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Modulation of Nicotine-Evoked [^{3}H]Dopamine Release from Rat Striatal Synaptosomes by Voltage-Sensitive Calcium Channel Ligands

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ABSTRACT. The calcium channel subtypes mediating nicotine-evoked [^{3}H]dopamine release from rat striatal synaptosomes were probed with L-, N-, and P-type calcium channel ligands. Responses to nicotine were blocked by the peptides ω-conotoxin GVIA and ω-agatoxin IVA. The affinity constants for these compounds were consistent with their actions at N- and P-type channels, respectively. Together, these channels mediate at least 90% of the calcium-dependent response to nicotine. The L-type antagonists nifedipine, verapamil, and nicardipine were also effective blockers of nicotine-evoked release with maximal effects of 80–100% inhibition. However, these effects occurred at concentrations 2–3 orders of magnitude higher than those necessary to block L-type channels. Moreover, Bay K8644, an L-type agonist, also blocked nicotine-evoked release. Together, these findings argue strongly against the involvement of L-type channels. BIOCHEM PHARMACOL 52;4:613–618, 1996.

KEY WORDS. nicotinic acetylcholine receptor; ω-conotoxin GVIA; ω-agatoxin IVA; dihydropyridine; neurotransmitter release; verapamil

The stimulation of [^{3}H]dopamine release from striatal synaptosomes has been used extensively as a functional assay of the actions of central nicotinic agonists [1, 2]. Previous studies have shown this modulation to be calcium dependent [1, 3], implying the involvement of a vesicular neurotransmitter pool. Calcium flux has been presumed to occur through VSCC§ that are activated by the depolarization evoked by cation flux through presynaptic nicotinic acetylcholine receptors. However, a detailed mechanism for this process has not been characterized. In particular, the role of calcium and other ions in nicotine-evoked release is poorly understood.

To date, six VSCC types have been described: T, L, N, P, Q, and R [4–6]. These differ in pharmacology, distribution, and voltage sensitivity. Several authors have attempted to characterize the VSCC types involved in striatal dopamine release and have generally concluded that N-type channels account for approximately 40% of the calcium flux, and that there is little involvement of L-type channels [3, 7, 8]. This is in contrast to preliminary observations in our laboratory that nicardipine, an L-type antagonist, at a concentration of 10 μM, is capable of inhibiting striatal [^{3}H]dopamine release to a degree similar to that of the N-type antagonist ω-conotoxin GVIA (conotoxin) [9]. More recently, it was demonstrated that for K^+-evoked release, P-type channels are also involved and that the release process can be reduced greatly by a combination of P- and N-type antagonists [7, 10]. However, the relationship between the synaptosomal population which releases dopamine in response to nicotine and that which is capable of a depolarization-evoked release is not known. It is possible that nicotine acts only upon a subpopulation of dopaminergic terminals that may differ markedly in VSCC pharmacology. Moreover, the calcium permeability of the nicotinic receptor is well documented [11–13], and direct flux of calcium through this channel may also play a role in nicotine-evoked dopamine release.

In the present study, we have attempted to characterize the VSCC pharmacology of the nicotine-evoked dopamine release process. In contrast to previous studies, we have examined concentration–effect relationships in an attempt to correlate our results with extant binding and electrophysiological studies. In addition, we have examined the effects of a recently characterized specific P-type antagonist, ω-agatoxin IVA (agatoxin) [14], which provides a powerful new tool for dissection of the nicotine-evoked release process.

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§ *Abbreviation:* VSCC, voltage-sensitive calcium channel.
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MATERIALS AND METHODS

[^{3}H]Dopamine (40–50 Ci/mmol) was obtained from NEN-DuPont (Boston, MA). S(−)-Bay K8644, ω-conotoxin GVIA, verapamil, and nifedipine were obtained from RBI (Natick, MA). Synthetic ω-agatoxin IVA was provided by RBI as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract N01MH30003, and was also provided by Pfizer Research (Groton, CT). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) and were of the highest grade available.

