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Brain voltage-sensitive calcium channel subtypes differentiated by ω-conotoxin fraction GVIA

(calcium uptake/calcium antagonist/neurotransmitter release/fura-2)

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ABSTRACT **We have studied the voltage-activated influx of Ca^{2+} into synaptosomes. Rapid $^{45}Ca^{2+}$ influx into synaptosomes, measured at 1 sec, was blocked by predepolarization and by low concentrations of cadmium (IC_{50}, 1 μM), as anticipated for voltage-sensitive calcium channels (VSCCs). However, fluxes were insensitive to dihydropyridine drugs that block or activate VSCCs, including nitrendipine, Bay K 8644, and (+)- and (−)-PN202-791. Phenylalkylamine calcium antagonists, including verapamil and desmethoxyverapamil, blocked $^{45}Ca^{2+}$ uptake in a nonspecific fashion. The peptide ω-conotoxin fraction GVIA (ω-CgTx GVIA) blocked $^{45}Ca^{2+}$ uptake in a biphasic fashion, with a 30% reduction at 50 pM toxin and a further decrease at concentrations > 5 nM. The toxin inhibited neurotransmitter release from synaptosomes in nanomolar concentrations, corresponding to its low-affinity effects on $^{45}Ca^{2+}$ influx. ω-CgTx GVIA also inhibited depolarization-induced increases in intracellular Ca^{2+} concentration in single hippocampal and striatal neurons. These findings indicate that ω-CgTx GVIA blocks VSCCs in both cell bodies and nerve terminals and that the predominant form of VSCC in nerve terminals is the dihydropyridine-insensitive N type.**

Synaptosomes have been widely used for the study of voltage-sensitive calcium channels (VSCCs) in nerve terminals (1–7) and their role in the control of neurotransmitter release (6, 8, 9). This preparation has also been employed for the study of calcium antagonist receptors, using tritiated ligands such as the dihydropyridines (DHPs) nitrendipine and Bay K 8644 (4, 7, 10–12) and the verapamil analogue (−)-desmethoxyverapamil (13, 14). Such ligands bind to receptors with high affinity, specificity, and stereoselectivity. However, in most studies these drugs do not alter Ca^{2+} fluxes (1, 3, 5, 7, 11) or neurotransmitter release (15, 16) in neuronal preparations. Both DHP-sensitive and -insensitive VSCCs occur in neurons (17), and their relative roles in mediating Ca^{2+} influx and stimulus secretion coupling in synaptosomes has been unclear.

Recently, a series of peptide neurotoxins have been isolated from the venom of the cone snail *Conus geographus* (18). These toxins include agents that block voltage-sensitive sodium channels, nicotinic receptors, and VSCCs. Electrophysiological studies in peripheral neurons have demonstrated that ω-conotoxin (ω-CgTx) GVIA blocks both L-type (DHP-sensitive) and N-type (DHP-insensitive) VSCCs but not T-type (DHP-insensitive) VSCC (19, 20). The ^{125}I-labeled toxin labels high-affinity binding sites in synaptosomes (21).

In the present study, we have examined the drug and toxin sensitivity of voltage-dependent $^{45}Ca^{2+}$ fluxes in synaptosomes, using methods which minimize fluxes via the Na^+/Ca^{2+} exchange system. This has enabled us to determine the type of VSCC involved in $^{45}Ca^{2+}$ uptake and neurotransmitter release.

MATERIALS AND METHODS

Materials. Drugs were obtained from the following sources: PN200-110 and PN202-791 isomers were the gift of Sandoz Pharmaceuticals; nitrendipine and Bay K 8644 were provided by Miles Pharmaceuticals (New Haven, CT); verapamil, methoxyverapamil, and desmethoxyverapamil were provided by Knoll (Ludwigshafen, F.R.G.); and MDL 12,330A was supplied by Merrell Dow (Cincinnati, OH). ω-CgTx GVIA was prepared from the venom of *Conus geographus* as previously described (22). All choline used in uptake and release buffers was obtained from Calbiochem and was recrystallized from ethanol before use and stored under reduced pressure. All other reagents were from commercial sources.

