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Widespread Distribution of Dihydropyridine-Sensitive Calcium Channels in the Central Nervous System

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SUMMARY

We have used microspectrofluorimetry and the Ca^{2+}-sensitive dye fura-2 to analyze the effects of dihydropyridine drugs on the voltage-sensitive influx of Ca^{2+} into central nervous system neurons grown in primary culture. Depolarization, induced by raising $[K^+]_o$, produced a rapid increase in $[Ca^{2+}]_i$ measured in single neurons from the cortex, hippocampus, striatum, septum, and cerebellum. These increases were slightly attenuated when external Na^+ was replaced by choline$^+$ and were absent in Ca^{2+}-free (1 mM EGTA) media. The depolarization-induced uptake of Ca^{2+} by neurons from all regions of the brain was enhanced by the dihydropyridine agonist BAY K8644 (1 μM). The dihydropryidine antagonist nitrendipine (1 μM) inhibited calcium influx into hippocampal and striatal neurons by 79% and 31%, respectively, indicating that different routes of voltage-dependent calcium influx predominate in neurons from different brain regions. The results establish the widespread distribution of functional dihydropyridine-sensitive Ca^{2+} channels in the central nervous system.

Several classes of VSCCs have been identified in a variety of excitable tissues (1–3). One type, designated L in the terminology of Nowycky *et al.* (3, 4), is sensitive to modulation by DHP drugs. Radiolabeled DHPs can be used to biochemically identify and characterize these VSCCs (5). In the brain for example, there is a widespread and heterogeneous distribution of ^{3}H-DHP-binding sites (6). However, although there are a few reports of effects of DHPs on neurons (7–12), the vast majority of studies have been unable to demonstrate actions of these drugs (reviewed in Refs. 1, 2, and 5). Consequently, there are questions at this time as to whether ^{3}H-DHP-binding sites in the brain really represent functional VSCCs or, if so, how widespread these VSCCs might be and, ultimately, what their functions are. In the present study, we have used microspectrofluorimetry to measure $[Ca^{2+}]_i$ in single CNS neurons in an attempt to clearly answer the first two of these questions. Surprisingly, we have found that virtually all CNS neurons possess functional DHP-sensitive VSCCs.

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Materials and Methods

Cell culture. Neurons were cultured as described below. The dissection followed that described by Hemmendinger *et al.* (13). Pregnant mice (C57BL/6J, The Jackson Laboratory) were sacrificed by cervical dislocation on the 14th or 15th day of gestation. Pregnant rats (Sprague-Dawley, Holtzman) were sacrificed by decapitation on the 18th day of gestation. The embryos were staged according to the method of Gruneberg (14). Embryos with visible deformities were discarded. The dissection and dissociation were carried out entirely in CMF-EMEM. Mouse striatal, septal, and whole brain cells were removed at embryonic (E) days E14 or E15. Cerebellar cells were removed at day E14. Rat hippocampal cells were removed at day E18. Like tissues were combined. Pieces (1–2 mm) of tissue were washed three times to remove meningeal fibroblasts and other small debris. The tissues were incubated in 0.67% (1% for cerebellum) trypsin (Worthington) for 35 min and then inactivated with 1.5 ml of FBS. The inactivated trypsin solution was removed and the tissue (still in pieces) was washed three times with the CMF-EMEM. The tissue was dissociated by gentle flushing through fine bore Pasteur pipettes. Dissociated cells were counted to accommodate a density of 1200 cells/mm^2 and plated on coverglasses (No. 1, 25 mm round) which had been coated overnight with polylysine (Sigma, 2 μg/ml), washed, coated with laminin (Collaborative Research, Inc., 6 μg/ml dissolved in CMF-EMEM) for 6 hr, and then washed with CMF-EMEM. Cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Hazelton KC

ABBREVIATIONS: VSCC, voltage-sensitive calcium channel; DHP, dihydropyridine; CNS, central nervous system; CMF-EMEM, Ca^{2+}/Mg^{2+}-free Earle's minimal essential medium buffered with HEPES; FBS, fetal bovine serum; araC, arabinosylcytosine; fura-2, 2-(6-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy-2-benzofuranyl)5-oxazole carboxylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BAY K8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate.

