Calcium Channels in Rat Brain Synaptosomes: Identification and Pharmacological Characterization

High Affinity Blockade by Organic Ca^{2+} Channel Blockers¹

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Abstract

Rat brain synaptosomes are shown to contain functional voltage-sensitive Ca^{2+} channels that are inhibited by organic Ca^{2+} channel blockers. Depolarization of synaptosomes with high K^{+} stimulates uptake of $^{45}\text{Ca}^{2+}$ which is biphasic in its time course. Replacement of external Na^{+} with choline eliminates the slower phase of depolarization-stimulated Ca^{2+} uptake, leaving only a rapid uptake process which terminates within 1 sec. This rapid, tetrodotoxin-insensitive Ca^{2+} uptake can be inactivated by prior depolarization of the synaptosomes. Depolarization has no effect on the rate of synaptosomal $^{22}\text{Na}^{+}$ efflux. These results are interpreted as ruling out $\text{Na}^{+}/\text{Ca}^{2+}$ exchange as a mediator of the rapid phase of depolarization-stimulated Ca^{2+} uptake.

A portion (30 to 50%) of the rapid phase of depolarization-stimulated Ca^{2+} uptake is inhibited by nitrendipine, as is depolarization-stimulated [^3H]norepinephrine release from synaptosomes. In external Na^{+} , the inhibition constant (K_{app}) for nitrendipine inhibition of Ca^{2+} uptake is 56 nM. The potency of nitrendipine is increased in the absence of external Na^{+} ($K_{\text{app}} = 1.7$ nM), such that inhibition correlates more closely with the equilibrium dissociation constant for [^3H] nitrendipine binding to synaptosomes ($K_d = 0.35$ nM). Other organic channel blockers (nifedipine, verapamil, D600, and diltiazem) inhibit the rapid Ca^{2+} uptake. The potencies of all Ca^{2+} channel blockers tested by us are in reasonable agreement with their potencies, observed in other laboratories, as blockers of Ca^{2+} channels in smooth and cardiac muscle.

These data demonstrate the existence of active voltage-sensitive Ca^{2+} channels in synaptosomes. The results are shown to be consistent with nitrendipine blockade of Ca^{2+} channels as a result of occupancy of the [^3H]nitrendipine-binding site and suggest a minimum single-channel flux of 1.4×10^6 ions/sec.

The release of neurotransmitter from presynaptic nerve terminals is initiated by an increase in intraterminal Ca^{2+} concentration (Miledi and Slater, 1966; Miledi, 1973; Llinàs and Heuser, 1977). In certain large terminals that are accessible to electrophysiological measurements (Katz and Miledi, 1967; Tillotson, 1979; Byerley and Hagiwara, 1982) and in neurosecretory cells (Fenwick et al., 1982; Hagiwara and Ohmori, 1983), a voltage-sensitive Ca^{2+} current has been observed that is pharmacologically distinct from the action potential Na^{+} current. This Ca^{2+} current is slower in onset and longer in duration than the Na^{+} current mediated by tetrodotoxin-sensitive Na^{+} channels and is mediated by voltage-sensitive Ca^{2+} channels in the presynaptic terminals.

The small size of neurons and their processes and intricate neuronal interconnections have precluded direct electrophysiological measurement of Ca^{2+} channels in the mammalian central nervous system. An alternative biochemical approach to characterization of neuronal Ca^{2+} channels has been to measure $^{45}\text{Ca}^{2+}$ accumulation by mammalian brain synaptosomes, the subcellular fraction of brain homogenates rich in presynaptic nerve terminals (Whittaker, 1969). Synaptosomes have been shown to possess many functional features of intact neurons, such as maintenance of membrane potentials (Blaustein and Goldring, 1975), neurotransmitter uptake, and depolarization-stimulated neurotransmitter release (Blaustein, 1975; Raiteri and Levi, 1978). A depolarization-dependent accumulation of $^{45}\text{Ca}^{2+}$ by synaptosomes produced by equimolar substitution of K^{+} for external Na^{+} has been observed (Blaustein, 1975; Nachsen and Blaustein, 1979). However, it is unclear whether this accumulation is mediated by the voltage-sensitive Ca^{2+} channel, since the specific Ca^{2+} channel blocker nifedipine failed to inhibit Ca^{2+} uptake (Nachsen and Blaustein, 1979) and catecholamine release (Ebstein and Daly, 1982). Furthermore, it has been well established in electrophysiologically accessible excitable cells that Ca^{2+} currents decay minutes after disruption of cellular integrity (Kostyuk et al., 1981; Byerley and Hagiwara, 1982); such disruption is the first step in the preparation of synaptosomes.

