Nicotine Enhancement of Dopamine Release by a Calcium-Dependent Increase in the Size of the Readily Releasable Pool of Synaptic Vesicles

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A major factor underlying compulsive tobacco use is nicotine-induced modulation of dopamine release in the mesolimbic reward pathway (Wise and Rompre, 1989). An established biochemical mechanism for nicotine-enhanced dopamine release is by activating presynaptic nicotinic acetylcholine receptors (nAChRs) (Wonnacott, 1997). Prolonged application of $10^{-7}$ to $10^{-5}$ M nicotine to striatal synaptosomes promoted a sustained efflux of $[^3$H]dopamine. This nicotine effect was mediated by non-$\alpha_7$ nAChRs, because it was blocked by 5 $\mu$M mecamylamine but was resistant to 100 nM $\alpha$-bungarotoxin ($\alpha$BgTx). Dopamine release was diminished by omitting Na$^+$ or by applying peptide calcium channel blockers, indicating that nAChRs trigger release by depolarizing the nerve terminals. However, because $\alpha_7$ receptors rapidly desensitize in the continuous presence of agonists, a repetitive stimulation protocol was used to evaluate the possible significance of desensitization. This protocol produced a transient increase in $[^3$H]dopamine released by depolarization and a significant increase in the response to hypertonic solutions that measure the size of the readily releasable pool (RRP) of synaptic vesicles. The nicotine-induced increase in the size of the readily releasable pool was blocked by $\alpha$BgTx and by the calmodulin antagonist calmidazolium, suggesting that Ca$^{2+}$ entry through $\alpha_7$ nAChRs specifically enhances synaptic vesicle mobilization at dopamine terminals. Thus, nicotine enhances dopamine release by two complementary actions mediated by discrete nAChR subtypes and suggest that the $\alpha_7$ nAChR-mediated pathway is tightly and specifically coupled to refilling of the RRP of vesicles in dopamine terminals.

Key words: striatum; acetylcholine; presynaptic; kinetics; $\alpha_7$ nAChR; $\alpha$-bungarotoxin

Introduction
Presynaptic ionotropic receptors are gaining recognition for their role in modulating synaptic activity (Miller, 1998; MacDermott et al., 1999). Unlike presynaptic metabotropic receptors that typically diminish the probability of release, presynaptic ionotropic receptor activity generally enhances neurotransmitter release. As observed by recording synaptic events in postsynaptic neurons, the enhancement appears as an increase in the frequency of quantal, or miniature, events. Examples of such enhancement include nicotinic acetylcholine receptor (nAChR) modulation of glutamate release in hippocampus (Gray et al., 1996) and of GABA release in thalamus (Léna and Changeux, 1997), NMDA receptor-mediated glutamate, GABA, and 5-HT release, and 5-HT$_3$ receptor-mediated increases in GABA release in amygdala (Koyama et al., 2000). In addition, biochemical evidence indicates that presynaptic ionotropic receptor activation promotes the release of the monoamines dopamine (Rapier et al., 1988; Grady et al., 1992) and norepinephrine (Clarke and Reuben, 1996).

The mechanisms responsible for coupling presynaptic ionotropic receptor activity to an enhanced probability of release remain incompletely understood. On the basis of the biophysical properties of ionotropic receptors, there are at least two likely possibilities to be considered. First, inward current mediated by presynaptic receptors, or secondarily by presynaptic Na$^+$ channels, could activate voltage-gated Ca$^{2+}$ channels to trigger exocytosis. Second, many presynaptic ionotropic receptors have considerable permeability to Ca$^+$, providing a direct path for Ca$^{2+}$ entry through the presynaptic receptor. Receptor-mediated Ca$^{2+}$ entry, through some unidentified intracellular pathway, could lead to increases in the frequency of quantal events.

In this report, I focused on nicotine-stimulated dopamine release from striatal nerve terminals in an effort to differentiate the relative contribution of these two mechanisms to presynaptic modulation of neurotransmitter release. Synaptosomes, a highly reduced preparation enriched in nerve terminals, was used to study presynaptic actions of nicotine in isolation of postsynaptic or “preterminal” effects. The ability of nicotine to modulate $[^3$H]dopamine secretion from synaptosomes was measured using two distinct presynaptic activities: nicotine-evoked secretion and hypertonic sucrose-induced exocytosis of the readily releasable pool (RRP) of synaptic vesicles. Consistent with previous reports (Rapier et al., 1988; Grady et al., 1992), prolonged application of nicotine at pharmacologically relevant concentrations produced modest amounts of Ca$^{2+}$-dependent $[^3$H]dopamine.
release that required neuronal calcium channel activity. In addition, two different conditioning protocols were used to demonstrate that nicotine enhanced striatal $[^{3}H]$dopamine release, as assessed by measuring both depolarization-evoked and hypertonic sucrose-induced release. The selective blockade of the nicotine-induced enhancement of the response to sucrose by $\alpha$-bungarotoxin ($\alpha$BgTx) and by calmidazolium suggests that Ca$^{2+}$ entry through a7 nAChR regulates synaptic vesicle dynamics via a calmodulin-dependent pathway.