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Biological), penicillin (GIBCO, 5 μg/ml), and streptomycin (GIBCO, 5 μg/ml). After 24 hr, the FBS was replaced with horse serum (Hazelton KC Biological). The medium was changed every other day thereafter, using horse serum/Dulbecco's modified Eagle's medium. Glial growth was controlled with 10 μM araC (Upjohn). Striatal, cerebellar, and septal cultures were treated from days 5 to 9 with araC. Cortical, hippocampal, and whole brain cultures were treated from days 6 to 9 with araC.

Measurement of $[Ca^{2+}]_i$. Cytosolic free Ca^{2+} was determined by using a microspectrofluorimeter, which has been described previously (15), to monitor the Ca^{2+}-sensitive, fluorescent chelator, fura-2 (16). Neurons were loaded with the dye by incubation in 5 μM fura-2 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR), which is membrane permeant, for 1 hr at 37° in HEPES-buffered Hanks' balanced salt solution, pH 7.45, containing 0.5% bovine serum albumin. Following the loading incubation, during which time the dye ester is hydrolyzed by cytosolic esterases to the membrane-impermeant polycarboxylate anion that is fura-2, the cells were washed twice in the HEPES-Hanks' solution and incubated for 30 min. Complete hydrolysis of the fura-2 ester to fura-2 was confirmed by comparing excitation spectra run on individual dye-loaded cells, treated with 10 μM ionomycin to raise $[Ca^{2+}]_i$, with the spectrum of the fura-2 ester run in calibration buffer (which contains, in mM: KCl, 120; NaCl, 5; KH_2CO_4, 1; $NaHCO_3$, 5; HEPES, 20) and an equivalent concentration of Ca^{2+}. The spectrum of the fura-2 ester peaked at wavelengths slightly greater than 380 nm and was Ca^{2+} insensitive. Spectra of ionomycin-treated, dye-loaded cells peaked at less than 350 nm as expected for the fura-2-free acid in the presence of Ca^{2+} and fluoresced at an intensity near background (<10% of intensity at 340 nm) at wavelengths greater than 380 nm, indicating that there is not a significant concentration of the dye ester present in these loaded and washed central neurons.

The coverslips containing the loaded and washed cells were then mounted in a flow-through chamber for viewing. The chamber consisted of a 3 ml stainless-steel-lined well, surmounted by a rubber O-ring against which the inverted overslip was sealed by pressure. The bottom of the well was covered by a Plexiglas plate for substage illumination to localize cells. The temperature in the well was monitored by a thermocouple probe and maintained at 37° by circulating warm water in a separate surrounding cavity.

Fura-2 fluorescence from individual cells was monitored with a microspectrofluorimeter operating in the epi-illumination mode. Light from a xenon arc lamp was passed through a double-grating monochromator to the Ploem illuminator of a Leitz Orthoplan microscope. A × 54 fluorite objective was used and a rectangular diaphragm was set to limit fluorescence measurements to individual cells. The fluorescence emission was analyzed by a second double-grating monochromator and a Leitz MPV I photomultiplier tube coupled to a Princeton Applied Research photon counter. The accumulated counts were displayed as analog signals on a chart recorder and collected by an LSI-11 microcomputer. Monochromator settings were also controlled by this microcomputer, enabling the rapid (approximately 1 sec) switching between two wavelengths. Thus, quantification of cytosolic Ca^{2+} was determined by measuring the ratio of the fura-2 fluorescence detected at 510 nm when excited at either 340 or 380 nm. The fura-2 fluorescence intensity measured at 340 nm increases upon binding Ca^{2+}, whereas the 380-nm intensity reflects the dye which has not bound Ca^{2+}, therefore, the ratio of these two measurements is related to the free Ca^{2+} concentration in a manner which is not dependent on optical pathlength, dye concentration, or dye bleaching (16). A standard curve was determined for the fura-2 pentapotassium salt in calibration buffer containing Ca^{2+} and EGTA in ratios calculated to give Ca^{2+} concentrations ranging from 0 to 1000 nM. In these buffers we assume that the dye behaves like the intracellular dye. The standard curve was closely approximated ($r > 0.99$) by the equation $[Ca^{2+}] = -70.8 + 133.9\ R + 45.0\ R^2]$ in which R is the 340/380 nm fluorescence ratio. This equation was chosen for its close fit to the calibration data and enabled us to calculate Ca^{2+} concentrations from experimentally determined fluorescence ratios. Depolarization-induced uptake was produced by changing the perfusing solution from low K^+ (5 mM) to high K^+ (50 mM) with K^+ exchanged for Na^+ reciprocally. In Ca^{2+}- and Na^+- free media, 1 mM EGTA was substituted for Ca^{2+} and choline$^+$ was substituted for Na^+, respectively.