An alternate process that may generate depolarization-stimulated Ca^{2+} uptake in synaptosomes is $\text{Na}^{+}/\text{Ca}^{2+}$ exchange. $\text{Na}^{+}/\text{Ca}^{2+}$ exchange is thought to be electrogenic (Blaustein, 1977; Reeves and Sutko, 1980; Pitts, 1981), exchanging 3 to 4 Na^{+} per Ca^{2+} , thus using both the inward Na^{+} concentration gradient and the internally negative membrane potential of the nerve terminal in its resting state to drive Ca^{2+} out of the cytoplasm against its large concentration gradient. This $\text{Na}^{+}/\text{Ca}^{2+}$ exchange is bidirectional; thus, increases in intraterminal Na^{+} , decreases in external Na^{+} , and depolarization can all increase net Ca^{2+} uptake.

The goal of this study was to determine whether voltage-sensitive Ca^{2+} channels are functional in synaptosomes. We show that Ca^{2+} channels can indeed mediate depolarization-stimulated $^{45}\text{Ca}^{2+}$ uptake in synaptosomes and that, under appropriate conditions, the

Received June 12, 1984; Revised August 7, 1984;
Accepted August 31, 1984

¹ We would like to thank Kathleen Sweadner and Steven King for critical reviews of the manuscript. This work was supported by United States Public Health Service Research Grant NS 15236 from the National Institutes of Health and awards from the McKnight Foundation and the Searle Scholars Program (to S. M. G.). T. J. T. was supported by Pharmacological Sciences Training Grant GM-07306 from the National Institutes of Health.

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synaptosomal Ca^{2+} channels are blocked by a variety of organic Ca^{2+} channel blockers at concentration levels comparable to their potencies observed in electrophysiological studies on intact cardiac cells (Hachisu and Pappano, 1983; Lee and Tsien, 1983; Janis and Triggle, 1983). Furthermore, the blockade of depolarization-stimulated synaptosomal Ca^{2+} uptake by these drugs in the absence of external Na^+ is potentiated so that the inhibition produced by nitrendipine correlates with [^3H]nitrendipine binding to a saturable, high affinity site in synaptosomes. This high affinity [^3H]nitrendipine-binding site appears to be identical to the dihydropyridine-binding site observed in the whole brain particulate fraction, a site hypothesized to be associated with the voltage-sensitive Ca^{2+} channel (Ehlert et al., 1982; Glossmann et al., 1982; Gould et al., 1982; Murphy et al., 1983).

Materials and Methods

Materials. [5-methyl- ^3H]Nitrendipine (87 Ci/mmol), [N-methyl- ^3H]verapamil (75 Ci/mmol), *levo*-[2,5,6- ^3H]norepinephrine (52.9 Ci/mmol), $^{45}\text{CaCl}_2$ (32 to 38 mCi/mg), and $^{22}\text{NaCl}$ (120 mCi/mg) were obtained from New England Nuclear (Boston, MA) and were stored at -30°C under N_2 . The organic Ca^{2+} channel blockers were obtained from the following sources: diltiazem hydrochloride, Warner-Lambert Co. (Ann Arbor, MI); D600 hydrochloride, Knoll A.G. (Ludwigshafen, West Germany); nifedipine, Pfizer, Inc. (Brooklyn, NY); nitrendipine, Miles Laboratories, Inc. (New Haven, CT); and verapamil hydrochloride, Searle and Co. (Chicago, IL). Desmethylinipramine was kindly provided by Dr. R. E. Zigmond, Harvard Medical School. All other chemicals were of reagent grade.

Preparation of synaptosomes. Synaptosomes were prepared from the brains of adult CD male rats (Charles River Breeding Laboratories, Cambridge, MA) as described previously (Hajos, 1975). The 0.8 M sucrose layer was collected and diluted with 2.5 vol of buffer A (in millimolar concentration: NaCl, 145; KCl, 5; MgCl_2 , 1.2; CaCl_2 , 1.2; NaH_2PO_4 , 2.4; HEPES, 10; titrated to pH 7.4 with Tris base). The synaptosomes were sedimented by centrifugation at $10,000 \times g$ for 10 min, and were resuspended in an appropriate volume of buffer A. Protein was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

Determination of free drug concentration. The free drug concentration was determined for each drug in each type of experiment. This was accomplished by centrifugation for 5 min in a Beckman microfuge of parallel samples from each experiment to separate the synaptosomes from the supernatant. The supernatant was then analyzed for drug content using liquid scintillation spectroscopy of [^3H]nitrendipine (for both dihydropyridines) and [^3H]verapamil (for both phenylalkylamines), or by UV spectroscopy of diltiazem using a molar absorptivity value of 2.2×10^4 liters/cm-mol at a wavelength of 236 nm.