Synaptosomal Calcium Influx. Rat brain synaptosomes were prepared for calcium uptake as follows. Male Sprague–Dawley rats (150–200 g, Hilltop Farms, Scottsdale, PA) were sacrificed by cervical dislocation and decapitation. Brains, with the cerebellum and brainstem removed, were placed on ice. All further procedures were performed at 4°C. Brains were homogenized in 8 vol of 0.32 M sucrose, using 10 up-and-down strokes of a loose-fitting motor-driven Teflon–glass homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was decanted, diluted 1:1 with basal buffer (composition, in mM: choline chloride, 145; KCl, 3; $MgCl_2$, 1; $CaCl_2$, 0.5; Hepes, 10; pH adjusted to 7.4 with Tris base), and centrifuged at 10,000 × *g* for 15 min. The pellet was resuspended in 4 ml of basal buffer per brain, using four strokes of the homogenizer. Synaptosomes were then stored on ice until use, always within 30 min, to minimize loss of uptake activity. Drugs, when used, were added during this incubation period. Uptake was initiated by injecting 50 μl of the tissue stock solution that had been briefly prewarmed to tubes containing 950 μl of basal or depolarizing buffer (50 mM KCl isoosmotically substituted for choline) with $^{45}CaCl_2$ at 0.5–1 μCi/ml (1 Ci = 37 GBq) and drugs as appropriate. The reaction was timed with a electronic metronome and terminated by the addition of 3 ml of ice-cold stop buffer (160 mM choline chloride, 2 mM $CaCl_2$, 20 mM Tris·HCl, 2 mM $LaCl_3$, pH 7.4), usually after 1 sec. The synaptosomes were then collected on glass-fiber filters (number 32, Schleicher & Schuell) using a Brandel cell harvester (Brandel, Gaithersburg, MD), and washed twice with 4 ml of ice-cold buffer. Retained radioactivity was determined by liquid scintillation spectrophotometry. Voltage-dependent $^{45}Ca^{2+}$ uptake was

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Abbreviations: ω-CgTx GVIA, ω-conotoxin fraction GVIA; DHP, dihydropyridine; VSCC, voltage-sensitive calcium channel.

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defined as the difference between uptake in depolarizing and basal buffers.

Neurotransmitter Release. Synaptosomes for neurotransmitter release were prepared in a similar fashion except that basal buffers contained 95 mM NaCl and 50 mM choline chloride, while depolarizing buffers contained 95 mM NaCl and 50 mM KCl. Sodium was added to permit neurotransmitter uptake. Ascorbic acid (100 μM) and pargyline (10 μM) were also present in release buffers. Synaptosomes were incubated for 5 min at 30°C with [^{3}H]norepinephrine or [^{3}H]serotonin at 5 μCi/ml, diluted with 10 ml of cold basal buffer, and centrifuged at 10,000 × *g* for 5 min. The tissue was then resuspended and kept on ice until use. To measure release, 25-μl aliquots of tissue were added to tubes and incubated briefly at 30°C. Synaptosomes were then loaded onto a soaked filter in a Brandel cell harvester and washed three times with 8 ml each of basal buffer, which was discarded. A fraction collector was attached to the cell harvester, and 2 ml of basal or depolarizing buffer was passed over the synaptosomes, resulting in a 1-sec exposure to basal or stimulating buffers. This was followed by a wash with 3 ml of basal buffer, all at 30°C. The radioactivity levels released and retained on the filter were both estimated, and the tritium release was expressed as a proportion of the total radioactivity present.

Fura-2 Measurements. Neurons were cultured as described below. The dissection followed that described by Hemmendinger *et al.* (23), and culture conditions were essentially those of Messer *et al.* (24). Modifications to the growth conditions consisted of the inclusion of 10% fetal bovine serum in the media for the first 24 hr and its replacement with 10% horse serum for the remaining days in culture. Additionally, cells were cultured on no. 1, 25-mm-diameter coverslips that had been coated with polylysine (2 μg/ml, Sigma), rinsed, and coated with laminin (6 μg/ml, Collaborative Research, Waltham, MA) for 6 hr.

Intracellular calcium ($[Ca^{2+}]_i$) was determined in single cells by previously described methods (25), using the calcium-sensitive dye fura-2 (30). Cells were incubated with 5 μM fura-2 acetoxymethyl ester for 1 hr at 37°C in Hepes-buffered Hanks' balanced salt solution, pH 7.4, containing 0.5% bovine serum albumin. Following two 3-ml washes with buffer (without bovine serum albumin) the cells were incubated for 30 min at 37°C. The coverslips with attached cells were mounted in the microspectrofluorimeter for $[Ca^{2+}]_i$ measurement. $[Ca^{2+}]_i$ was determined by measuring the ratio of fluoresence emission at 510 nm after excitation at either 340 or 380 nm and quantitated by using a calibration curve over the range 1–1000 nm. Cells were depolarized by changing the perfusing solution from 5 mM KCl to 50 mM KCl with potassium exchanged isoosmotically for choline. In calcium-free buffer, calcium was replaced by 1 mM sodium EGTA.