**Materials and Methods**

For the preparation of synaptosomes, striatal tissue from adult mice (20–30 gm) was dissected and then homogenized in 4 ml of 0.32 M sucrose and 1 mM EDTA in an “AA” Wheaton glass-Teflon homogenizer (Thomas Scientific, Swedesboro, NJ). The supernatant fraction remaining after low-speed centrifugation (1500 x g, 7 min) was divided into two equal portions and applied to two identical discontinuous Percoll (Amerham Biosciences, Piscatway, NJ) gradients, which were centrifuged at 50,000 x g for 5 min (Dunkley et al., 1986). The material that migrated to the 10/15 and 15/23% Percoll boundaries was collected and washed in basal buffer (in mM: 145 NaCl, 2.7 KCl, 1.0 MgCl$_{2}$, 10 glucose, and 10 HEPES-Tris, pH 7.4) by two 7 min centrifugation steps at 12,000 x g. The final pellet was resuspended in basal buffer and stored on ice until use.

In a typical experiment, a 40 $\mu$L portion of the synaptosomal suspension containing $\sim$ 50 $\mu$g of protein was combined with 10 $\mu$L of $[^{3}H]$dopamine (28.0 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) that had been prepared by evaporating the aqueous stock solution (2% ethanol) under a stream of N$_{2}$ and dissolving the residue in basal buffer at a concentration of $1\mu$Ci/$\mu$L. The loading reaction was stopped by adding 750 $\mu$L of basal buffer that contained 1 mg/ml BSA, which improved retention of the synaptosomes on the filter. This suspension was applied to a filtration sandwich composed of cellulose ester and glass fiber filters, as described previously (Turner et al., 1989).

Release was measured using a superfusion device in conjunction with a fraction collector modified from a phonograph turntable (Forbush, 1984; Turner and Goldin, 1989). Synaptosomes were superfused with an appropriate stimulus buffer, and fractions ranging from 15 msec (78 rpm) to 100 msec (12 rpm) were collected on the turntable. Alternatively, longer fractions were collected by advancing the turntable platter manually, using a quartz metronome (Seiko Corporation, Mahwah, NJ) for timing. Radioactivity in each fraction and the amount remaining on the filter at the end of the experiment were determined by adding 1.5 ml of liquid scintillation mixture (BioSafe II; Research Products, Mt. Prospect, IL) and counting in a Tri-Carb 2100TR liquid scintillation analyser (Packard Bioscience, Pangbourne, UK).

Stimulus buffers designed to depolarize the synaptosomal membrane were prepared by combining two solutions, the basal buffer and an otherwise identical KCl solution in which NaCl had been substituted with KCl (final KCl concentration was 147.7 mM). These solutions were combined in the appropriate ratio to yield a stimulus buffer with the desired KCl concentration. Ca$^{2+}$-dependent release was calculated as the net difference between release at a given Ca$^{2+}$ concentration and “Ca$^{2+}$-independent” release evoked with a stimulus buffer that contained no added Ca$^{2+}$ (free [Ca$^{2+}$] = 3 mM). The small Ca$^{2+}$-independent component of release has been shown previously to be mediated by thermo-dynamic reversal of the Na$^{+}$-dependent transporter that normally concentrates dopamine in the nerve terminal cytoplasm under resting ionic conditions. Data were analyzed using Microsoft (Seattle, WA) Excel. Results were expressed as the ratio of counts per minute in each fraction to the total radioactivity remaining on the filter ($\times 100$%); error bars represent the SEM. Data reported are the average of at least three separate experiments performed on different days with freshly prepared synaptosomes. To account for any time-dependent changes in release rates, the order of the experimental conditions was randomized for each experiment.

The peptide toxins were prepared as 1.0 mM stock solutions in water, divided into 10 $\mu$L portions, and stored at $-70^\circ$C. Working stocks were prepared by diluting the concentrated solutions 10-fold with basal buffer that contained 1 mg/ml bovine serum albumin. These working solutions could be subjected to multiple freeze–thaw cycles without apparent loss of activity. $\alpha$-Bungarotoxin was obtained from Sigma (St. Louis, MO), $\alpha$-agatoxin IVA ($\alpha$AgA IVA) and $\alpha$-conotoxin GVIA ($\alpha$CoTX GVIA) were purchased from Peptides International (Frankfort, KY), and $\alpha$-conotoxin GVIA ($\alpha$CoTX GVIA) was purchased from Bachem (Torrance, CA). Other reagents, unless indicated otherwise, were purchased from Fluka (Neu-Ulm, Germany).

