Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7389–7393, October 1988
Neurobiology

Characterization of dihydropyridine-sensitive calcium channels in rat brain synaptosomes

(Bay K 8644/calcium uptake/dopamine release)

JOHN J. WOODWARD*, MARTHA E. COOK, AND STEVEN W. LESLIE

Division of Pharmacology and Toxicology, College of Pharmacy and Institute for Neurological Sciences Research, University of Texas at Austin, Austin, TX 78712

Communicated by Karl Folkers, June 13, 1988

ABSTRACT **We examined the effects of dihydropyridine Ca^{2+}-channel agonists on synaptosomal voltage-dependent Ca^{2+} entry and endogenous dopamine release. The (−) isomer of Bay K 8644 and the (+) isomer of Sandoz compound 202-791 were 100–1000 times more potent than their respective opposite enantiomers in enhancing Ca^{2+} uptake and dopamine release from striatal synaptosomes. The active isomer of each of these compounds increased Ca^{2+} entry and dopamine release to the same extent at a concentration of 1 nM. Fast-phase Ca^{2+} entry into synaptosomes isolated from cerebellum, cortex, and hippocampus was sensitive to nanomolar concentrations of Bay K 8644. No effect of Bay K 8644 was observed in synaptosomes isolated from brainstem. Bay K 8644 increased synaptosomal Ca^{2+} uptake and endogenous dopamine release from striatal synaptosomes only during the initial seconds of KCl-induced depolarization. The greatest increase was observed during the first second of depolarization. No effect was observed after ≥5 sec of depolarization. Bay K 8644 did not alter Ca^{2+} uptake or dopamine release under resting conditions (5 mM KCl) or in response to KCl at >15 mM. The activity of Bay K 8644 was also attenuated by lowering the concentrations of divalent cations in the incubation medium. Agonist activity was observed at Mg^{2+} concentrations >500 μM (Ca^{2+} held at 100 μM) and Ca^{2+} concentrations >100 μM (Mg^{2+} held at 1000 μM). These results suggest that the Ca^{2+} channels present in synaptosomes are sensitive to nanomolar concentrations of dihydropyridine agonists under a narrow range of experimental conditions.**

The translocation of Ca^{2+} through membrane-associated Ca^{2+} channels is an important signaling mechanism in many tissues (1, 2). The identification and characterization of these channels has been greatly aided by the development of Ca^{2+}-antagonist drugs (3). These compounds alter Ca^{2+} movement by specifically binding to and interacting with the Ca^{2+}-channel protein (4, 5). Studies with the 1,4-dihydropyridines (DHPs), one of three major groups of organic Ca^{2+} antagonists, have demonstrated a striking homogeneity of high-affinity DHP binding across many tissue types (6). The physiological response of the particular tissue to this binding, however, differs markedly. Ca^{2+}-dependent processes such as contraction in cardiac muscle and smooth muscle and secretion in cultured neuron-like cells (e.g., chromaffin, pheochromocytoma PC12, and neuroblastoma cells) are blocked by nanomolar concentrations of DHP Ca^{2+} antagonists such as nifedipine and nitrendipine (7–11). Ca^{2+} entry and neurotransmitter release in brain synaptosomes have been reported to be insensitive to these compounds despite the presence of high-affinity DHP binding sites (12–14).

Slight modifications in the structure of DHP Ca^{2+} antagonists yield compounds, such as Bay K 8644 [methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate], that enhance rather than inhibit Ca^{2+} flux in smooth and cardiac muscle (15, 16). In chick dorsal root ganglion, Bay K 8644 increased the frequency and duration of Ca^{2+}-channel activity and augmented KCl-stimulated release of substance P (17, 18). Bay K 8644 also potentiated release of [^{3}H]norepinephrine release from cultured PC12 cells and adrenal medullary cells (19, 20). The effects of Bay K 8644 on brain Ca^{2+} channels is less clear. Bay K 8644 augmented [^{3}H]serotonin release from rat cortical slices and increased fast-phase Ca^{2+} uptake and endogenous dopamine release by rat striatal synaptosomes (21, 22). In both studies, the effect of Bay K 8644 was antagonized by DHP antagonists. Similarly, Bay K 8644 increased Ca^{2+} uptake and endogenous amino acid release by rat cortical synaptosomes and enhanced the K^+-stimulated release of endogenous γ-aminobutyrate from cultured striatal neurons (23, 24). Other investigators reported no effect of Bay K 8644 on synaptosomal Ca^{2+} entry (25–28). These conflicting reports suggest that DHP-sensitive Ca^{2+} channels may exist in brain but may be masked by DHP-insensitive channels or possibly by certain unfavorable experimental conditions. We undertook this study to rigorously examine the question of whether DHP-sensitive Ca^{2+} channels exist in brain synaptosomes.