Results and Discussion

Neurons were cultured from embryonic mice and rats and grown in culture on glass coverslips. Cells were loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 and mounted in the microspectrofluorimeter as illustrated for a typical cell in Fig. 1a.

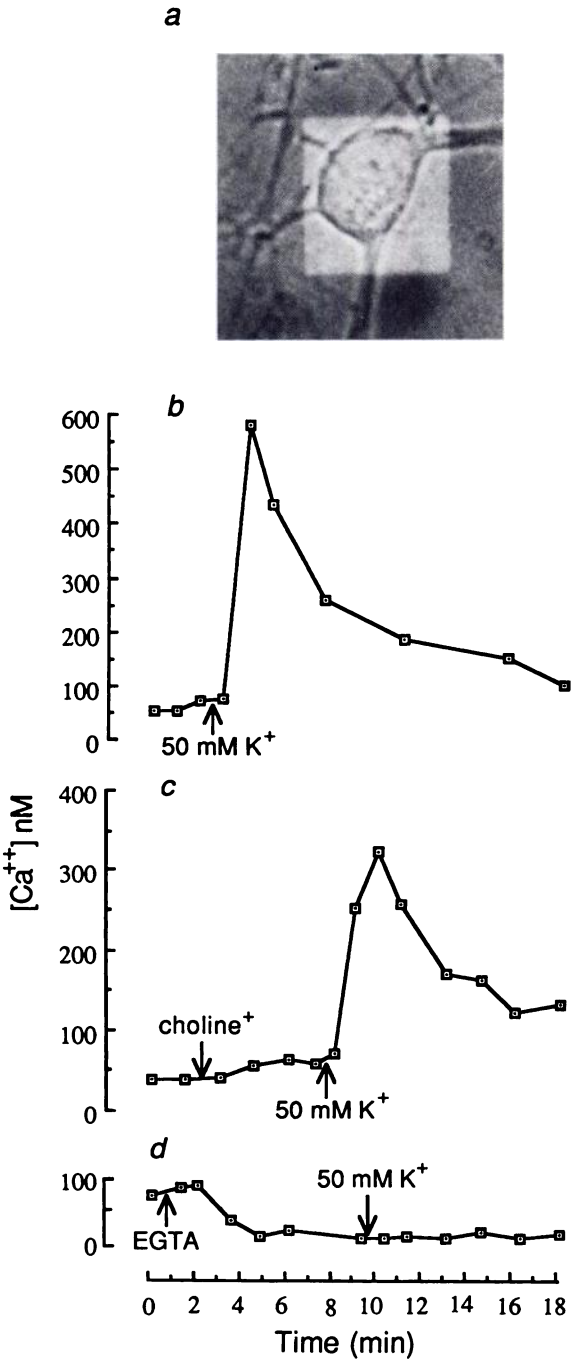

Fig. 1. a, Single hippocampal neuron mounted in the microspectrofluorimeter illustrating the depolarization (50 mM K^+)-induced increase in $[Ca^{2+}]_i$ from; b) the same cell; c, a neuron in Na^+-free (choline$^+$ substituted) medium; and d, a neuron in Ca^{2+}-free (1 mM EGTA) medium.