Female Sprague–Dawley rats (100–200 g) were decapitated after anesthesia with 70% CO_2. Striatal tissue was dissected out rapidly and homogenized in ice-cold 0.32 M sucrose containing 5 mM HEPES (pH 7.4) (7.5 mL/striatum) using a glass/glass homogenizer. All subsequent manipulations were carried out at 4°. The tissue was then centrifuged at 1000 *g* for 10 min, and the pellet was discarded. The supernatant was centrifuged at 12,000 *g* for 20 min. The resultant pellet was resuspended in superfusion buffer (128 mM NaCl, 1.2 mM KH_2PO_4, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 1 mM ascorbic acid, 0.01 mM pargyline HCl and 10 mM glucose, pH 7.4) and centrifuged for 15 min at 25,000 *g*. The final pellet was resuspended in superfusion buffer, 1 mL/striatum, and kept on ice until required.

The synaptosomal suspension was incubated for 10 min, at 37° to restore metabolic activity. [^{3}H]Dopamine (sp. act. 40–50 Ci/mmol) was added directly to the tissue to yield a final concentration of 0.1 μM, and the suspension was incubated at 37° for a further 10 min. Ten 100-μL aliquots of tissue were added to microtiter plate wells containing 250 μL of superfusion buffer and harvested onto Gelman AE filters (6 mm diameter) using an Inotech cell harvester. Then the tissue was washed with 2 mL of superfusion buffer.

[^{3}H]Dopamine release was measured essentially as described by Grady *et al.* [2]. Tissue-loaded filters were placed onto 13 mm diameter Gelman A/E filters on an open-air support. Superfusion buffer, containing various drugs as required, was dripped onto the tissue through 0.03 in. steel tubing at a rate of 4 ml/min using a Brandel Versaflow peristaltic pump. The buffer was drawn through the filter using a second pump with an off-flow rate of 5 mL/min. All superfusion experiments were performed at room temperature. After a 5-min wash period, fractions were collected every 10 sec, using a 10 channel Gilson FC204 fraction collector. After the collection of 4 or 5 fractions to establish the baseline release, nicotine or superfusion buffer with 40 mM KCl (isotonically substituted for NaCl) was applied in the superfusion stream. Further fractions were collected after nicotine or KCl application to re-establish the baseline. Any change in the baseline observed after the removal of stimulator was assumed to have occurred in a linear fashion with time. In experiments that involved chronic application, the VSCC ligands were present during the wash period as well as during exposure to nicotine or KCl. When acute exposure was required, the drugs were present only in the stimulating solution. The limited availability of peptide toxins necessitated a modified protocol in experiments with these antagonists. Tissue was superfused at a rate of 1 mL/min for 10 min and then for 1 min at 4 mL/min before the collection of fractions. In all experiments, the superfusate was collected directly into scintillation vials, and released radioactivity was quantified using conventional liquid scintillation techniques. Nifedipine, verapamil, Bay K8644, and nicardipine were dissolved initially in DMSO which was present in the assay at a concentration of 0.1%. Control channels were exposed to identical concentrations of solvent.

Agonist-induced dopamine release was expressed relative to the basal release to allow comparison between experiments. The peak to baseline ratio was calculated as follows: % increase = 100 × (integrated peak—baseline)/baseline. Curves were fitted to the Hill equation using Inplot 4.0 (GraphPad Software, San Diego, CA).

RESULTS

Nicotine concentration dependently increased the release of [^{3}H]dopamine with an EC_{50} (concentration giving 50% of maximal response) of 104 ± 6 nM (N = 10) (Fig. 1A). A maximally effective concentration (10 μM) typically gave a release of 500% above baseline. Figure 1B shows a sample release profile. As demonstrated by previous workers [1, 3], this effect could be blocked by mecamylamine and was dependent upon the presence of Ca^{2+} (Fig. 2). Addition of 2 μM tetrodotoxin to the buffer did not alter significantly responses to 10 μM nicotine, implying that voltage-gated sodium channels do not contribute to nicotine-evoked depolarization (Fig. 2).

Release evoked by isotonically substituting KCl for NaCl in the superfusion medium was also concentration dependent with an EC_{50} of 29 ± 6 mM (N = 4) and a Hill slope of 2.4 ± 0.2. Maximal release evoked by KCl was 3700 ± 300% above baseline, around 8-fold higher than that evoked by nicotine.

