). Certain assays are known to measure a particular subtype of nAChR. Binding of L-[3H]NIC to brain membranes correlates well with the α2β2 form of neuronal nAChR (Whiting et al., 1991; Flores et al., 1992; Lindstrom et al., 1995; Gopalakrishnan et al., 1996) and binding of [125I]BTX is a measure of the alpha-7 subunit (Vernallis et al., 1993; Gotti et al., 1994). Parameters measuring effects of nicotinic agonists on [3H]DA release, K_F, K_P (table 2) and K_A (fig. 7), were compared with K_I values for inhibition of binding of L-[3H]NIC to mouse thalamic membranes (Marks et al., 1996) and inhibition of binding of [125I]BTX to mouse brain membranes (unpublished data) by regression analysis (table 4). High correlations (0.97–0.98) with slopes near 1 were seen for all [3H]DA release parameters compared with inhibition of L-[3H]NIC binding. Correlations to [125I]BTX binding were lower (0.82–0.87) and similar to that seen for a comparison of the two binding assays (0.85). A significant correlation between inhibition of transient release of [3H]DA from rat striatal synaptosomes and L-[3H]NIC binding has also been reported for rat brain (Lippiello et al., 1995). Because high-affinity L-[3H]NIC binding measures primarily the α4β2 form of neuronal nAChRs, it is likely that receptors mediating [3H]DA release in rodent striata contain alpha-4 and beta-2 subunits.

Another subtype-specific technique is measurement of inhibition by nBTX. Oocyte experiments with specific nAChR subtypes have shown that receptors potently inhibited by nBTX are most likely of α3β2 composition (Duvoisin et al., 1989; Luette and Patrick, 1991; Luette et al., 1990, 1993). Previous experiments have shown that [3H]DA release is completely inhibited by low concentrations of nBTX (Grady et al., 1992; Schultz and Zigmond, 1989). These data indicate that alpha-3 and beta-2 subunits are involved in mediating agonist-evoked [3H]DA release. By functional measurements of receptors expressed in oocytes, the beta-2 subunit is associated with partial agonist activity of cytisine (Luette and Patrick, 1991).

It has been established that individual neurons can express multiple subtypes of nAChR (Horch and Sargent, 1995; Sargent, 1993; McGehee and Role, 1995). In addition, the existence of receptors containing more than one type of alpha or beta subunit have been proposed for other systems (Colquhoun et al., 1993; Vernallis et al., 1993; Mandelzys et al., 1995). The strong pharmacological correlations between the transient and persistent phases of [3H]DA release from striatal synaptosomes presented here indicate that both phases are likely mediated by one type of receptor. The correlations of [3H]DA release and L-[3H]NIC binding parameters implicate an α4β2 composition whereas sensitivity to nBTX favors α3β2 subunit containing receptors. It seems possible that this nAChR contains both an alpha-3 and an alpha-4 as well as beta-2 subunits. Although correlations of pharmacological parameters can suggest that receptors mediating two processes are identical, confirmation of the subunit composition of these receptors must be achieved by other methods.

Acknowledgments

The authors wish to thank Scott Robinson for assistance with surgery and chronic treatments, and Jerry Stitzel and Amy Clark for assistance with L-[3H]NIC binding assays.

References


| Table 4 | Comparison of parameters for [3H]DA release and membrane binding for nine agonists |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Inhibition of | Inhibition of |
|                  | l-NIC binding   | αBTX binding    |
| Activation of   | (K<sub>s</sub>)  | (K<sub>s</sub>)  |
| transient       | R = 0.97        | R = 0.82        |
| release (K<sub>r</sub>) | S = 1.05 | S = 0.94 |
| Inhibition of   | (K<sub>r</sub>)  | (K<sub>r</sub>)  |
| transient       | R = 0.98        | R = 0.87        |
| release (K<sub>r</sub>) | S = 1.00 | S = 0.89 |
| Inhibition of   | (K<sub>50</sub>)| (K<sub>50</sub>)|
| L-NIC binding   | R = 0.98        | R = 0.86        |
| (K<sub>50</sub>) | S = 0.87        | S = 0.87        |

S = slope; R = correlation coefficient. K<sub>r</sub> and K<sub>s</sub> values are from table 2; K<sub>50</sub> values are from fig. 7. K<sub>50</sub> values for inhibition of [3H]L-NIC binding are from Marks et al. (1996). K<sub>r</sub> values for inhibition of [125I]αBTX binding are from unpublished data (Marks, M.J.).


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