**Results**

Nicotine depolarizes synaptic terminals

A biochemical approach was used to study nicotine-evoked dopamine secretion in mouse brain, using striatal synaptosomes metabolically labeled with $[^{3}H]$dopamine. $[^{3}H]$Dopamine release was measured using a superfusion device that combined high flow (2 ml/sec) through a small chamber (50 $\mu$L) to provide subsecond temporal resolution ($t \approx $ volume/flow = 25 msec) (Turner et al., 1993) (Fig. 1). The synaptosomes were superfused with a basal buffer that contained 2.5 mM Ca$^{2+}$, and basal efflux rates of $[^{3}H]$dopamine were measured at 1 sec intervals. After 6 sec (where $t$ is indicated as 0), the superfusate was switched to a stimulation buffer that contained 1 $\mu$M nicotine or 10 $\mu$M acetylcholine. Both agonists produced a rapid, long-lasting increase in
the rate of dopamine release. The net, nicotine-stimulated dopamine release rate, calculated by subtracting the basal rate obtained in the absence of agonist, reached a peak within 1 sec of agonist application and subsequently decreased to a slowly decaying plateau. The cumulative release calculated by summing the release over time (Fig. 1B) was well described as the sum of an exponential and linear process. When the nicotine-containing stimulus buffer had Na⁺ replaced by the impermeant cation N-methylglucammonium⁺, the exponential phase of the fit was eliminated, leaving only the slow linear component.

Pharmacological experiments were performed that were designed to address the mechanisms that underlie nicotine-evoked release from striatal terminals (Fig. 1C). Mecamylamine at 10 μM, a nicotinic antagonist that selectively blocks α7BgtX-insensitive nAChRs at this concentration (McQuiston and Madison, 1999), inhibited nicotine-evoked dopamine release by 81 ± 11% (n = 12). Conversely, 100 nM αBgtX, the α7-selective toxin, had no effect. These results argue that nicotine-stimulated dopamine release was mediated by non-α7 nAChR subtype(s). The Na⁺ requirement (Fig. 1B) suggested that membrane depolarization was a requisite element of nicotine action. To confirm that voltage-gated Ca²⁺ channels were involved (Prince et al., 1996), synaptosomes were pretreated with selective antagonists of neuronal calcium channels to determine whether they could inhibit nicotine-stimulated dopamine release. The P/Q channel antagonist ωAgA IVA (1 μM) and the N channel blocker ωCTX GVIA (1 μM) each reduced nicotine-stimulated dopamine release, by 57 and 64%, respectively. When both peptides were combined, nicotine action was inhibited by 84%. Together, these experiments indicate that prolonged nicotine exposure stimulates dopamine secretion by activating non-α7 nAChRs, causing depolarization-gated calcium entry through neuronal calcium channels that trigger exocytosis.

Repetitive stimulation with nicotine reveals a role for the α7 nAChR subtype

Despite the evidence that non-α7 receptors were the sole mediators of nicotine in triggering striatal dopamine release, it remained possible that α7 nAChR activity might be obscured by rapid receptor desensitization caused by the prolonged application of agonist. To evaluate this hypothesis, a repetitive stimulation protocol was used based on the work of Radcliffe and Dani (1998). The objective of this approach was to activate α7 receptors with a brief pulse of nicotine, allowing sufficient time for the receptors to recover from desensitization before the next pulse was applied. The protocol consisted of a series of four 500 msec depolarizing pulses (using 60 mM KCl) that allowed us to measure the amount of dopamine secretion triggered by neuronal Ca²⁺ channels. The first two depolarizing pulses were delivered at 20 sec intervals, an interpulse interval that was sufficient to maintain a constant response to KCl depolarization (Turner and Goldin, 1989). One second after the second depolarizing pulse, a series of five 200 msec conditioning pulses containing 3 μM nicotine (or basal saline as a control) was delivered at 10 sec intervals. One second after the fifth conditioning pulse, another pair of 60 mM KCl-containing depolarizing pulses was delivered at 20 sec intervals to assess any change in the amount of depolarization-evoked dopamine release that was produced by the conditioning protocol. As a control, basal buffer was used as the stimulus solution in the conditioning protocol. Under this condition, there was no change in dopamine release produced by the third or fourth depolarizing pulse (Fig. 2). However, when basal buffer plus 3 μM nicotine was used, dopamine release evoked by the third depolarizing pulse was significantly increased (44.5% increase; p < 0.02; n = 8). This enhancement was transient, because dopamine release during the fourth depolarizing pulse was slightly reduced (84.4% of control values). This result suggested that brief pulses with nicotine transiently enhanced [³H]dopamine release.

Nicotine enhances dopamine release by altering the size of the readily releasable pool of synaptic vesicles

In addition to triggering exocytosis, Ca²⁺ influences several other steps in the synaptic vesicle cycle. Specifically, moderate increases in Ca²⁺ concentrations have been shown to enhance the size of the RRP of vesicles that are docked and fusion competent in chromaffin cells (Smith et al., 1998) and accelerates the refilling of the RRP in autaptic neurons (Stevens and Wesseling, 1998). Thus, Ca²⁺ entry via nAChRs could modulate the equilibrium between the reserve and readily releasable pools of synaptic vesicles, enhance refilling by activating exocytosis, or regulate some other unidentified process. To test whether the brief pulses with nicotine enhanced dopamine release by modulating synaptic vesicle dynamics, the size of the RRP was measured by applying saline made hypertonic by the addition of 0.5 m sucrose. This technique has been used previously to measure the size of the RRP of glutamatergic vesicles in synaptosomes (Lonart and Sudhof, 2000) and in autaptic cultures (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996).