Superfusion of synaptosomes for 10 min in the presence of ω-conotoxin GVIA or ω-agatoxin IVA resulted in a concentration-dependent decrease in the [^{3}H]dopamine release stimulated by 100 nM nicotine (Fig. 3). Conotoxin gave a fitted maximal inhibition of 45 ± 5% with an IC_{50} (concentration giving half-maximal inhibition) of 9.3 ± 3.2 nM (N = 4). The values for agatoxin from 4 experiments were 34 ± 8.6% and 7.9 ± 2.7 nM, respectively. Responses to 10 μM nicotine, however, were inhibited less strongly and less consistently by agatoxin or conotoxin. When both toxins were added simultaneously, the decrease in responses to 100 nM nicotine was approximately the sum of the separate effects of the toxins. In contrast, simultaneous addition of agatoxin and conotoxin resulted in synergistic inhibition

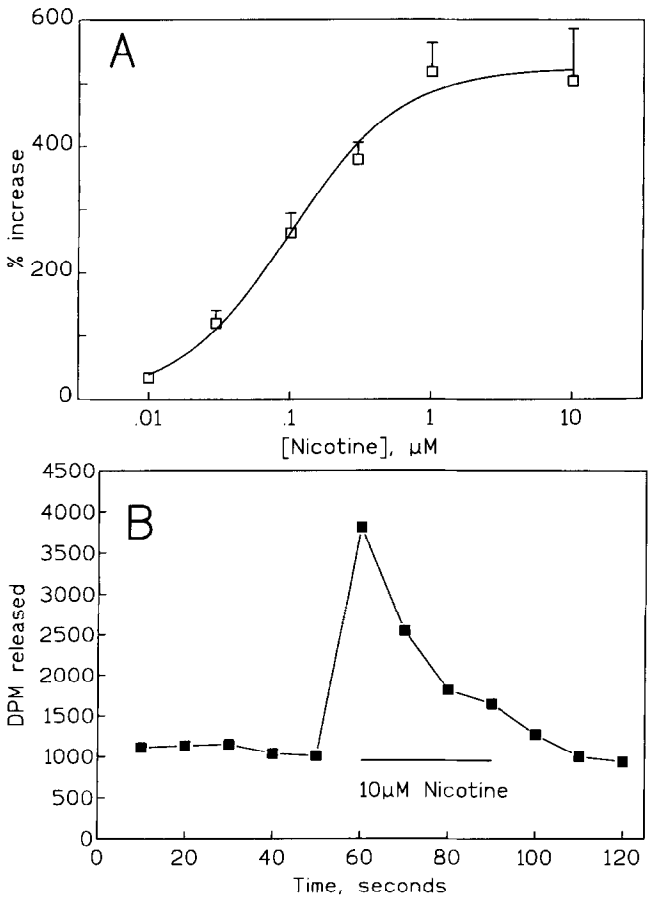

FIG. 1. Characteristics of nicotine-evoked dopamine release. (A) Representative concentration–effect curve for nicotine stimulation of [^{3}H]dopamine release. Each point is the mean ±SEM of triplicate determinations. (B) Sample superfusion profile showing the response to 10 μM nicotine applied for 30 sec (bar).