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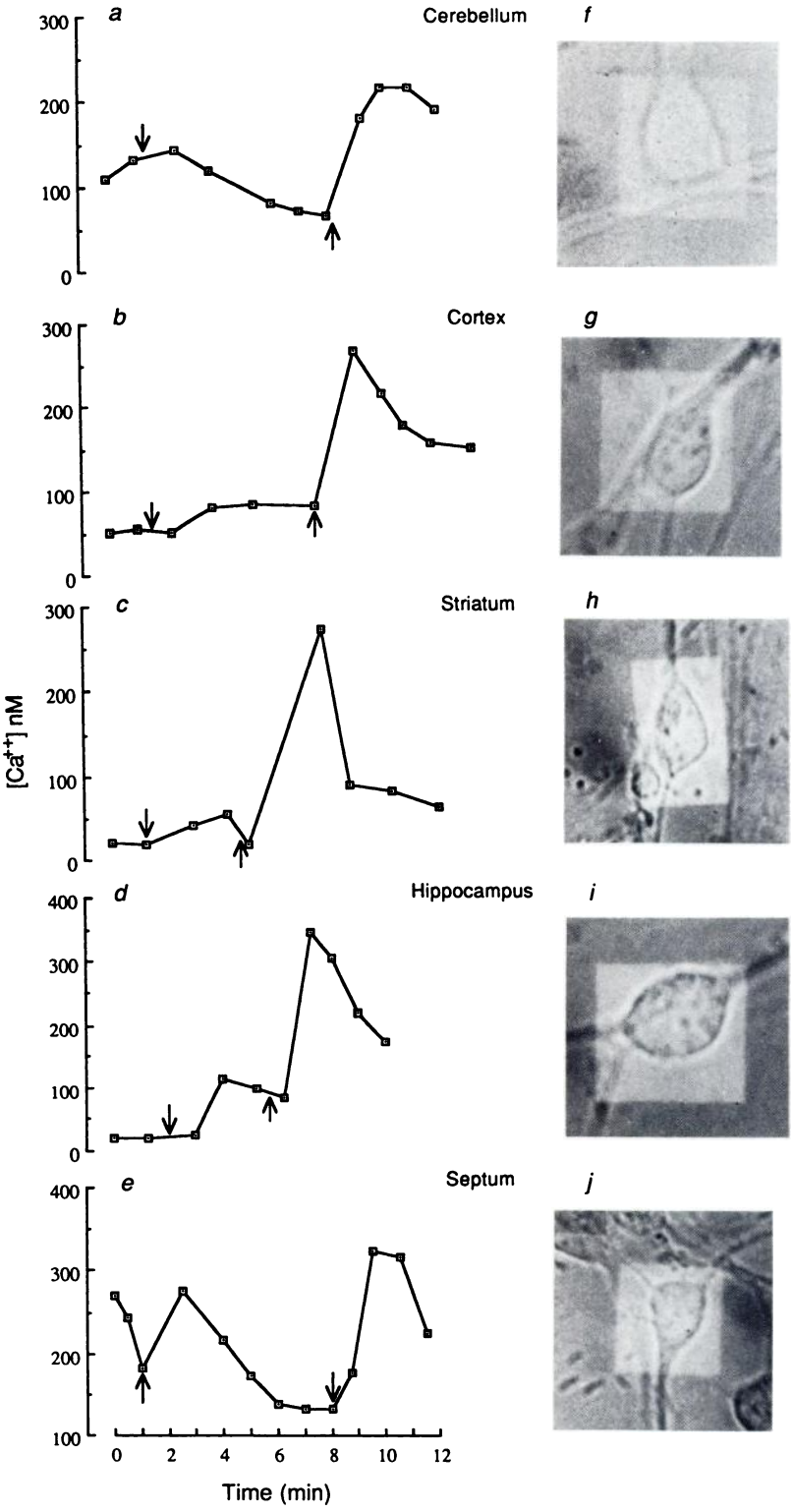

Fig. 2. BAY K8644-induced increase in $[Ca^{2+}]_i$ in central neurons. Neurons cultured from: a, cerebellum; b, cortex; c, striatum; d, hippocampus; and e, septum were perfused with 10 mM K^+ (↓) and then 1 μM BAY K8644 was added (↑). The actual cells used in these experiments are shown in f–j. Cell cultures and microspectrofluorimetry are described in Materials and Methods.

The rectangular image of the backlit photomultiplier diaphragm defines the area from which photon counts were recorded. The fura-2 fluorescence from this cell, for example, registered approximately 1000 photon cps over a typical background autofluorescence of 25 cps. In Fig. 1b the response of this hippocampal neuron to perfusion with a depolarizing 50 mM K^+ solution is shown. This response is typical. The vast majority of cells challenged with 50 mM K^+ responded with large rapid increases in $[Ca^{2+}]_i$. Eighty-cells were tested and 67 of them (84%) responded with at least a doubling of the resting $[Ca^{2+}]_i$ when stimulated with 50 mM K^+ (basal $[Ca^{2+}]_i$ = 68 ± 6 nM; 50 mM K^+-stimulated $[Ca^{2+}]_i$ = 404 ± 29). Cells that did not respond to 50 mM K^+ may have been astrocytes in some cases as it is not always easy to distinguish these from neurons on purely morphological grounds. Incubation of cells with buffers in which the extracellular Na^+ was replaced with cho-