^{45}Ca Uptake. Ca^{2+} uptake experiments were performed as described previously (Nachsen and Blaustein, 1980). Synaptosomes were divided into several equal portions and preincubated with or without drugs at 30°C for 30 min. All drugs were added by a 1:1000 dilution of an ethanolic stock solution, so that the final ethanol concentration did not exceed 0.1% (16 mM). This ethanol concentration was included in the control samples and by itself had no significant effect on Ca^{2+} uptake, which is consistent with previous reports (Leslie et al., 1983). To initiate Ca^{2+} uptake, 100 μl of the synaptosomes (1 to 2 mg of protein) were added to the uptake buffer which contained $^{45}\text{CaCl}_2$ (1 to 2 $\mu\text{Ci}/\mu\text{mol}$). "Basal" uptake buffer was buffer A, and the "depolarizing" uptake buffer was buffer A with KCl substituted isotonicity for NaCl so that the final concentration of K^+ was 57.5 mM. After various time periods, uptake was halted by addition of 4 ml of ice-cold buffer A, and the solution was rapidly filtered under vacuum through Whatman GF/C glass fiber filters, followed by three 4-ml rinses with cold buffer A. Radioactivity trapped on the filters was determined by liquid scintillation spectroscopy of the samples solubilized in 5 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ) at a counting efficiency of 30 to 40%. A background value of $^{45}\text{Ca}^{2+}$, accumulated on the filters using a sample that did not contain synaptosomes, was subtracted from each data point. This filter background was not significantly different from the background obtained when synaptosomes that had been boiled for 5 min were passed through the filter. Net K^+ -stimulated Ca^{2+} uptake was defined as the difference between uptake in basal and depolarizing buffers. In some experiments, 1 μM tetrodotoxin was included to block voltage-dependent Na^+ channels; tetrodotoxin had no effect on Ca^{2+} uptake. All experiments in which dihydropyridines were used were performed in dim light.

Uptake experiments were also performed in a Na^+ -free buffer which was identical to buffer A except that choline was substituted isotonicity for Na^+ .

The choline/ K^+ depolarizing buffer had K^+ substituted isotonicity for choline so that the final K^+ concentration was 57.5 mM. Na^+ concentration in these buffers was $<50 \mu\text{M}$ as determined by flame photometry.

^{22}Na efflux. Synaptosomes were preincubated at 30°C with buffer A containing 5 μCi of $^{22}\text{Na}^+$ (4 nCi/ μmol) for 30 min. Synaptosomes were then divided into 100- μl portions (1 to 2 mg of protein) and pelleted by centrifugation for 2 min at top speed in an IEC model CL clinical centrifuge. The efflux was initiated by resuspension of the synaptosomal pellet in 1 ml of the choline-substituted basal or depolarizing buffer maintained at 30°C . After various incubation times, the reaction was quenched with 4 ml of ice-cold choline-substituted buffer, filtered over Whatman GF/C filters, and washed twice with 4 ml of the cold buffer. Radioactivity trapped on the filters was determined by liquid scintillation spectroscopy using 5 ml of Hydrofluor. Efflux was calculated as the percentage of counts remaining on the filters compared to a zero point determined by resuspending a synaptosomal pellet in 4 ml of ice-cold choline buffer and filtering as usual.

[^3H]Nitrendipine binding. Equilibrium binding of [^3H]nitrendipine to synaptosomes was performed as described previously in other systems (Gould et al., 1982). One to 2 mg of synaptosomal protein in 1 ml of buffer A were added to 10 to 2000 pmol of [^3H]nitrendipine dissolved in ethanol (final ethanol concentration, 0.1%). Variation of the ethanol concentration used for [^3H]nitrendipine delivery between 0.1 and 1.0% had no significant effect on the results of the binding studies. Incubation was carried out for 60 min at 30°C in the dark. After dilution with 4 ml of ice-cold buffer A, bound radioligand was separated from free ligand by rapid filtration through Whatman GF/C filters and rinsed twice with 4 ml of ice-cold buffer A. Radioactivity trapped on the filters was determined by liquid scintillation spectroscopy in 5 ml of Hydrofluor after 20 min of solubilization. Nonsaturable binding was defined as counts bound in the presence of 1 μM nitrendipine. A competitive binding assay was also performed to estimate the K_d for nifedipine; 0.2 nM [^3H]nitrendipine and various concentrations of unlabeled nifedipine or nitrendipine were treated as for saturation binding. K_d values were calculated from the IC_{50} values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