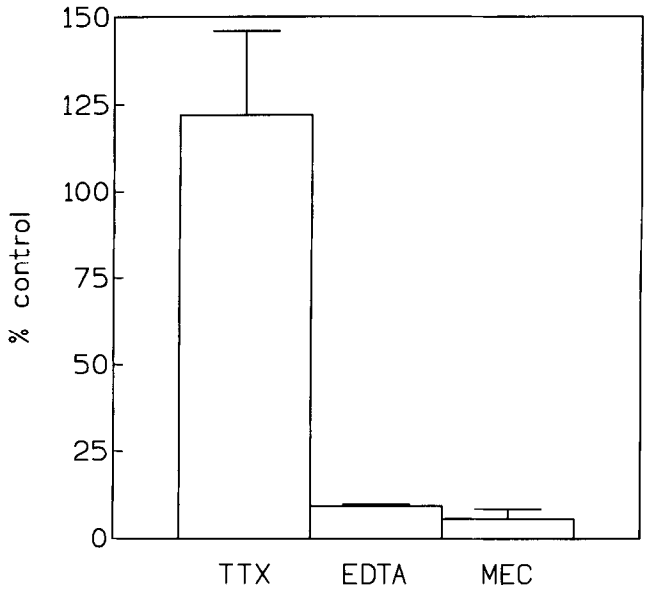

FIG. 2. Characterization of nicotine-evoked [^{3}H]dopamine release. Responses to a 30-sec stimulus with 10 μM nicotine were measured in the presence of 2 μM tetrodotoxin (TTX), 50 μM mecamylamine (MEC), or under Ca^{2+}-free conditions (EDTA). The mean control release was 538 ± 110% above baseline. The mean control baseline release was 1490 ± 420 cpm/10-sec fraction. Data are the means ± SEM of 3 determinations.

of responses to 10 μM nicotine. These results are summarized in Table 1.

The actions of L-type ligands were examined by exposing synaptosomes to various concentrations of drug for 5 min prior to stimulation with either nicotine or K^+. Nifedipine, nicardipine, and verapamil concentration-dependently inhibited the release evoked by 10 μM nicotine with IC_{50} values in the low micromolar range and maximal effects near 100% inhibition (Fig. 4A). The actions of nifedipine and verapamil on the release evoked by 40 mM K^+ were also determined. For verapamil, the IC_{50} and maximal inhibition were similar to those for inhibition of nicotine-evoked release. Determination of concentration–effect parameters for nifedipine was hampered by solubility limitations. However, fitted inhibition curves indicate that while the maximal inhibition of K^+ responses was somewhat lower than that of nicotine responses, the IC_{50} values for these two effects were similar (Fig. 4B). Table 2 provides a summary of these data. No effect of Bay K8644 upon nicotine-evoked responses was observed at concentrations of 1 μM or less, the range in which previous studies have indicated it to be an "agonist" at L-type channels, causing direct or enhanced Ca^{2+} flux [15, 16]. At higher concentrations, Bay K8644 inhibited responses to 10 μM nicotine in a concentration-dependent manner (Fig. 5 and Table 2). As direct stimulation of [^{3}H]dopamine release during chronic application of Bay K8644 might potentially lead to a reduction in the vesicular dopamine pool, and thus to a

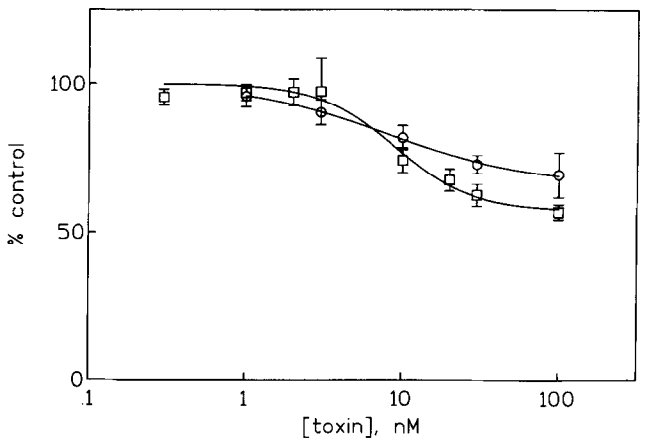

FIG. 3. Inhibition of nicotinic responses by N- and P-type VSCC antagonists. After superfusion with ω-conotoxin GVIA (□) or ω-agatoxin IVA (○) for 10 min, the synaptosomes were stimulated with 100 nM nicotine for 30 sec. Data are the means ± SEM of 4 determinations. In this series of experiments, the mean control response to 100 nM nicotine was 301 ± 38% above baseline (N = 8). Control baseline release was 1370 ± 280 cpm/10-sec fraction.