The validity of using hypertonic sucrose to measure the RRP of dopaminergic vesicles in synaptosomes was evaluated by comparing the properties of sucrose-evoked [³H]dopamine release with the results observed previously (Fig. 3). The kinetics of sucrose-evoked release of [³H]dopamine were remarkably similar to those observed in autaptic cultures of hippocampal neurons. Superfusion of synaptosomes with saline containing 0.5 m
Hypertonic sucrose stimulates secretion of synaptosomal dopamine. A, Strial synaptosomes were loaded with $[^3H]$dopamine and superfused with basal saline for 1.8 sec to establish baseline release rates. At the time indicated by the horizontal bar, the superfusate was switched to saline made hypertonic by the addition of 0.5 M sucrose. The release rate from control synaptosomes (C) increased gradually to a maximum rate observed after ~500 msec, and it subsequently decayed within 2 sec to a sustained plateau rate when compared with synaptosomes continuously superfused with isotonic basal saline (*—*). Pretreating synaptosomes with 3 μM PDBu (●) resulted in an increase in the amount of dopamine release, whereas pretreatment with 10 μM reserpine (△) greatly diminished sucrose-evoked release. B, The net sucrose-evoked release was integrated and fit to the sum of an exponential component plus a linear phase (smooth lines); the amplitude of the exponential component (dashed lines), defined as the RRP, was increased by 57% by PDBu treatment and decreased by 71% by reserpine treatment. Data are averages of three experiments performed in quadruplicate.

Sucrose produced a gradual increase in the rate of $[^3H]$dopamine release that reached a peak within 500 msec and decayed over ~2 sec to a sustained plateau level (Fig. 3A). The cumulative release was adequately described as the sum of an exponential component and a linear component. The amplitude of exponential component was defined as the readily releasable pool size. This parameter was appropriately modulated by 12,13-phorbol dibutyrate (PDBu), an activator of protein kinase C that has been shown to increase the size of the RRP of glutamatergic vesicles in hippocampal neurons (Stevens and Sullivan, 1998) and secretory granules in chromaffin cells (Gillis et al., 1996). After phorbol treatment, the RRP of $[^3H]$dopamine was increased by 57%, quantitatively similar to the increase in glutamate release observed at glutamatergic autapses and in chromaffin cells. Finally, treatment with 10 μM reserpine, an inhibitor of the H+-dependent vesicular monoamine transporter required for dopamine storage in synaptic vesicles (Schuldiner, 1994), markedly diminished the size of the RRP, indicating that sucrose-induced release of transmitter is vesicular in origin.

To determine whether Ca$^{2+}$ influx through nAChRs might regulate the size of the RRP, nicotine was delivered using repetitive stimulation (five 200 msec pulses at 10 sec intervals), followed 1 sec after the end of the fifth nicotine pulse by a test pulse with 0.5 M sucrose to measure the size of the RRP (Fig. 4A). The conditioning protocol significantly increased (41.9 ± 6.3%) $[^3H]$dopamine release evoked by a hypertonic sucrose test pulse. The increase was quantitatively similar to the effect of the conditioning protocol on KCl-evoked release (Fig. 2). The pharmacology of nicotinic receptors involved in the enhanced response to sucrose was investigated by using the nAChR antagonists mecamylamine and αBgTx. One set of synaptosomes was preincubated with 100 nM αBgTx before the nicotine conditioning protocol, whereas a second set was conditioned with 200 msec pulses that contained both 3 μM nicotine and 5 μM mecamylamine, a concentration of antagonist that is relatively selective for non-α7 nAChRs (McQuiston and Madison, 1999). The enhanced response to sucrose produced by nicotine was essentially blocked in αBgTx-treated synaptosomes, whereas mecamylamine, at a concentration that blocked nicotine-evoked release of dopamine, had no significant effect.

The effect of nicotine on the response to sucrose was strictly Ca$^{2+}$ dependent (Fig. 4B). When the Ca$^{2+}$ in the conditioning stimulus was replaced by Ba$^{2+}$, there was no nicotine-induced enhancement despite the fact that α7 nAChRs are quite permeant to Ba$^{2+}$ (Sands et al., 1993). Unlike the nicotine-evoked release of $[^3H]$dopamine, neuronal calcium channels were not involved in the nicotine-induced enhancement of the sucrose-evoked release. This action of nicotine was unaltered by the combination of αCTx GVIA and αCTx MVIIIC (1 μM each), inhibitors of N and P/Q calcium channels that strongly reduced nicotine-evoked release.