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TABLE 1

Sensitivity of central neurons to DHPs

In all brain areas, BAY K8644 produced a significant increase in $[Ca^{2+}]_i$ above basal (10 mM K^+) levels ($p \leq 0.05$; paired t test). A cell was considered sensitive if BAY k8644 produced a 2-fold increase in $[Ca^{2+}]_i$. Cells which failed to produce at least a 2-fold increase in $[Ca^{2+}]_i$ in response to 50 mM K^+ (16%, see text) were omitted from these calculations. Experiments were performed as described in Fig. 2.

Brain area	BAY K8644 insensitive	BAY K8644 sensitive	$[Ca^{2+}]_i$	
			Basal	Stimulated
			nM	
Cerebellum	0	7	46 ± 14	199 ± 56
Cortex	0	4	74 ± 17	200 ± 39
Striatum	1	6	48 ± 12	152 ± 39
Hippocampus	1	4	98 ± 34	303 ± 113
Septum	0	6	68 ± 18	225 ± 37
Whole brain	1	6	66 ± 13	215 ± 53

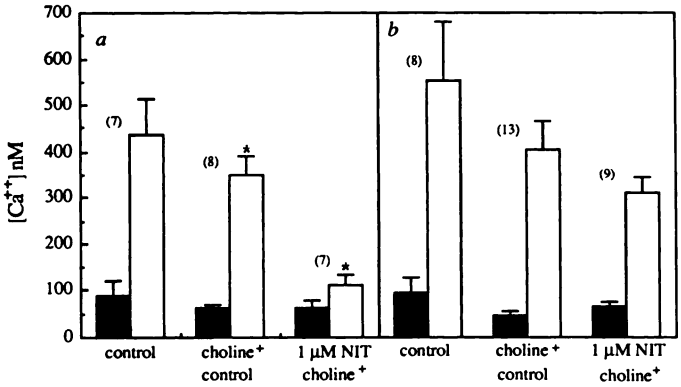

Fig. 3. Depolarization-produced increases in $[Ca^{2+}]_i$ in: a, hippocampal and b, striatal neurons before and after pretreatment with 1 μM nitrendipine (*NIT*). Cell culture ard microfluorimetry are described in Figs. 1 and 2. Control data in the presence and absence of choline$^+$ were determined as described in Fig. 1, b and c, respectively. The last data point (■) prior to the addition of 50 mM K^+ was taken as the resting $[Ca^{2+}]_i$ and the peak rise (□) following depolarization was defined as the stimulated $[Ca^{2+}]_i$. Nitrendipine was added to cells that had been perfused with choline$^+$ media for 5 min and were allowed to incubate for an additional 5 min before stimulation with 50 mM K^+. Stimulated $[Ca^{2+}]_i$ in the hippocampus was significantly lower in nitrendipine-treated cells than in the choline controls (*, $p < 0.01$).

line$^+$ usually lead to a small increase in $[Ca^{2+}]_i$ (Fig. 1c). Following this rise, $[Ca^{2+}]_i$ usually returned to previous levels with time. Replacement of extracellular Na^+ with choline$^+$ only altered the response to 50 mM K^+ slightly (Figs. 1c and 3), indicating that the depolarization-induced increase in $[Ca^{2+}]_i$ was probably not due to activation of Na^+/Ca^{2+} exchange (10, 17). Furthermore, depolarization with 50 mM K^+ in Ca^{2+}-free medium (supplemented with 1 mM EGTA) failed to produce an increase in $[Ca^{2+}]_i$ (Fig. 1d). These data indicate that depolarization produces an increase in $[Ca^{2+}]_i$ which is primarily due to the entry of Ca^{2+} through VSCCs, although some entry of Ca^{2+} through Na^+ channels may also occur (17).