TABLE 1. Inhibition of responses to 10 μM and 100 nM nicotine by ω-conotoxin GVIA and ω-agatoxin IVA applied alone or in combination

	Inhibition of nicotine response (% of control)	
	10 μM Nicotine response	100 nM Nicotine response
ω-Conotoxin GVIA, 100 nM	77 ± 10 (3)	61 ± 3 (8)
ω-Agatoxin IVA, 30 nM	96 ± 10 (3)	69 ± 7* (3)
ω-Agatoxin IVA, 30 nM, + ω-conotoxin GVIA, 100 nM	33 ± 9† (3)	23 ± 9† (4)

Data are expressed as the means ± SEM of (N) experiments.

* Significantly different ($P < 0.05$), as determined by Student's *t*-test, from response to 10 μM nicotine.

† Significantly different ($P < 0.05$), as determined by Student's *t*-test, from response in the presence of either 100 nM conotoxin or 30 nM agatoxin alone.

reduction in subsequent responses to nicotine, the effect of acute application of Bay K8644 was also examined. When applied simultaneously with either 100 nM or 10 μM nicotine, no significant effect was observed with 100 nM Bay K8644. At a concentration of 10 μM Bay K8644, acute exposure reduced responses to 100 nM and 10 μM nicotine by 18.4 ± 5 and 17.5 ± 0.5%, respectively (N = 3 for both). Alone, neither concentration of Bay K8644 caused a significant increase in [^{3}H]dopamine release.

DISCUSSION

Previous studies aimed at characterizing nicotine-evoked dopamine release from striatal tissue have reached two main conclusions: (i) the process is largely Ca^{2+} dependent [1, 3], and (ii) around 40% of the response is mediated by voltage-sensitive calcium channels of the N type [3]. Similarly, in the current experiments, around 90% of the nicotine-evoked response was abolished in the absence of Ca^{2+}, and a maximum of 45% of the release was inhibited by the N-type antagonist ω-conotoxin GVIA. Concentration–effect measurements yielded an IC_{50} for conotoxin of 9 nM. Although this value is likely to be higher than the true K_d as complete equilibration is unlikely to have occurred at lower toxin concentrations, studies in other systems have yielded similar values for the actions of conotoxin on N-type channels [10, 16, 17].

To dissect the remaining portion of the Ca^{2+}-dependent response, we turned to the P-type selective antagonist ω-agatoxin IVA. This peptide inhibited nicotine-evoked release to an extent similar to that of conotoxin. Concentration–effect analysis yielded an IC_{50} of 8 nM, a value in the same range as affinity constants determined in other studies [6, 10, 18]. In addition to actions at P-type channels, agatoxin has also been reported to inhibit the recently characterized Q-type VSCC. Randall and Tsien [6] have reported that the agatoxin-sensitive Ca^{2+} current in cerebellar granule cells could be resolved into a P-type component with an affinity of 1–3 nM and a dominant Q-type current with an affinity of 90 nM. As with conotoxin, our data for agatoxin probably yield an underestimation of the true affinity due to non-equilibration at lower concentrations. Our results, therefore, are consistent with the presence of P-type rather than Q-type VSCCs on nicotine-sensitive dopaminergic terminals in the striatum.

When applied simultaneously, conotoxin and agatoxin inhibited around 80% of the [^{3}H]dopamine release elicited by 100 nM nicotine. This corresponds to a reduction of 90% in the Ca^{2+}-dependent release. The remaining 10% non-blockable component may reflect Ca^{2+} flux through a non-N-, non-P-type channel, direct flux through calcium permeable nicotinic receptors, or perhaps, because of the