The relative potency of nicotine in triggering dopamine release (persistent agonist application) and in enhancing the size of the RRP (pulsatile application) was compared to further distinguish the properties of these two activities (Fig. 4C). Concentration–response data indicated an EC$_{50}$ value for nicotine of

Figure 3. Hypertonic sucrose stimulates secretion of synaptosomal dopamine. A, Strial synaptosomes were loaded with $[^3H]$dopamine and superfused with basal saline for 1.8 sec to establish baseline release rates. At the time indicated by the horizontal bar, the superfusate was switched to saline made hypertonic by the addition of 0.5 M sucrose. The release rate from control synaptosomes (C) increased gradually to a maximum rate observed after ~500 msec, and it subsequently decayed within 2 sec to a sustained plateau rate when compared with synaptosomes continuously superfused with isotonic basal saline (*—*). Pretreating synaptosomes with 3 μM PDBu (●) resulted in an increase in the amount of dopamine release, whereas pretreatment with 10 μM reserpine (△) greatly diminished sucrose-evoked release. B, The net sucrose-evoked release was integrated and fit to the sum of an exponential component plus a linear phase (smooth lines); the amplitude of the exponential component (dashed lines), defined as the RRP, was increased by 57% by PDBu treatment and decreased by 71% by reserpine treatment. Data are averages of three experiments performed in quadruplicate.

Figure 4. Nicotine enhances the size of the RRP in dopaminergic terminals. The ability of nAChRs to modulate synaptic vesicle dynamics in dopaminergic terminals was assessed by applying five 200 msec conditioning pulses at 10 sec intervals, followed 1 sec after the end of the fifth nicotine pulse by a test pulse that contained 0.5 M sucrose to measure the size of the RRP. A, At $t = 0$, the superfusate was switched to an identical solution (*—*) to measure baseline release rates or a hypertonic saline containing 0.5 M sucrose. When 3 μM nicotine was included in the conditioning pulses (●), the size of the RRP (assessed as described in Fig. 2) was increased by 41.9 ± 6.3% when compared with control samples in which no nicotine was included in the conditioning pulses (C). The effect of nicotine was essentially blocked when the synaptosomes were pretreated with 100 nM αBgTx before the nicotine exposure (△). In contrast, the α7 nAChR antagonist mecamylamine (5 μM in both the preincubation and conditioning solutions) had no effect against nicotine-induced increases in the RRP (▲). B, The same conditioning protocol was used to evaluate the Ca$^{2+}$ requirement for nicotine-induced increases in the size of the RRP. Compared with control (C), 3 μM nicotine increased the size of the RRP by 40% when 1 mM Ca$^{2+}$ was included in the conditioning solution (●). Incubating the synaptosomes in saturating concentrations (1 μM each) of neuronal calcium channels blockers α-conotoxin GVIA and α-conotoxin MVIIIC (▲) had no significant effect on the ability of nicotine to potentiate the sucrose response, but Ba$^{2+}$ substitution for Ca$^{2+}$ in the nicotine conditioning pulses abolished the effect (△). Data were averaged from three experiments performed in quadruplicate. C, Concentration–response data were obtained for nicotine-evoked release (●), calculated as described in Fig. 1 or for the enhanced response to sucrose induced by the conditioning protocol (●, as described above) and plotted on the same axis for comparison. Results are the mean ± SEM of 10 and 6 independent measurements, respectively.
proximted by a first-order process with a time constant of 8.6 sec, suggesting that this nicotinic effect is short-lived.

Choline selectively enhances the size of the RRP
Choline, a precursor to and a hydrolytic product of acetylcholine, has been shown to be a selective (albeit low-potency) agonist of α7-containing nAChRs (Alkondon et al., 1997). The ability of αBgTx to block the nicotine-induced enhancement of the size of the RRP suggested that an α7-selective agonist such as choline should also produce enhancement. This possibility was tested using a two-pulse protocol consisting of a 7.5 sec pulse with a stimulation buffer (3 μM nicotine, 10 mM choline Cl, or control saline), followed by a 9 sec pulse with 0.5 M sucrose saline to measure the RRP (Fig. 6). When the superfusate was switched from basal saline to a stimulus buffer that contained 2.5 mM Ca2+ (at t = 0), there was a transient increase in [3H]dopamine release even in the absence of a nicotinic agonist (Fig. 6A, inset). This transient release event has been observed previously (Bower and Weiner, 1990) and is probably dependent on Ca2+ entry via neuronal calcium channels via a poorly understood mechanism. When 3 μM nicotine was included in the stimulus buffer, there was a modest increase in [3H]dopamine release that was insensitive to 100 nM αBgTx, similar to the release event described in Figure 1. After exposing the synaptosomes to nicotine, the response to the second pulse containing hypertonic saline was significantly enhanced compared with saline control. This enhancement was essentially blocked by 100 nM αBgTx. In contrast, a 7.5 sec pulse with a stimulus buffer that contained 10 mM choline Cl had no measurable effect on the rate of [3H]dopamine release when compared with control. However, like nicotine, choline treatment produced a significant increase in the response to hypertonic saline that was eliminated by 100 nM αBgTx. Thus, choline selectively enhanced the sucrose response without directly evoking [3H]dopamine release, consistent with a selective activation of α7-containing nAChRs and suggesting that α7 nAChRs are expressed at relatively low levels compared with non-α7 receptors.