[^3H]Norepinephrine release. Synaptosomes were loaded with 10^{-7} M *levo*-[^3H]norepinephrine (2 to 3 $\mu\text{Ci}/\text{ml}$) in buffer A at 30°C for 30 min. Subsequently, 1 μM nitrendipine or an equivalent amount of ethanol vehicle was added to the synaptosomes, which were then divided into 100- μl aliquots (1 to 2 mg of protein) and sedimented at top speed for 10 min in an IEC model CL clinical centrifuge. The supernatant was aspirated, and the pellets were preincubated at 30°C for 5 min. Release was measured by addition of 1 ml of basal or depolarizing buffer that contained 10^{-5} M desmethylinipramine to prevent reuptake of norepinephrine. After various incubation periods, release was terminated by dilution of the samples with 2 ml of ice-cold, Ca^{2+} -free buffer A, filtration through GF/C filters, and a wash with 2 ml of Ca^{2+} -free buffer A. The filtrate (5 ml) was collected and combined with 5 ml of Hydrofluor to determine [^3H]norepinephrine release. The filters were also counted in 5 ml of Hydrofluor to determine the unreleased radioactivity. All solutions contained 5×10^{-4} M ascorbic acid and 10^{-5} M pargyline to retard oxidation of norepinephrine.

Results

^{45}Ca uptake into synaptosomes. The initial step in characterization of synaptosomal Ca^{2+} fluxes was to compare the basal rate of $^{45}\text{Ca}^{2+}$ uptake in 5 mM external K^+ with that stimulated by depolarization by substitution of 57.5 mM external K^+ on an equimolar basis for Na^+ . The time course of this accumulation is shown in Figure 1A. The initial amount of uptake during depolarization is 3-fold greater than the initial amount of basal uptake; the difference between the basal and depolarized uptake is defined as the net K^+ -stimulated Ca^{2+} uptake. The net Ca^{2+} uptake is biphasic in character, as described by Nachsen and Blaustein (1980). An initial rate for the rapid component could not be resolved; this rapid uptake largely occurs within 1 sec, the minimum time interval employed.

It is possible that some or all of the net K^+ -stimulated Ca^{2+} uptake is mediated by $\text{Na}^+/\text{Ca}^{2+}$ exchange. If, as described in other systems (Blaustein, 1977; Reeves and Sutko, 1980; Pitts, 1981), synaptosomal $\text{Na}^+/\text{Ca}^{2+}$ exchange is electrogenic with respect to Na^+ (3 to 4 Na^+ per Ca^{2+}), a reduction of the membrane potential would reduce Na^+ entry and hence would increase net accumulation of Ca^{2+} by reduction of Ca^{2+} efflux. The inhibitory effect of depolarization on $\text{Na}^+/\text{Ca}^{2+}$ exchange has been observed previously in squid axons (Blaustein et al., 1974; Baker, 1976). Furthermore, a reduction of Ca^{2+} efflux via $\text{Na}^+/\text{Ca}^{2+}$ exchange could also result simply from the

Figure 1. Time course of $^{45}\text{Ca}^{2+}$ uptake by synaptosomes. A, Uptake in basal ($\bullet$) and depolarizing ($\circ$) buffers in the presence of external Na^+ (>95 mM). The net depolarization-stimulated Ca^{2+} uptake ($\blacktriangle$) is the difference between basal and depolarized uptake. B, Uptake in choline-substituted buffer ($\text{Na}^+ < 50$ μM). The symbols correspond to those in A. C, Net depolarization-stimulated Ca^{2+} uptake in choline-substituted buffer for depolarized ($\blacktriangle$) and pre-depolarized ($\triangle$) synaptosomes. Error bars denote standard error of measurement. See "Materials and Methods" for experimental details.

reduction of external Na^+ , as has been observed previously (Blaustein and Oborn, 1975).

To eliminate processes dependent on external Na^+ as possible effectors of Ca^{2+} uptake, the isotopic flux experiments were repeated using buffer in which choline was substituted for external Na^+ . The time course of the uptake under these conditions is shown in Figure 1B. The net K^+ -stimulated Ca^{2+} uptake during the first second is about 2-fold higher in the absence of external Na^+ , and it is monophasic, reaching a plateau within 1 sec. Blockade of voltage-sensitive Na^+ channels with tetrodotoxin (1 μM) had no effect on Ca^{2+} uptake (data not shown).