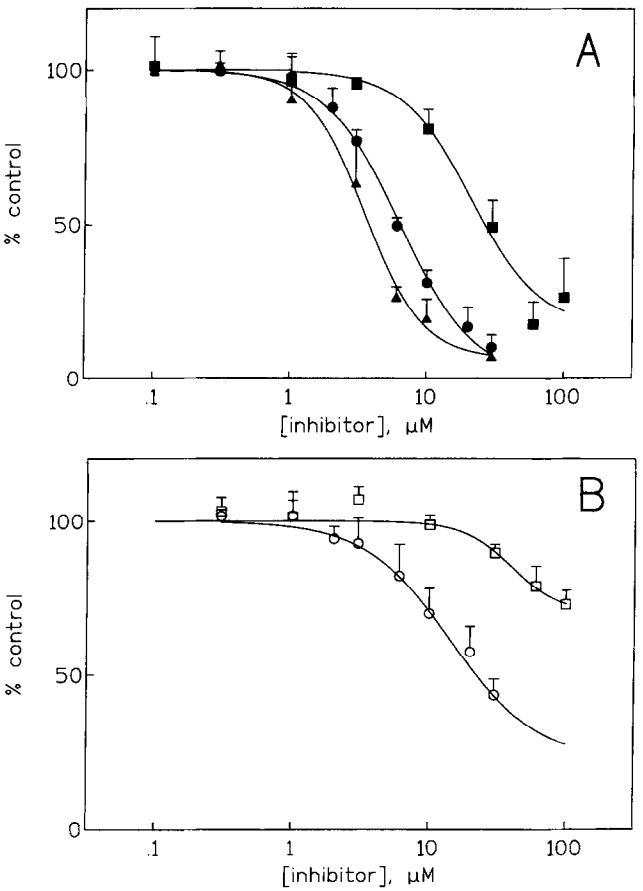

FIG. 4. Actions of L-type calcium channel antagonists upon nicotine- and potassium-evoked [^{3}H]dopamine release. Synaptosomes were exposed to various concentrations of nifedipine (squares), nicardipine (triangles), or verapamil (circles) for 5 min prior to a 30-sec stimulation with either (A) 10 μM nicotine or (B) 40 mM KCl. In both panels, data are the means ± SEM of 3 experiments. Control responses to nicotine were 539 ± 65% above a mean baseline release of 1070 ± 300 cpm per 10-sec fraction (N = 9). The mean response to KCl was 2290 ± 180% above a mean baseline of 1070 ± 270 cpm per 10-sec fraction (N = 6).

TABLE 2. Inhibition of responses to 10 μM nicotine and 40 mM KCl by L-type calcium channel ligands

	Stimulator	IC_{50} (μM)	I_{max} (%)	n_H
Verapamil	10 μM Nic	6.9 ± 0.7	100	−1.4 ± 0.2
Nifedipine	10 μM Nic	22 ± 0.5	85 ± 11	−1.6 ± 0.2
Nicardipine	10 μM Nic	3.9 ± 1.6	98 ± 2	−1.9 ± 0.6
S(−)-Bay K8644	10 μM Nic	8.3 ± 0.7	100	−1.6 ± 0.4
Verapamil	40 mM K^+	16 ± 1.3	81 ± 10	−1.5 ± 0.3
Nifedipine	40 mM K^+	41 ± 11	30 ± 8	−2 ± 0.2

Data are the means ± SEM of 3 experiments. Synaptosomes were incubated with various concentrations of L-type VSCC ligands for 5 min prior to stimulation with either nicotine or KCl. Inhibition curves were fit to the Hill equation, where n_H is the Hill slope, I_{max} is the fitted maximal inhibition, and IC_{50} is the concentration giving 50% of maximal inhibition.

slow association rates of the toxins, a small residue of unbound N- and P-type channels.

Interestingly, responses to 10 μM nicotine were blocked less effectively by conotoxin or agatoxin alone than were responses to 100 nM nicotine. Similar results were noted for the inhibition of K^+-evoked [^{3}H]dopamine release in a study by Turner *et al.* [10]. These authors suggested that this may reflect the presence of an excess of calcium channels beyond that required to elicit a maximal release. At high levels of depolarization, such as may be induced by 10 μM nicotine, most of the calcium channels would be activated, and blockade of one type alone would not be sufficient to greatly reduce the response. Blockade of two or more classes of channels, however, would result in apparent synergy as the buffering capacity of the excess channels would be removed. This hypothesis is consistent with the synergistic effects of agatoxin and conotoxin in the inhibition of responses to 10 μM nicotine, and additive effects for inhibition of release induced by 100 nM nicotine.