RESULTS

Addition of synaptosomes to incubation medium containing 50 mM K^+ resulted in a rapid influx of $^{45}Ca^{2+}$ that was maximal within 20 sec. As reported by others (3, 7), this influx was reduced approximately 65% after predepolarization in the absence of $^{45}Ca^{2+}$ (control uptake, 2.4, predepolarized uptake 0.8 nmol of $^{45}Ca^{2+}$/mg of protein, measured at 1 sec). $^{45}Ca^{2+}$ influx was also blocked by Cd^{2+} (Fig. 1*A*) with an IC_{50} of approximately 1 μM when fluxes were measured at 1 sec. In contrast, when fluxes were measured at 10 sec, Cd^{2+} was about 1/10th as potent. We have found that Na^+/Ca^{2+} exchange in these synaptosomal preparations is 1/30th to 1/50th as sensitive to blockade by Cd^{2+} as voltage-stimulated uptake measured at 1 sec is (unpublished observations). These findings, like those of Nachshen (26), suggest that Na^+/Ca^{2+} exchange takes place even in nominally Na^+-free buffers, possibly due to a residual intracellular sodium. This effect can be minimized by measuring fluxes at 1 sec. Accordingly, drug actions were studied on $^{45}Ca^{2+}$ uptake terminated after 1 sec.