To strengthen the pharmacological identity of the nAChR that mediated the actions of nicotine in this assay, the same experimental paradigm was repeated including dihydro-β-erythroidine (100 nM), a selective inhibitor of β2-containing nAChRs, or methyllycaconitine (30 nM), a high-potency inhibitor of α7 receptors, in the nicotine-containing stimulus buffer. Dihydro-β-erythroidine eliminated nicotine-evoked dopamine release without altering the enhancement of sucrose-evoked release; methyllycaconitine had the converse action (data not shown).

Signaling pathways that couple nAChRs to changes in synaptic vesicle dynamics
The observation that Ca2+ entry mediated by α7 nAChRs enhanced the response to hypertonic saline suggested that an intracellular calcium signaling pathways linked receptor activity with synaptic vesicle mobilization. An obvious candidate for an intracellular mediator of such a pathway was calmodulin, a prominent Ca2+ binding protein that is ubiquitous in the brain. A two-pulse protocol consisting of a sustained (7.5 sec duration) nicotine pulse followed immediately by a pulse with hypertonic saline (Fig. 7) was used to evaluate the role of calmodulin in the nicotine-induced enhancement. As described previously (Figs. 1, 6), sustained application of 3 μM nicotine produced a biphasic increase in dopamine release rate. Subsequent application of hy-
pertonic saline revealed that, compared with control release rates, the nicotine pulse enhanced the amount of dopamine released.

When synaptosomes were pretreated with the calmodulin inhibitor calmidazolium, two prominent effects were observed (Fig. 7A). First, dopamine release evoked by the sustained application of nicotine was diminished, such that 300 nM calmidazolium selectively blocked the linear, Na⁺-dependent component without altering the fast, exponential component. Second, the calmidazolium pretreatment blocked the nicotine enhancement of sucrose-evoked release. Control experiments (data not shown) demonstrated that 300 nM calmidazolium had no effect on sucrose-evoked release from synaptosomes that had not been stimulated with nicotine. It should be noted that these actions of calmidazolium were unlikely to be attributable to a direct blockade of the nAChRs because the synaptosomes were only pretreated with calmidazolium for 4 min immediately before the release experiment. This protocol allowed the drug to gain access to the cytoplasmic compartment, but external drug was removed by an extensive wash with basal saline that was administered immediately before the two-pulse protocol.

Because the selectivity of calmidazolium, even at submicromolar concentrations, is questionable, a second calmodulin inhibitor was tested. Trifluperazine also inhibits calmodulin-mediated signaling, albeit at lower potency than calmidazolium. An examination of the effects of these two agents on nicotine-induced increases in the response to hypertonic sucrose indicated that calmidazolium was at least 10-fold more potent than trifluperazine and that both agents were effective at submicromolar concentrations.

The selective calmodulin kinase inhibitor KN-62 (1-[NO-bis-1,5-isooquinolinesulfonyl]-N-methyl-t-tyrosyl-4-phenylpipera-zine) was used to test the possible role of this kinase in mediating the actions of calmodulin (Fig. 7B). As with calmidazolium, KN-62 (1 μM) blocked the linear component of nicotine-evoked dopamine release. However, at this concentration, KN-62 had no discernable effect on the sucrose-evoked release. The results with calmidazolium and KN-62, together with the inability of Ba²⁺ to substitute for Ca²⁺ in the nicotine enhancement of sucrose-evoked release, indicate that Ca²⁺ entry through α7 nAChRs alters synaptic vesicle dynamics via a calmodulin-dependent signaling pathway that does not require calmodulin kinase activity. Furthermore, these results suggest that the linear phase of nicotine-evoked release may involve a calmodulin kinase-dependent mechanism, because both calmidazolium and KN-62 inhibited this process. Although the accuracy of conclusions made using a pharmacological approach is limited by the uncertain specificity of these drugs, it offers the ability to access intracellular signaling pathways that are otherwise difficult to study. More specific approaches will be required to help confirm the intracellular events that couple Ca²⁺ entry to synaptic vesicle mobilization in striatal dopamine terminals.