If this rapid, net K^+ -stimulated Ca^{2+} uptake in Na^+ -free medium is mediated by Ca^{2+} channels, it should be inactivated by prior depolarization of the synaptosomes. The experiment depicted in Figure 1C demonstrates that this was the case. The synaptosomes were added to an Na^+ -free buffer that contained unlabeled Ca^{2+} and either 5 mM K^+ or 57.5 mM K^+ . After 15 sec, this suspension was transferred to a solution of either 5 mM K^+ or 57.5 mM K^+ that contained $^{45}\text{Ca}^{2+}$. No net K^+ -stimulated Ca^{2+} uptake was resolved when the synaptosomes were previously depolarized in the absence of $^{45}\text{Ca}^{2+}$, whereas the synaptosomes preincubated in 5 mM K^+ retained the same degree of $^{45}\text{Ca}^{2+}$ uptake when exposed to 57.5 mM K^+ and $^{45}\text{Ca}^{2+}$ simultaneously. This demonstrates that in the absence of external Na^+ , the net Ca^{2+} uptake rapidly inactivates as a result of K^+ -induced depolarization.

Inactivation of Ca^{2+} uptake by prior depolarization, although consistent with the expected properties of voltage-sensitive Ca^{2+} channels, does not rule out the possibility that depolarization-stimulated Ca^{2+} uptake in the absence of external Na^+ could be mediated by $\text{Na}^+/\text{Ca}^{2+}$ exchange. Internal Na^+ can drive Ca^{2+} uptake by reversal of the normal direction of $\text{Na}^+/\text{Ca}^{2+}$ exchange. The rapid inactivation of Ca^{2+} uptake could be due to rapid efflux of Na^+ via the electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchange pathway, because of the effect of depolarization on the electrochemical driving force for Na^+ . As is shown in Figure 2, this is not the case; the rates of $^{22}\text{Na}^+$ efflux from $^{22}\text{Na}^+$ -preloaded synaptosomes under basal versus depolarized conditions do not differ significantly. Even if the preponderance of $^{22}\text{Na}^+$ efflux were via other pathways, the data indicated that there has been no significant change in internal Na^+ concentration as a consequence of depolarization.

Binding of nitrendipine and nifedipine to synaptosomes. Saturable binding of the Ca^{2+} channel blockers nitrendipine and nifedipine to synaptosomes was observed, under the same conditions used to characterize Ca^{2+} uptake. Bound [^3H]nitrendipine was separated

from free radioligand using the rapid filtration technique, and the free radioligand concentration was determined by centrifugation of parallel samples of the synaptosomes to assay the supernatant. Unlike Ca^{2+} uptake, binding of [^3H]nitrendipine to synaptosomes is essentially identical in the presence or absence of external Na^+ . Scatchard analysis of these data (Fig. 3) yields a linear plot consistent with binding of ligand to a single high affinity site. Linear regression analysis of these data gives the following values: $K_d = 0.35$ nM, $B_{\text{max}} = 36$ fmol/mg of protein in external Na^+ ; $K_d = 0.36$ nM, $B_{\text{max}} = 39$ fmol/mg of protein for choline-substituted external medium. A competitive binding assay was also used to determine the apparent K_d values for nitrendipine and nifedipine in external Na^+ . The K_d values obtained were 0.38 and 1.5 nM, respectively (data not shown).

Inhibition of Ca^{2+} uptake by dihydropyridine Ca^{2+} channel blockers. The [^3H]nitrendipine binding studies indicate the presence of high affinity dihydropyridine receptors in synaptosomes. The effect of these drugs on the uptake of Ca^{2+} by synaptosomes was assessed in the presence and absence of external Na^+ . In the presence of external Na^+ , dihydropyridines had no effect on the basal rate of accumulation but were able to inhibit a portion of the K^+ -stimulated Ca^{2+} uptake. The log dose-response curve shown in Figure 4A illustrates the effect of nitrendipine and nifedipine on the net K^+ -stimulated Ca^{2+} uptake after 10 sec. Eadie-Hofstee plots (Fig. 4B) of these data are linear, which is consistent with action of the drug at a single site. Linear regression analysis of this curve yields a K_{app} of 56 ± 20 nM ($n = 3$) for nitrendipine and 63 ± 28 nM ($n = 3$) for nifedipine. The I_{max} values, the fractions of the net Ca^{2+} uptake that are sensitive to these drugs, are 0.29 ± 0.10 and 0.38 ± 0.06 , respectively.