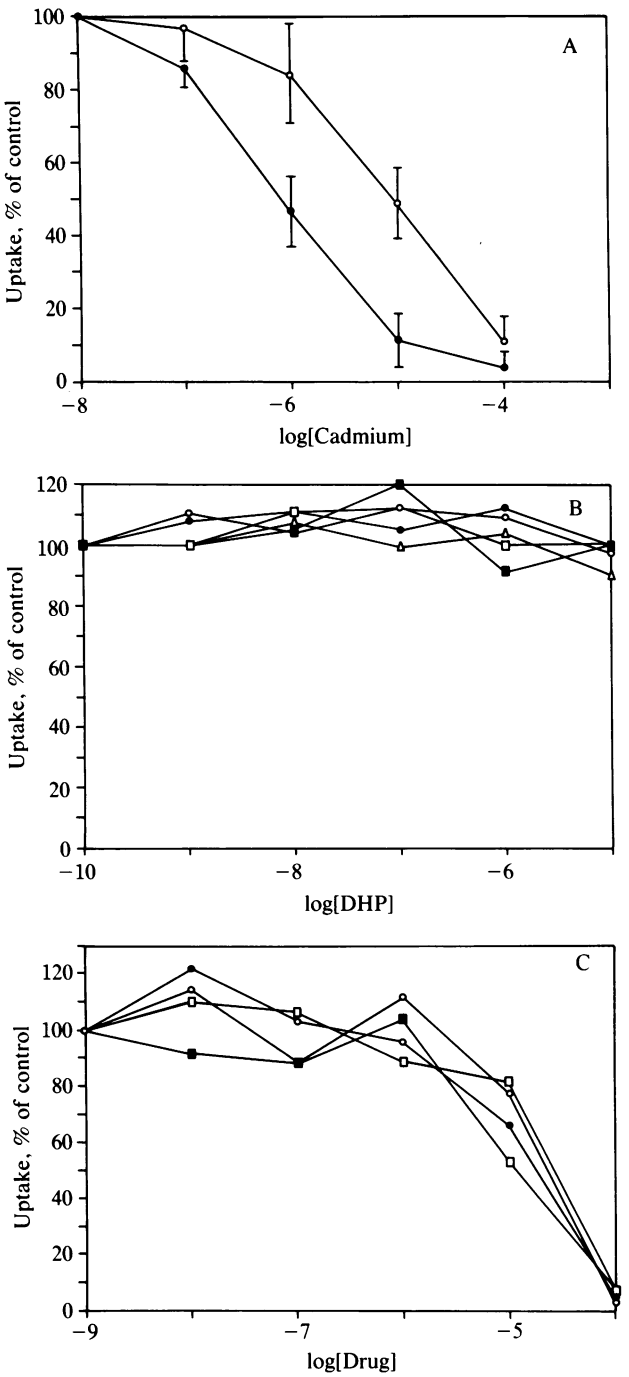

FIG. 1. Effects of cadmium, DHPs, and verapamil derivatives on voltage-sensitive calcium uptake into synaptosomes. $^{45}Ca^{2+}$ influx was measured as described in the text. Control values represent the difference between basal and stimulated uptake in the absence of drugs. (*A*) Effects of Cd^{2+} on uptake measured at 1 (●) and 10 sec (○). (*B*) Effects of DHP agonists and antagonists Bay K 8644 (△), nifedipine (■), nitrendipine (□), (+)-PN202-791 (○), and (−)-PN202-791 (●) on uptake. (*C*) Effects of verapamil derivatives (−)-desmethoxyverapamil (□), (+)-desmethoxyverapamil (●), methoxyverapamil (○), and verapamil (■). Results shown are mean (±SEM in *A*) of three to six experiments performed in quadruplicate. In *B* and *C*, SEM values are less than 15% of the values shown.

DHP agonists and antagonists did not alter $^{45}Ca^{2+}$ influx over a wide concentration range (Fig. 1*B*). Thus, the antagonists nitrendipine, nifedipine, and (−)-PN202-791 did not

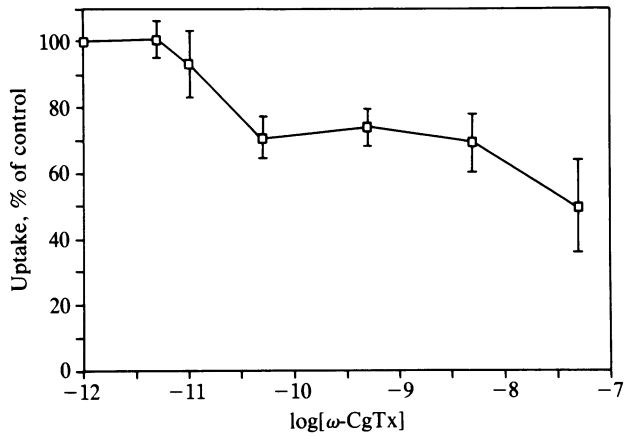

FIG. 2. Effects of ω-CgTx GVIA on voltage-stimulated calcium uptake. Uptake was measured as described in the text. Control values represent the difference between basal and stimulated uptake in the absence of drugs. The results shown are the mean of four experiments performed in quadruplicate. Bars represent SEM.

inhibit influx of $^{45}Ca^{2+}$, and the agonists Bay K 8644 and (+)-PN202-791 did not enhance uptake. Furthermore, drug effects were not increased by 1 μM MDL 12,330A, a potent diltiazem-like drug that increases the affinity of DHPs for their binding sites (27). Phenylalkylamine drugs of the verapamil class, including (−)- and (+)-desmethoxyverapamil, methoxyverapamil, and verapamil, inhibited uptake only in concentrations exceeding 1 μM, without stereoselectivity and with relative potencies unlike those found in binding studies (Fig. 1*C*) (13, 14). Though calcium antagonist drugs failed to influence $^{45}Ca^{2+}$ flux in a receptor-relevant fashion, [^{3}H]nimodipine and (−)-[^{3}H]desmethoxyverapamil did bind to receptors in tissue preparations identical to those employed for these $^{45}Ca^{2+}$ flux studies.

ω-CgTx GVIA very potently blocked depolarization-induced synaptosomal $^{45}Ca^{2+}$ influx (Fig. 2). This effect was biphasic, with 30% of voltage-stimulated $^{45}Ca^{2+}$ influx inhibited by 50 pM toxin and a second phase of inhibition at toxin concentration >5 nM. ω-CgTx GVIA also decreased voltage-stimulated release of [^{3}H]norepinephrine and [^{3}H]serotonin from synaptsomes (Fig. 3). This effect was observed only at concentrations above 5 nM toxin and therefore apparently involves the low-affinity component of toxin actions on $^{45}Ca^{2+}$ influx.