Discussion

Defining the role of nAChRs in the physiology and pharmacology of CNS neurons is an important task given the widespread distribution of cholinergic systems in the brain and the social significance of chronic tobacco use (McGehee and Role, 1995; Wonnacott, 1997). It is believed that cholinergic interneurons in the ventral striatum and cholinergic projection neurons that connect the pedunculopontine tegmental nucleus to the ventral tegmental area supply the acetylcholine that modulates presynaptic dopamine release and the firing of dopamine neurons, respectively. However, the physiological actions of nicotine appear to be quite complex and cannot be explained solely on the basis of acute activation of nAChRs. Chronic nicotine exposure, similar to what might be expected for smokers, primarily results in nAChR desensitization because the effect of nicotine is comparable with that of receptor antagonists, such as mecamylamine (Zhou et al., 2001). Furthermore, recent results obtained from striatal slices indicate that this desensitization may play an important role in the behavioral reinforcement observed for tobacco users. Rice and Cragg (2004) showed that nicotine suppressed striatal dopamine levels in response to low-frequency stimuli but augmented them at high (25–100 Hz) frequencies. Zhang and Sulzer (2004) demonstrated a similar frequency dependence of dopamine release and further showed that nicotine (or mecamylamine) could produce a short-term, Ca²⁺-dependent facilitation of electrically evoked dopamine release. The results reported here expand the complexities of presynaptic regulation of dopamine release prob-
ability by invoking an α7-mediated regulated change in the size of the releasable pool of dopamine.

**Two distinct actions of nicotine found in striatal synaptosomes**

Biochemical measurement of radiolabeled dopamine release from mouse brain synaptosomes was used to examine the actions of nicotine on dopamine nerve terminals. These results confirm previous findings that nicotine stimulates dopamine release by activating presynaptic, non-α7 nAChRs. These receptors act to depolarize the terminals, triggering dopamine release by activating neuronal calcium channels. Those findings were extended by the demonstration that nicotine stimulated a Ca²⁺-dependent increase in synaptic vesicle availability, as assessed by the ability of hypertonic saline to evoke exocytosis of dopamine-containing vesicles. Unexpectedly, the ability of nicotine to modulate synaptic vesicle mobilization was blocked by αBgtX and methyllycaconitine but not by 5 µm mecamylamine or dihydro-β-erythroidine, indicating that the ability of nicotine to enhance the response to hypertonic saline was mediated by an α7-containing nAChRs. This view was reinforced by the finding that choline, a selective α7 agonist, did not evoke [³H]dopamine release but selectively enhanced the response to sucrose in an αBgtX-sensitive manner.

Calmodulin (but not a CaM kinase) inhibitors selectively blocked the effects of nicotine on the response to hypertonic saline, suggesting a role for calmodulin in the signaling pathway. Both classes of inhibitor blocked the linear, or late, phase of nicotine-evoked release. Although this may represent a nonselective effect of these relatively hydrophobic agents, it is also consistent with the observation that the selective α7 nAChR agonist choline specifically enhanced synaptic vesicle availability without directly evoking exocytosis. Evidence that in situ application of αBgtX (but not mecamylamine) into the nucleus accumbens suppressed nicotine-stimulated dopamine release (Fu et al., 2000) is consistent with this view.

Although it has been well documented that the α7 nAChR has a large Ca²⁺ permeability, there is surprisingly little evidence for a functional role for Ca²⁺ entry through the α7 nAChRs in neurons. α7 nAChRs have been shown to be necessary for reliable synaptic transmission during early development in the chick (Chang and Berg, 1999), and the same group has demonstrated α7-dependent calcium transients in dendritic spines (Shoop et al., 2001). The data presented here demonstrate that Ca²⁺ entry via αBgtX-sensitive nAChRs is selectively coupled to synaptic vesicle dynamics and that the signaling pathway that mediates this effect likely involves calmodulin. The ability of the α7-selective agonist choline to selectively enhance the sucrose-evoked response without directly triggering dopamine release is consistent with the view that α7 nAChRs are relatively sparse in the dopamine terminals. Morphological evidence suggests that α7 nAChR targeting and localization is tightly specified by protein–protein interactions between the second intracellular loop of nAChRs and various cytoskeletal elements (Williams et al., 1998). Additional evidence supports the possibility that α7 nAChRs are localized in lipid raft signaling domains (Oshikawa et al., 2003; Roth and Berg, 2003). Together with the results in this Ca²⁺-dependent modulation of synaptic vesicle dynamics occurs via a tight “nanodomain” coupling of the α7 nAChRs into the signaling machinery at dopamine terminals and is consistent with the observation that the selective α7 nAChR agonist choline specifically enhanced synaptic vesicle availability without directly evoking exocytosis. Evidence that in situ application of αBgtX (but not mecamylamine) into the nucleus accumbens suppressed nicotine-stimulated dopamine release (Fu et al., 2000) is consistent with this view.

Nicotine activates two distinct signaling pathways in striatal dopamine terminals