MATERIALS AND METHODS

Bay K 8644 and its isomers were the gift of Alexander Scriabine (Miles Institute for Preclinical Pharmacology). The isomers of compound 202-791 were generously provided by R. P. Hof (Sandoz). All other materials were purchased from commercial sources.

Synaptosomes from the P_2 pellet were prepared from male Sprague–Dawley rats (250–300) according to the method of Cotman (29). Incubation medium contained 136 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM EGTA, 10 mM glucose, and 20 mM Tris base. The pH of the solution was adjusted to 7.55 (room temperature) with 1.0 M maleic acid. Duplicate portions of the P_2 synaptosomal suspension (1.5–2.0 mg of protein per ml) were incubated for 14 min at 30°C with the DHP or vehicle prior to the addition of $^{45}Ca^{2+}$. DHP solutions were prepared fresh daily by diluting a portion of a 1 mM stock solution stored in polyethylene glycol 400 (PEG-400) to the appropriate concentration with warm incubation medium. The final PEG-400 concentration (0.001–1.0%) by itself had no effect on Ca^{2+} influx or dopamine release. All experiments were performed in a darkened laboratory to prevent photooxidation of the DHP compounds.

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Abbreviations: DHP, 1,4-dihydropyridine; Bay K 8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate.

*To whom reprint requests should be addressed.

Table 1. Bay K 8644 enhances $^{45}Ca^{2+}$ uptake and endogenous dopamine release by striatal synaptosomes

Depolarization interval, sec	Ca^{2+} uptake, pmol/mg of protein		Dopamine release, pmol/mg of protein	
	Control	Bay K 8644	Control	Bay K 8644
1	17.9 ± 0.7	31.2 ± 4.1*	0.38 ± 0.04	0.58 ± 0.12*
3	33.9 ± 3.5	50.5 ± 8.0*	1.38 ± 0.06	1.75 ± 0.22*
5	77.7 ± 9.2	82.2 ± 11.5	3.04 ± 0.46	3.15 ± 0.26
15	139.2 ± 7.6	151.5 ± 7.2	5.74 ± 0.78	5.98 ± 0.74
30	131.5 ± 11.2	146.2 ± 12.2	6.91 ± 0.71	7.09 ± 1.38

Synaptosomes isolated from striata were depolarized with 15 mM KCl for various times after 14 min of incubation with Bay K 8644 (1 nM) or vehicle (0.001% PEG-400). Values represent the mean (± SEM) net voltage-dependent Ca^{2+} uptake and dopamine release from six experiments performed in duplicate.
*Significantly different ($P < 0.05$) from corresponding control value by paired t test.