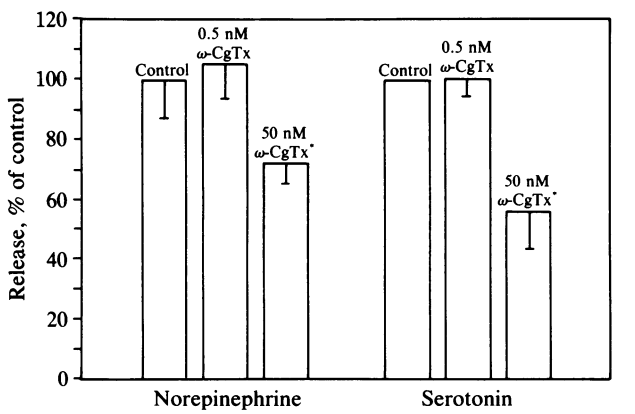

FIG. 3. Effects of ω-CgTx GVIA on depolarization-induced neurotransmitter release. Release of [^{3}H]norepinephrine and [^{3}H]serotonin was measured as described in the text. Control release is the fraction of the total radioactivity released by depolarization, which was set at 100%. Results are the mean of four experiments performed in triplicate. Vertical bars represent SEM.
*Significantly different from control ($P < 0.05$).

These results indicate that ω-CgTx GVIA blocks VSCCs in nerve terminals. To determine whether the toxin blocks VSCCs in other portions of the neuron, we examined the voltage-sensitive uptake of Ca^{2+} into cell bodies of single striatal and hippocampal cells in culture, using fura-2 microspectrofluorimetry (Fig. 4). Perfusion of the cell with Na^+-free choline$^+$-containing buffer, to minimize the contribution of Na^+/Ca^{2+} exchange, produced a small transient rise in $[Ca^{2+}]_i$. Subsequent depolarization with 50 mM K^+

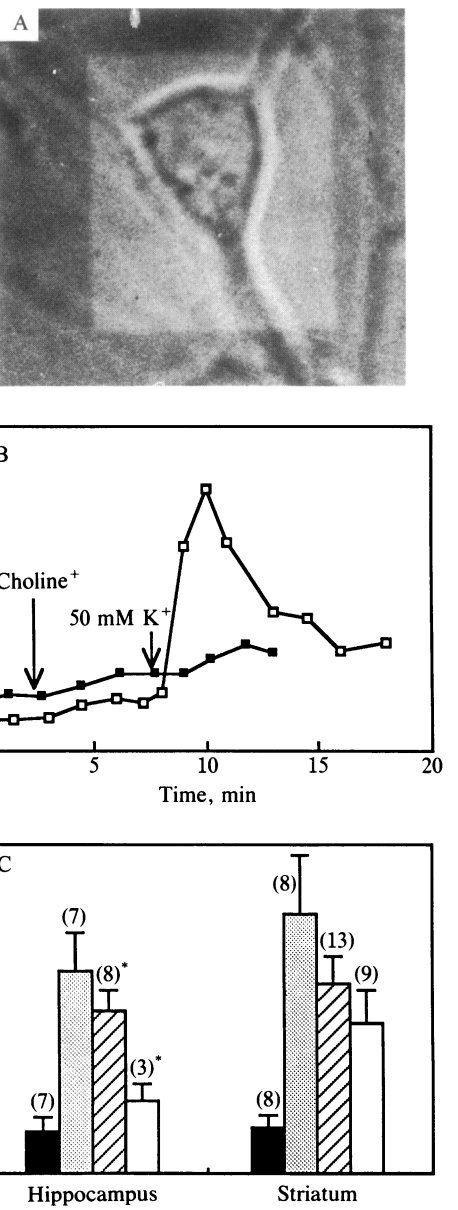

FIG. 4. Effects of nitrendipine and ω-CgTx GVIA on depolarization-induced increases in $[Ca^{2+}]_i$ measured with fura-2. (*A*) Hippocampal neuron mounted in microspectrofluorimeter. The rectangular image of the backlit photomultiplier field diaphragm defines the area from which photons were counted in this cell. (*B*) Time course of typical experiments run in the absence of ω-CgTx GVIA (□) or after 1-hr pretreatment with the toxin at 10 nM (■). (*C*) Histogram showing results of experiments using hippocampal and striatal neurons performed as shown in *B*. The data show resting $[Ca^{2+}]_i$ (black bar), $[Ca^{2+}]_i$ following depolarization by 50 mM K^+ in Na^+- (stippled bar) and choline- (hatched bar) based buffers, and depolarization in choline-based buffer after 1-hr pretreatment with 10 nM ω-CgTx GVIA (open bar). Fura-2 measurements were made as described in the text. The results shown are mean values (+SEM) with the number of cells shown in parentheses.
*Significantly different from results with 50 mM K^+.