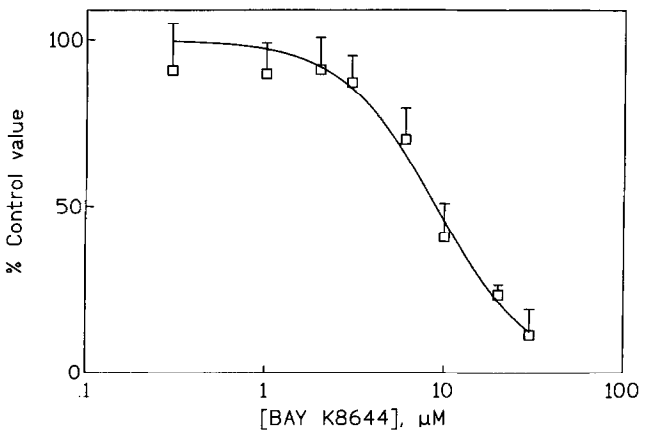

FIG. 5. Inhibition of responses to 10 μM nicotine by S(−)-Bay K8644. Synaptosomes were superfused with Bay K8644 for 5 min prior to stimulation with nicotine for 30 sec. Data are the means ± SEM of 3 experiments. In this series of experiments, the mean response to nicotine was 428 ± 33% above a mean baseline release of 1040 ± 90 cpm per 10-sec fraction.

The second part of this paper represents an extension of our preliminary report that nicardipine is an effective inhibitor of [^{3}H]dopamine release from striatal synaptosomes [9]. These findings are in contrast to other studies in which L-type dihydropyridine ligands were noted to be without effect [3, 8]. However, the actions of these ligands on L-type channels generally have been described as occurring at concentrations 2–3 orders of magnitude lower than the effects seen in the present study. For example, Collins and Lile [19] determined an EC_{50} of 11 nM for the potentiation by Bay K-8644 of K^+-mediated neuron survival, and Triggle [4] reported an IC_{50} for nifedipine in ileal muscle of 30 nM. These values contrast with our affinity determinations of 8.3 and 22 μM, respectively, arguing against an L-type mediation of dopamine release. Further evidence against the involvement of these channels comes from the inhibitory effect of Bay K8644 during both chronic and acute application. This L-type "agonist" would be expected to enhance or directly stimulate [^{3}H]dopamine release if L-type VSCCs played a significant role in the release process [15, 16]. Finally, we have demonstrated [^{3}H]dopamine release to be largely dependent upon the activation of N- and P-type channels, with a maximum of 10% of Ca^{2+}-dependent dopamine efflux unaccounted for. It is difficult, therefore, to envision a scheme that could also accommodate complete inhibition of release by L-type ligands acting at L-type VSCCs. Several reports have indicated that L-type ligands have actions at a much wider range of sites than originally supposed. Verapamil has been demonstrated to block ATP-dependent K^+ currents [20], while dihydropyridines have been shown to act at the *N*-methyl-D-aspartate (NMDA) receptor channel [21] and at both adenosine receptors and transporters [22]. Many of these effects occur at concentrations in the same range as the inhibition of dopamine release noted in the present study. Additionally, it has been demonstrated that L-type ligands are noncompetitive inhibitors of peripheral nicotinic receptors [23, 24] and that these effects, too, occur in the low micromolar range. In the light of these latter studies, it is tempting to infer that our results are due to similar actions at the striatal nicotinic receptor or perhaps to low affinity competition at the nicotine binding site. However, the inhibitory effects of verapamil and nifedipine upon K^+-evoked release tend to preclude this and suggest that the site of action of these compounds lies downstream from receptor activation in the release cascade. Given the complexity of the release process, there are a large number of candidate targets for this effect. Investigation of the exact site of action of L-type ligands will require the use of assay techniques capable of dissecting dopamine release into its component processes.

In summary, we have demonstrated that the release of dopamine from striatal synaptosomes is mediated by a combination of P- and N-type voltage-sensitive calcium channels. While L-type calcium channel ligands are also effec-

tive blockers of this release process, the evidence suggests that this inhibition is not mediated by actions at L-type channels. Instead, we hypothesize that these compounds act at some other, unidentified component of the release cascade.

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