Synaptosomal Ca^{2+} entry and endogenous dopamine release from rat striatal synaptosomes were measured as described (30). In brief, a 200-μl aliquot of depolarizing medium (in which NaCl was replaced isosmotically with KCl) containing $^{45}Ca^{2+}$ (75 μCi of $^{45}Ca^{2+}$/μmol of $^{40}Ca^{2+}$; 1 μCi = 37 kBq) was added to the synaptosomal aliquot to give a final K^+ concentration ranging from 5 to 60 mM. Solutions with $^{45}Ca^{2+}$ also contained the appropriate concentrations of the drug or vehicle to maintain the desired drug concentration in the synaptosomal suspension. Ca^{2+} influx and dopamine release were terminated by the rapid addition of 1 ml of ice-cold EGTA stopping solution (136 mM NaCl/5 mM KCl/1.3 mM $MgCl_2$/3 mM EGTA/10 mM glucose/20 mM Tris base, pH adjusted to 7.00 at room temperature with 1.0 M maleic acid). The diluted synaptosomal preparation was filtered through Whatman GF/B filters in a Hoefer manifold with reduced pressure (25 cm Hg; 1 cm Hg = 1330 Pa). Filters were washed twice with 2-ml portions of ice-cold incubation medium. The dopamine-containing filtrate was collected in a test tube (mounted under the filter) that contained 500 pg of the internal standard dihydroxybenzylamine and 200 μl of 1 M perchloric acid. Filters were assayed for radioactivity by liquid scintillation counting. Dopamine released from the synaptosomes was analyzed by liquid chromatography with electrochemical detection as described (30). Protein concentrations were determined by the method of Oyama and Eagle (31).

RESULTS

Table 1 shows the effects of Bay K 8644 (1 nM) on net voltage-dependent (15 mM KCl) Ca^{2+} uptake and endogenous dopamine release by rat striatal synaptosomes. This concentration of Bay K 8644 had been found to enhance KCl-dependent fast-phase $^{45}Ca^{2+}$ entry and dopamine release of striatal synaptosomes by 30–50% (22). Bay K 8644 (1 nM) significantly enhanced both Ca^{2+} uptake and dopamine release only during the 0- to 1-sec and 0- to 3-sec depolarization intervals. This was due to an increase in KCl-stimulated uptake and release, as no effect was noticed under resting (5 mM) K^+ concentrations.

Since the DHPs are asymmetric molecules, we investigated the effects of the (+) and (−) isomers of two DHP agonists on Ca^{2+} entry and dopamine release. These isomers have been reported to produce opposite effects on smooth muscle tension, one being an agonist and the other an antagonist (32, 33). Nanomolar concentrations of the (−) isomer of Bay K 8644 and the (+) isomer of Sandoz compound 202-791 enhanced synaptosomal Ca^{2+} entry and endogenous dopamine release during submaximal (15 mM) KCl depolarization (Table 2). There was no obvious concentration–response relationship observed with these compounds above 1 nM, suggesting that the threshold concentration for increasing Ca^{2+} flux with these compounds may be subnanomolar. The (+) isomer of Bay K 8644 and the (−) isomer of 202-791 enhanced Ca^{2+} entry, but only at 100–1000 times the concentrations as their respective enantiomers. No effect on dopamine release was observed with (+)-Bay K 8644 or (−)-202-791. Subsequent experiments were performed with the racemic mixture of Bay K 8644 unless otherwise noted.

In the absence of drug, increasing the external K^+ concentration from 7.5 mM to 60 mM produced a 20- to 50-fold increase in synaptosomal Ca^{2+} entry and dopamine release during the 3-sec stimulation (Table 3). Bay K 8644 (1 nM) significantly enhanced both Ca^{2+} entry (33%) and dopamine release (53%) at 15 mM KCl only. In addition, release was also significantly enhanced by Bay K 8644 at 7.5 mM KCl. Synaptosomes in subsequent experiments were thus depolarized with 15 mM KCl unless otherwise noted.

Net voltage-dependent Ca^{2+} entry was increased 25-fold by increasing the external Ca^{2+} concentration from 25 to 1000 μM (Fig. 1). Bay K 8644 (1 nM) significantly enhanced Ca^{2+} uptake (30–40%) at external Ca^{2+} concentrations of 100,500, and 1000 μM (Mg^{2+} concentration, 1000 μM). No significant effects were observed when the external Ca^{2+} concentration was decreased to ≤50 μM. Net voltage-dependent Ca^{2+} uptake in control synaptosomes exhibited a biphasic response to external Mg^{2+} concentrations of 0–1000 μM (Fig. 2; Ca^{2+} concentration, 100 μM). Peak uptake occurred at a Mg^{2+} concentration of 100 μM. Bay K 8644 (1 nM) did not increase Ca^{2+} entry at 0–100 μM Mg^{2+}. Bay K 8644 (1 nM) increased Ca^{2+} entry by 36% and 42% as compared to control values when the external Mg^{2+} concentration was increased to 500 and 1000 μM, respectively.