elicited a large increase in $[Ca^{2+}]_i$ (Fig. 4*B*). In both hippocampus and striatum a portion of the 50 mM K^+-induced rise in $[Ca^{2+}]_i$was blocked by ω-CgTx GVIA (10 nM) (Fig. 4*C*). This portion was larger in hippocampal than striatal neurons. It has also been demonstrated that a portion of the flux in both striatal and hippocampal neurons can be blocked by nitrendipine (S.A.T., S. M. Murphy, and R.J.M., unpublished observations). Interestingly, the amount of block produced by ω-CgTx GIVA (10 nM) was similar in magnitude to that obtained with nitrendipine (1 μM) in each brain area.

DISCUSSION

Electrophysiological studies have shown that VSCCs are heterogeneous in peripheral neurons, where at least three types have been observed. The "L" type of VSCC (17) is sensitive to modulation by DHPs, whereas the VSCC subtypes designated "N" and "T" are not sensitive. ω-CgTx blocks N and L but not T-type VSCCs in chicken sensory neurons and rat sympathetic neurons (19, 20). The nature of VSCC subtypes in brain is less clear. Synaptosomal preparations, used to study $^{45}Ca^{2+}$ uptake into nerve terminals, contain high-affinity binding sites for DHPs (4, 7). However, most studies have shown that the VSCCs in synaptosomes are not sensitive to these drugs. The results reported here help clarify these questions. The VSCCs in synaptosomes monitored in this study by rapid $^{45}Ca^{2+}$ influx are (*i*) DHP insensitive; (*ii*) partially ω-CgTx sensitive; (*iii*) very sensitive to Cd^{2+}; and (*iv*) inactivated by prior depolarization. These properties coincide with those of the N-type VSCC (17, 19, 20).

ω-CgTx GVIA binds with high affinity to synaptosomal preparations (21). We find that the toxin blocks VSCCs in brain synaptosomes. The biphasic actions of the toxin on synaptosomal $^{45}Ca^{2+}$ flux suggest that more than one type of VSCC can be blocked by the toxin, fitting with recent observations of distinct high- and low-affinity binding sites for ω-CgTx in brain membranes (B.M.O., unpublished observations). We cannot say at this time whether the VSCCs that exhibit different toxin sensitivities exist in the same nerve terminals or whether they are found in separate populations of terminals.

Our data also indicate that ω-CgTx-sensitive VSCCs participate in neurotransmitter release from synaptosomes. The toxin inhibition of norepinephrine and serotonin release correlates with the lower affinity toxin effects on synaptosomal VSCCs. Conceivably, the high-affinity toxin sites are involved in the release of other transmitters. In cultured sympathetic neurons the evoked release of norepinephrine is partially blocked by subnanomolar concentrations of toxin (20). Our results implicate N-type VSCCs in voltage-sensitive $^{45}Ca^{2+}$ influx into synaptosomes and evoked neurotransmitter release. Could the [^{3}H]DHP binding sites found in these preparations, which presumably reflect L-type VSCCs, also play a role in these processes? The density of the toxin sites in synaptosomes is 10- to 20-fold higher than the number of DHP receptors (4, 7, 21). The difficulty in demonstrating DHP effects may reflect a great excess of N- over L-type VSCCs in these preparations, so that modification of 5% of the calcium flux by calcium antagonists would be hard to detect. However, Turner and Goldin (4) did report some effects of DHPs on synaptosomal Ca^{2+} flux and transmitter release.

Recent biochemical and biophysical studies have suggested that [^{3}H]DHP binding sites and DHP-sensitive VSCCs are preferentially localized in cell bodies rather than nerve terminals (28, 29). We show in this study that ω-CgTx GVIA can also block VSCCs localized on the soma of central nervous system neurons. It is not yet clear what types of VSCCs in the cell soma are being blocked by ω-CgTx GVIA. However, in contrast to influx in nerve terminals, some proportion of Ca^{2+} influx into cell bodies is DHP sensitive (S.A.T., S. M. Murphy, and R.J.M., unpublished observations). Electrophysiological studies have demonstrated that the toxin can block L- as well as N-type VSCCs. The consequences of L-type VSCC blockade in the cell soma are difficult to predict, as the function of these channels in neurons is not clear at this time.

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