The consensus view holds that presynaptic nAChRs regulating striatal dopamine release are non-α7, because nicotine-induced release is αBgtX insensitive and αBgtX binding site density is relatively low in this region. The evidence that α7 nAChRs on dopamine terminals can regulate synaptic vesicle dynamics adds to the complexity of presynaptic cholinergic mechanisms. Anatomical and pharmacological evidence in support of these findings has been reported recently. Reverse transcription–PCR amplification of RNA from individual cells in ventral midbrain that innervate the nucleus accumbens indicates that ~40% of the tyrosine hydroxylase-positive dopamine cells express mRNA encoding α7 along with other more common nAChR subunits (Klink et al., 2001). Low levels of α7 nAChRs in the dopamine terminals suggested by cytological studies may indicate that the α7 nAChR targeting and localization is tightly specified by protein–protein interactions between the second intracellular loop of nAChRs and various cytoskeletal elements (Williams et al., 1998). Additional evidence supports the possibility that α7 nAChRs are localized in lipid raft signaling domains (Oshikawa et al., 2003; Roth and Berg, 2003). Together with the results in this
This report documents how presynaptic depolarization-dependent gating of exocytotic calcium channels increases the size of the readily releasable pool (RRP) of dopamine in striatal neurons. It is well established that non-7 α7 nAChRs in regulating striatal dopamine release. It is well established that non-7 α7 nAChRs in regulating striatal dopamine release. It is well established that non-7 α7 nAChRs in regulating striatal dopamine release. It is well established that non-7 α7 nAChRs in regulating striatal dopamine release. It is well established that non-7 α7 nAChRs in regulating striatal dopamine release.

**Measuring the releasable vesicle pool with hypertonic saline**

The conclusions made regarding changes in the size of the RRP rely on the technique first described in the 1950s (Fatt and Katz, 1952; Furshpan, 1956) and more recently refined by Stevens and colleagues. The mechanisms by which hypertonic solutions promote vesicular release remain obscure but presumably involve mechanical forces imposed on the active zone by the gradient in osmotic pressure, perhaps by differential dehydration of the cytoplasmic space relative to the synaptic vesicle. With respect to this study, the relevant question is whether these observations of the action of hypertonic sucrose on synaptosomal [3H]dopamine release are a meaningful measure of the docked, fusion-competent population of synaptic vesicles in dopamine terminals. Despite some obvious differences between the two systems, the similarities between these results and the sucrose-evoked glutamatergic currents in hippocampal autaptic neurons are striking. First, the time course of sucrose-evoked dopamine release from synaptosomes and the autaptic EPSP events in hippocampal neurons are nearly indistinguishable (Rosenmund and Stevens, 1996, compare their Figs. 1B, 3A). Second, phorbol esters promoted an increase in the size of the RRP and in the slope of the “linear” phase of release thought to reflect refilling rates in both cases. Phorbol treatment increased the size of the RRP by 57% in synaptosomes, comparable with the 65% increase reported previously (Stevens and Sullivan, 1998). Finally, reserpine, a selective inhibitor of the vesicular monoamine transporter, was used to demonstrate that osmotically evoked release of dopamine originated from a vesicular pool, because reserpine treatment reduced the size of the sucrose response by 71% but only diminished dopamine content by 36%. The ability to make rapid measurements on transmitters such as dopamine that do not activate ionotropic receptors underscores the advantages of taking a biochemical approach to measuring radioisotope flux in striatal synaptosomes to study vesicle dynamics in monoaminergic terminals.

The regulation of synaptic vesicle dynamics by presynaptic signaling mechanisms has been studied extensively over the past decade. There is compelling evidence that the synaptic vesicle cycle is regulated at several steps and that multiple signaling pathways are involved. The size of the RRP of dopamine, and the rate of refilling of that pool, was increased by PDBu in a manner consistent with that described previously. Such results indicate that Ca2+ entry via the α7 nAChR regulates the RRP in a similar manner. The signaling pathways involved are yet to be delineated, but previous work on monoaminergic vesicles in chromaffin cells indicated that Ca2+ has multiple actions on vesicle dynamics in that system. Thus, there may be significant similarity between epinephrine-containing vesicles in the adrenal medulla and dopaminergic vesicles of nigral neurons.

**Physiological significance of transmitter regulation of synaptic vesicle dynamics**

These results support distinct roles for multiple subtypes of nAChRs in regulating striatal dopamine release. It is well established that non-7 α7 nAChRs trigger dopamine secretion by depolarization-dependent gating of exocytotic calcium channels. This report documents how presynaptic α7 nAChRs regulate the size of the RRP in a Ca2+-dependent manner. The observation that the α7-selective agonist choline activates this pathway suggests a novel mechanism for regulating the probability of dopamine release by increasing the size of readily releasable vesicles. According to this view, acetylcholine, or its hydrolysis product choline, would activate presynaptic α7 receptors, leading to a calcium-dependent enhancement of the amount of dopamine available for release. This enhancement might be accomplished by increasing the rate of synaptic vesicle mobilization from a reserve pool, allowing them to dock and to be primed for release. Because of the relatively low potency of acetylcholine on α7 nAChRs, it is possible that the two activities described here act sequentially. At low levels of cholinergic stimulation, the non-α7 pathway predominates, promoting modest amounts of dopamine release attributable to increased calcium channel activity. At higher levels of acetylcholine release, α7 nAChR-dependent increases in vesicle refilling could help replenish the RRP. Alternatively, extracellular choline produced by acetylcholinesterase could selectively activate the α7-dependent refilling pathway. Either of these scenarios could constitute a positive feedback loop opposing activity-dependent depletion, helping to maintain release probability under conditions of high exocytotic rates.

**References**


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