Radioligand binding studies have shown that DHP binding sites in the rat brain are not uniformly distributed, so that certain brain regions may be differentially sensitive to the

Table 2. Synaptosomal $^{45}Ca^{2+}$ uptake and endogenous dopamine release in the presence (0–1000 nM) of isomers of Bay K 8644 and 202-791 during a 3-sec depolarization

Isomer	Ca^{2+} uptake, pmol/mg					Dopamine release, pmol/mg				
	0 nM	1 nM	10 nM	100 nM	1000 nM	0 nM	1 nM	10 nM	100 nM	1000 nM
(+)-Bay K 8644	54.8 ± 2.6	49.9 ± 1.0	61.8 ± 1.4	57.1 ± 2.8	73.4 ± 2.0*	2.6 ± 0.3	2.7 ± 0.3	2.5 ± 0.4	2.4 ± 0.4	2.7 ± 0.6
(−)-Bay K 8644	55.5 ± 2.3	72.0 ± 2.8*	67.6 ± 1.9*	75.3 ± 3.1*	59.4 ± 2.5	3.8 ± 0.3	4.4 ± 0.3*	4.3 ± 0.3*	4.2 ± 0.3	3.8 ± 0.3
(−)-202-791	48.6 ± 2.4	53.8 ± 3.4	51.6 ± 2.6	59.5 ± 2.5*	70.7 ± 4.4*	2.1 ± 0.1	2.2 ± 0.3	2.2 ± 0.3	2.0 ± 0.3	2.1 ± 0.2
(+)-202-791	39.7 ± 7.1	66.3 ± 6.7*	59.7 ± 10.2*	70.1 ± 7.7*	63.0 ± 12.1*	2.6 ± 0.2	3.2 ± 0.3*	3.2 ± 0.3*	3.0 ± 0.2	3.3 ± 0.3*

Synaptosomes were incubated for 14 min with the appropriate concentration of the DHP isomer or vehicle (0.001% PEG-400) before a 3-sec depolarization with 15 mM KCl. Each value represents the mean (± SEM) net voltage-dependent Ca^{2+} uptake or dopamine release from five experiments done in duplicate.
*Significantly greater ($P < 0.05$) than corresponding control value by paired t test.

Table 3. Bay K 8644-induced increment in synaptosomal $^{45}Ca^{2+}$ uptake and endogenous dopamine release during a 3-sec depolarization with K^+ at various concentrations

KCl, mM	Ca^{2+} uptake, pmol/mg		Dopamine release, pmol/mg	
	Control	Bay K 8644	Control	Bay K 8644
7.5	22.2 ± 1.3	19.8 ± 1.3	0.30 ± 0.05	0.73 ± 0.09*
15	60.6 ± 2.7	80.6 ± 3.9*	2.05 ± 0.54	3.14 ± 0.72*
20	135.8 ± 9.2	125.4 ± 10.2	3.91 ± 0.31	4.02 ± 0.37
25	169.5 ± 31.3	157.4 ± 22.4	5.12 ± 0.41	5.38 ± 0.47
30	261.6 ± 13.7	241.0 ± 8.5	7.92 ± 1.30	7.81 ± 1.21
60	490.3 ± 16.5	487.9 ± 14.1	15.71 ± 1.85	16.44 ± 1.20

Synaptosomes isolated from striata were depolarized for 3 sec with various concentrations of KCl after 14 min of incubation with Bay K 8644 (1 nM) or vehicle (0.001% PEG-400). Values represent the mean (± SEM) net voltage-dependent Ca^{2+} uptake and dopamine release from six experiments done in duplicate.
*Significantly different ($P < 0.05$) from corresponding control value by paired t test.

actions of Bay K 8644 (34, 35). Table 4 shows the effects of Bay K 8644 on 15 mM KCl-stimulated $^{45}Ca^{2+}$uptake into synaptosomes isolated from various brain regions. There was no effect of Bay K 8644 on synaptosomal Ca^{2+} entry under resting K^+ conditions in any brain region tested. Control voltage-dependent Ca^{2+} entry was not significantly different between synaptosomes isolated from cerebral cortex and hippocampus, but uptake by synaptosomes from these regions was significantly greater than that measured with cerebellar and brain stem synaptosomes. Bay K 8644 at 1–1000 nM significantly enhanced (36–46%) voltage-sensitive Ca^{2+} entry into synaptosomes isolated from cerebral cortex, hippocampus, and cerebellum. No statistically significant increases in Ca^{2+} entry due to Bay K 8644 were observed in synaptosomes isolated from brainstem.