In order to investigate whether CNS neurons possessed DHP-sensitive VSCCs, we utilized the DHP agonist BAY K8644. If cells possess DHP-sensitive VSCCs, BAY K8644 should enhance Ca^{2+} uptake under the appropriate conditions. We utilized a paradigm which has been used previously to demonstrate the effect of BAY K8644 in smooth muscle preparations (18). Cells were primed with a small depolarizing concentration of K^+ (10 mM). This addition produced a small transient increase in $[Ca^{2+}]_i$ (Fig. 2). (Basal $[Ca^{2+}]_i$ = 67 ± 8 nM; 10 mM K^+-stimulated $[Ca^{2+}]_i$ = 109 ± 18 nM, n = 33.) Following this priming depolarization, BAY K8644 (1 μM) was added and its effect on $[Ca^{2+}]_i$ was monitored over time. We considered the drug to be effective if it produced a doubling of $[Ca^{2+}]_i$. In fact, as can be seen in Table 1, the increases produced by BAY K8644 were invariably larger than this. BAY K8644 produced no effects in Ca^{2+}-free medium. Fig. 2, a–e, illustrates typical experiments performed on cells from five separate areas of the brain. It is clear that, in all of these cases, DHP-sensitive VSCCs are present in the cells. We were surprised to observe that virtually every cell tested responded to BAY K8644 irrespective of the area of the brain we employed. This indicates that most CNS neurons may possess DHP-sensitive VSCCs. We wondered if we had fortuitously cultured areas of the brain that were particularly rich in these types of neurons. In order to answer this question, we prepared CNS cultures using mixed neurons from the whole brain. In seven such cultures we picked a neuron at random and examined its sensitivity to BAY K8644. Six of the seven neurons responded (Table 1).

These results clearly indicate that the vast majority of CNS neurons possess functional DHP-sensitive Ca^{2+} channels as previously suggested from biochemical ligand-binding studies using ^{3}H-DHPs. As demonstrated here, Ca^{2+} flux through these pathways can be easily observed when BAY K8644 is used. One of the main effects of this drug is to produce a hyperpolarizing shift in VSCC activation (19). We were also interested in knowing how much of the rise in $[Ca^{2+}]_i$ produced by 50 mM K^+ in the absence of BAY K8644 was due to influx through the DHP-sensitive pathway. We therefore examined the effect of the DHP antagonist nitrendipine (1 μM) on the increase in $[Ca^{2+}]_i$ produced by 50 mM K^+ in both hippocampal and striatal neurons. Choline$^+$ buffers were used to minimize the effects of Na^+/Ca^{2+} exchange. Nitrendipine produced inhibition of the 50 mM K^+-induced rise in $[Ca^{2+}]_i$ in both types of neurons. However, it was considerably more effective in hippocampal neurons (79% and 31% in hippocampus and striatum, respectively) (Fig. 3). This is interesting as K^+ depolarization has often been used as a stimulus in several types of investigation. Our results indicate that more than one channel type contributes to the total Ca^{2+} influx elicited by such a stimulus and that the proportion of DHP-sensitive to -insensitive channels is dependent on the cell type. This could be one reason for the variations in the DHP sensitivity of neuronal processes reported in different investigations. We imagine that the portion of the Ca^{2+} influx not blocked by nitrendipine is due to the activation of non-DHP-sensitive VSCCs which are also known to occur in neurons (3). Some Ca^{2+} may also enter through Na^+ channels (17). Other factors may be even more important in explaining previous discrepancies in the literature. It is interesting to note that most studies that have successfully demonstrated DHP-sensitive Ca^{2+} influx into neurons or neuronal cell lines have all used paradigms which have allowed measurement of Ca^{2+} influx into the cell soma (8, 9, 12, 20). Depolarization-induced Ca^{2+} influx into nerve terminal preparations (synaptosomes) has almost invariably been reported to be DHP insensitive, as has evoked neurotransmitter release (see Refs. 21 and 22 for some recent examples and Refs. 1, 2, and 5 for many further studies on this subject). This may indicate that DHP-sensitive VSCCs tend to be localized in the cell soma rather than in terminal regions. Recent biochemical studies on the distribution of ^{3}H-DHP-binding sites (23, 24) have supported this contention. Moreover, other recent studies in which

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we have combined measurement of neurotransmitter release, Ca^{2+} flux using the fura-2 technique, and whole cell voltage clamp recording of VSCCs in rat sympathetic neurons can also be interpreted by invoking such a differential distribution of DHP-sensitive and-insensitive VSCCs (25, 26).

In conclusion, the results reported here clearly establish the widespread existence of DHP-sensitive VSCCs in CNS neurons. It is now important to ascertain under what physiological conditions such VSCCs become activated and what neuronal functions they may influence. This could ultimately lead to the use of DHPs as novel therapeutic agents for treating dysfunctions of the CNS (27).

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