DISCUSSION

The results suggest that there may be synaptosomal Ca^{2+} channels linked to transmitter release that are sensitive to nanomolar concentrations of DHP Ca^{2+} agonists. These effects were observed with low nanomolar concentrations of Bay K 8644 and were stereospecific, indicating that the DHP

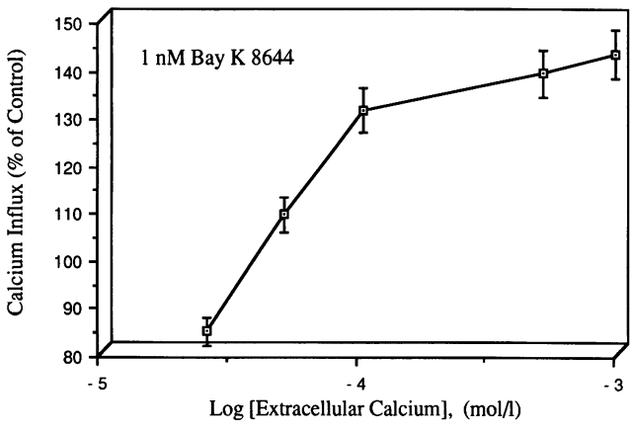

FIG. 1. Effects of extracellular Ca^{2+} on the Bay K 8644-induced increment in KCl-stimulated synaptosomal Ca^{2+} entry. Synaptosomes were preincubated for 14 min with Bay K 8644 (1 nM) or vehicle (0.001% PEG-400) in 5 mM KCl medium with various concentrations of Ca^{2+} before a 3-sec depolarization with 15 mM KCl. The Mg^{2+} concentration was maintained at 1000 μM. Data represent the mean ± SEM of eight experiments performed in duplicate and are expressed as a percent of the corresponding control value for each concentration of Ca^{2+}. Bay K 8644 significantly ($P < 0.05$, paired t test) increased net voltage-dependent Ca^{2+} influx at extracellular Ca^{2+} concentrations of 100, 500, and 1000 μM.

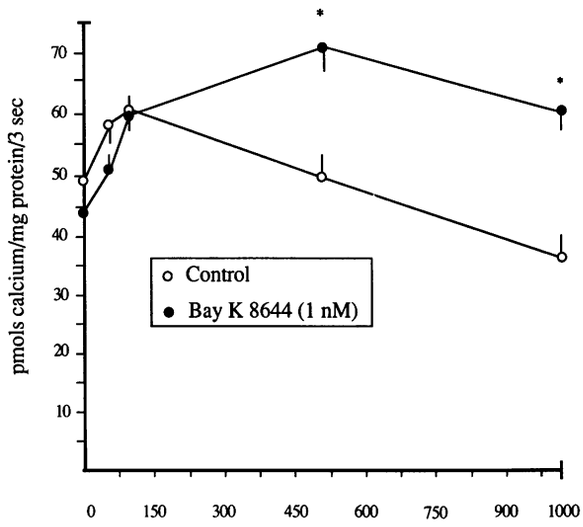

FIG. 2. Effects of Mg^{2+} on K^+-stimulated Ca^{2+} entry into striatal synaptosomes in the presence of Bay K 8644. Synaptosomes were incubated for 14 min in medium containing various amounts of Mg^{2+} and PEG-400 (0.001%) or 1 nM Bay K 8644 prior to a 3-sec depolarization with 15 mM KCl. The Ca^{2+} concentration was maintained at 100 μM. Data represent the mean and SEM of net voltage-dependent Ca^{2+} entry from four experiments performed in duplicate. Asterisks indicate values significantly different ($P < 0.05$) from corresponding control value by paired t test.

binding site was probably associated with the observed effects. Micromolar concentrations of DHP compounds have been reported to bind nonspecifically and with low affinity to other sites, not associated with Ca^{2+} channels, such as the nucleoside transport site and other ion channels (36–38). In the present study, Ca^{2+} entry into synaptosomes isolated from all of the brain regions tested with the exception of the brainstem was sensitive to Bay K 8644. These results indicate a widespread distribution of these DHP-sensitive Ca^{2+} channels in the brain and are in agreement with the reported distribution of DHP binding sites in rodent brain (39). By using the Ca^{2+} indicator fura-2, Thayer *et al.* (40) found that primary cultures of mouse brain neurons contain DHP-sensitive Ca^{2+} channels. All brain regions tested in that study were found to contain Ca^{2+} channels sensitive to Bay K 8644 in the presence of partial (10 mM) K^+ depolarization (brainstem was not reported). Although there is general agreement

Table 4. Effects of Bay K 8644 (0–1000 nM) on 15 mM KCl-stimulated $^{45}Ca^{2+}$ uptake by rat synaptosomes isolated from various brain regions

Brain region	Ca^{2+} uptake, pmol/mg				
	0 nM	1 nM	10 nM	100 nM	1000 nM
Cortex	114.9 ± 7.6	150.0 ± 5.2*	145.8 ± 7.9*	156.2 ± 10.1*	147.2 ± 7.7*
Hippocampus	90.1 ± 7.1	106.7 ± 8.9	114.7 ± 12.4	131.9 ± 9.1*	118.8 ± 5.5*
Cerebellum	59.3 ± 6.4	78.2 ± 4.6*	86.9 ± 4.5*	75.3 ± 4.8*	77.2 ± 3.3*
Brainstem	47.0 ± 8.2	52.2 ± 11.9	56.5 ± 7.5	54.5 ± 7.7	63.7 ± 7.7

Synaptosomes isolated from the indicated brain regions were depolarized with 15 mM KCl for 3 sec in the presence of Bay K 8644 or vehicle (0.001% PEG-400). Values represent mean (± SEM) net voltage-dependent Ca^{2+} uptake (15 mM KCl value − 5 mM KCl value) from duplicate determinations of six to eight experiments.
*Significantly different ($P < 0.5$) from corresponding control value by analysis of variance (Student–Newman–Keuls post-hoc test).

on the existence of DHP binding sites in brain, there has been difficulty in establishing any functional effects of the DHP compounds by using synaptosomes as a model of brain nerve terminals. In this study, three experimental factors were found to be particularly important for the expression of these DHP-sensitive channels in synaptosomes: (*i*) the concentration of divalent cations in the medium; reducing Ca^{2+} or Mg^{2+} levels to low micromolar concentrations attenuated the activity of Bay K 8644; (*ii*) the duration of the depolarization stimulus; Bay K 8644 potentiated Ca^{2+} entry and dopamine release during only the initial seconds of K^+ depolarization; and (*iii*) the strength of depolarization; Bay K 8644 activated Ca^{2+} channels under low-level K^+ stimulation only. However, it is important to note that the expression of these DHP-sensitive Ca^{2+} channels in synaptosomes is variable even under these carefully defined experimental conditions.

Cation Effects. The attenuation of the Bay K 8644 response at reduced divalent cation concentrations in the present study may be due in part to a reduction in the specific binding of Bay K 8644 to the synaptosomal membranes. Moderate concentrations of Ca^{2+} and/or Mg^{2+} are required for the binding of these compounds and the Ca^{2+}-channel antagonists verapamil and diltiazem to isolated brain membranes (6, 41). The divalent-cation chelators EGTA and EDTA reduce DHP binding to brain membranes, indicating an allosteric or permissive role of Ca^{2+} and Mg^{2+} in the binding of DHP (42, 43). These ions may convert low-affinity DHP binding sites to high-affinity sites associated with voltage-sensitive Ca^{2+} channels in brain and cardiac muscle (42). Diltiazem may utilize a similar mechanism to increase the number of high-affinity DHP binding sites (43). The modulation of low- and high-affinity binding sites for DHP on brain Ca^{2+} channels by divalent cations and perhaps endogenous ligands may be an important regulatory mechanism in controlling the flux of Ca^{2+} into neurons and may be similar to the guanine nucleotide-binding (G)-protein-coupled regulation of low- and high-affinity binding sites observed for many receptor ligands (44).

Time Dependence. A second major variable that was important in the demonstration of DHP-sensitive Ca^{2+} channels in brain synaptosomes in the current study was the duration of the depolarization stimulus. Synaptosomes exhibit a fast phase of Ca^{2+} influx and transmitter release, which terminates within 3–5 sec after KCl depolarization (30, 45–47). The fast phase is thought to correspond to the activation of voltage-sensitive Ca^{2+} channels that are linked to the release of endogenous transmitter. Ca^{2+} entry that occurs after this time is insensitive to prior channel inactivation and may involve another type of Ca^{2+} channel or processes that are not channel-mediated. In the current study, Bay K 8644 increased Ca^{2+} entry and dopamine release only during depolarization times of 1 and 3 sec. Longer exposures to K^+ attenuated the increase in the Ca^{2+} uptake produced by Bay K 8644. This is consistent with electrophysiological findings that suggest that DHP agonists transiently increase the amount of time that the channel remains open after depolarization (48). Measurement of Ca^{2+} flux into and transmitter release from brain synaptosomes during seconds of depolarization may represent only the net residual effects of channel activation and inactivation, which is estimated to occur over a span of several hundred microseconds (1, 2). Failure to observe increases in synaptosomal Ca^{2+} flux during prolonged K^+-induced depolarization probably reflects both the considerable damping of the signal that occurs after the transient activation of these channels and the involvement of intracellular processes that actively remove Ca^{2+} from the cell (49). Unfortunately, monitoring the influx of Ca^{2+} or the Ca^{2+}-dependent release of neurotransmitter from synaptosomes over shorter, more physiological time periods is difficult with existing techniques. For example, our minimum time for accurately measuring 15 mM KCl-induced Ca^{2+} entry and dopamine release by manual techniques was $\approx$1 sec, due to the relatively low net Ca^{2+} uptake and dopamine release obtained with submaximal depolarization. Our attempts to use machine-driven rapid-mixing systems to monitor shorter depolarization times met with limited success because of the considerable shear forces generated with these devices. This was indicated by the loss of significant amounts (30–50%) of endogenous dopamine during transport of synaptosomes through the rapid-mixer system and the inability to detect any net voltage-dependent release with this system (J.J.W., unpublished observations).

Voltage Dependence. The fast phase of synaptosomal Ca^{2+} influx and dopamine release increases dramatically as the KCl concentration is elevated and reaches a peak between 60 and 70 mM KCl (45, 47). Bay K 8644 enhanced Ca^{2+} entry and dopamine release in the present study only at very low (7.5 and 15 mM) KCl concentrations. This finding is consistent with our previous report on the actions of Bay K 8644 on synaptosomal Ca^{2+}-channel activity (22) and with the reported effects of Bay K 8644 on KCl-stimulated norepinephrine, acetylcholine, and serotonin release from rat brain slices and endogenous γ-aminobutyrate release from cultured striatal neurons (21, 24, 50). The effects of Bay K 8644 in these studies and the present one were apparent only at submaximal K^+ concentrations. These findings are also in agreement with reports suggesting that DHP agonists do not potentiate Ca^{2+} entry and transmitter release from brain synaptosomes with high levels of KCl depolarization (21, 22, 25–27). Bay K 8644 has also been reported to stimulate Ca^{2+} entry from other tissues in a similar voltage-dependent manner. For example, increasing the KCl concentration above the optimal level of 15–20 mM attenuated the Bay K 8644 effect on Ca^{2+} entry and the development of tension in isolated strips of smooth muscle (51). One possible explanation for this voltage sensitivity of Ca^{2+} channels to the actions of Bay K 8644 may be the existence of different types of Ca^{2+} channels with different gating kinetics that may determine the cell's sensitivity to the DHP compounds. Electrophysiological experiments support this hypothesis and have revealed the presence of three Ca^{2+}-channel subtypes (L, N, and T) in peripheral sensory neurons (17). Many other cell types have also been reported to contain distinct subtypes of voltage-sensitive Ca^{2+} channels (52). These channels are normally distinguished from one another by their activation and inactivation voltages and their sensitivity to pharmacological manipulation. The results of the present study show that nerve terminals may possess both DHP-sensitive (L) and DHP-insensitive (N or T) Ca^{2+} channels that are linked to transmitter release. The DHP-sensitive Ca^{2+} channels observed in this study appear to be somewhat different from the L-type channels described electrophysiologically in other neuronal tissue. For example, L channels in dorsal root ganglion neurons require a large depolarization (to -10 mV) from a moderately negative (-40 mM) holding potential for activation (52). Although electrophysiological measurements are not yet possible in mammalian central nerve terminals, resting membrane potentials in synaptosomes can be estimated according to the constant-field equation:

$$V_{m(30°C)} = 60 \text{ mV} \log \frac{P_{Na}/P_K[Na^+]_o + [K^+]_o}{P_{Na}/P_K[Na^+]_i + [K^+]_i},$$

where P_{Na}/P_K is estimated to be 0.05 (53). Estimates of intracellular Na^+ ($[Na^+]_i$) and K^+ ($[K^+]_i$) in incubated synaptosomes were taken from published values (54). By using these constants, the resting membrane potential of synaptosomes resuspended in normal Na^+-rich incubation medium was calculated to be approximately -52 mV. De-

polarizing the synaptosomes with 15 mM KCl (isotonically replacing Na^+ with K^+) reduces the membrane potential to a calculated value of approximately −38 mV. This decrease in potential represents the optimal depolarization found in this study for the activation of DHP-sensitive Ca^{2+} channels. Increasing K^+ to ≈60 mM further reduces the estimated membrane potential to between −10 and 0 mV, well within the activation range of the L-type DHP-sensitive channel described for neurons of dorsal root ganglia. Bay K 8644 appears to have no effect on synaptosomal Ca^{2+} entry or transmitter release under maximally depolarized conditions. These results are in agreement with those of Murawsky and Suszkiw (55), who found that synaptosomal Ca^{2+} channels exhibited gating kinetics and a sensitivity to Ca^{2+}-channel antagonists that were different from those described for L, N, or T channels in other cell types. We have recently determined that a large portion of voltage-sensitive synaptosomal Ca^{2+} influx and dopamine release is also insensitive to micromolar concentrations of ω-conotoxin (56), a blocker of N- and L-type Ca^{2+} channels. It is possible that many of these apparent discrepancies in the characteristics of Ca^{2+} channels from synaptosomes and other neuronal preparations are due to differences in the methods used to study them. Holz *et al.* (57) reported that DHP compounds differentially modulate release of substance P from cultured dorsal root ganglion neurons depending on whether K^+ or electrical-field stimulation was used as the depolarizing stimulus. Similar experiments with synaptosomes might resolve some of the confusion regarding the classification of the Ca^{2+}-channel subtypes present in brain. In addition, labile endogenous factors that maintain channel function in intact nerve cells may be lost during the preparation of synaptosomes, thereby altering the apparent kinetics of these channels. For example, compounds that promote the phosphorylation of the inner surface of the membrane (catalytic subunit of cAMP-dependent protein kinase, Bay K 8644, MgATP) are reported to prevent the rapid rundown of Ca^{2+}-channel activity recorded from cell-free patches from GH_3 rat pituitary tumor cells (58). Attempts to preserve channel function in isolated brain cells in this manner have been unsuccessful, presumably because of the inability of these compounds to gain access to the cell interior (59).

This study was supported by the Texas Advanced Technology Research Program (S.W.L. and R. E. Wilcox), National Institute of Alcohol Abuse and Alcoholism Grant 05809 (S.W.L.), a grant from the Miles Institute for Preclinical Pharmacology (S.W.L.), RSDA K02 AA00044 (S.W.L.), and a National Research Service Award from the National Institute of Mental Health (J.J.W.).

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