The dose-response relationship for inhibition of K^+ -stimulated Ca^{2+} uptake by these drugs in the absence of external Na^+ was also investigated. As in the presence of external Na^+ , the basal rate of Ca^{2+} accumulation was not affected by drug treatment. Again, both nitrendipine and nifedipine were able to inhibit a portion of the K^+ -stimulated Ca^{2+} uptake. The log dose-response curve in Figure 5A shows the inhibition produced by the two drugs; the Eadie-Hofstee plots (Fig. 5B) of these data are linear. The K_{app} values obtained by linear regression analysis are 1.7 ± 0.1 nM ($n = 3$) for nitrendipine and 4.4 ± 0.4 nM ($n = 3$) for nifedipine; the I_{max} values are 0.42 ± 0.08 and 0.47 ± 0.11 , respectively. Thus, the apparent potency of these drugs is greatly enhanced in the choline-substituted medium. There is a 30-fold increase in the potency of nitrendipine in the absence of external Na^+ . Furthermore, the K_{app} value obtained in the choline-substituted medium correlates more closely with the K_d

Figure 2. Time course of $^{22}\text{Na}^+$ efflux. $^{22}\text{Na}^+$ was loaded into synaptosomes and efflux was determined in basal (O) and depolarizing (●) buffers (see "Materials and Methods"). Efflux is expressed as percentage of $^{22}\text{Na}^+$ retained in synaptosomes as compared to an initial value. Values for each point are the average of three experiments, with each data point performed in triplicate. The error bars denote SEM.

Figure 3. $[^3\text{H}]$ Nitrendipine binding to synaptosomes. Scatchard analysis of specific binding of $[^3\text{H}]$ nitrendipine to synaptosomes in basal buffer in the presence (lower curve, ●) or absence (upper curve, O) of external Na^+ . Specific binding is defined as the difference between total and nonsaturable binding performed in the presence of $1 \mu\text{M}$ unlabeled nitrendipine. Linear regression analysis of the data in the presence or absence of external Na^+ indicates no significant difference in binding affinity or density. Data are from a representative experiment with each point performed in triplicate. Each experiment was repeated at least three times with similar results.

Figure 4. Dose-response for dihydropyridine inhibition of Ca^{2+} uptake in the presence of external Na^+ . A, Net K^+ -stimulated Ca^{2+} uptake in the presence of external Na^+ after 10 sec of incubation was determined in the presence of various concentrations of nifedipine (O) or nitrendipine (●). B, Eadie-Hofstee plots of the dose-response data shown in A. The data are averages of triplicate values; error bars represent SEM. This experiment was performed three times with comparable results. The K_{app} values were obtained by linear regression analysis of Eadie-Hofstee plots.

Figure 5. Dose-response for dihydropyridine inhibition of Ca^{2+} uptake in the absence of external Na^+ . A, Net K^+ -stimulated Ca^{2+} uptake in the absence of external Na^+ after 1 sec was determined in the presence of the indicated concentrations of nifedipine (O) or nitrendipine (●). B, Eadie-Hofstee plots of the dose-response data shown in A. Data are the averages of triplicate values; error bars represent SEM. This experiment was performed three times with comparable results. The K_{app} values were obtained by linear regression analysis of Eadie-Hofstee plots.

value of 0.35 nM obtained in the equilibrium binding experiment. Since the K_d for binding is identical in the presence and absence of external Na^+ , it appears that the effect of removal of Na^+ has no

direct effect on the interaction of the drugs with the dihydropyridine binding site.

Inhibition of K^+ -stimulated $[^3\text{H}]$ norepinephrine release by nitrendipine. The physiological response to depolarization-dependent Ca^{2+} entry in presynaptic nerve terminals is the release of neurotransmitter. Blockade of Ca^{2+} entry should lead to attenuation of neurotransmitter release. To test this, the K^+ -stimulated release of $[^3\text{H}]$ norepinephrine from synaptosomes was investigated. The time course of depolarization-dependent release of $[^3\text{H}]$ norepinephrine is shown in Figure 6. Nitrendipine ($1 \mu\text{M}$) is able to inhibit 40 to 60% of the K^+ -stimulated release of norepinephrine. This is comparable to the amount of inhibition of net Ca^{2+} uptake produced by equivalent concentrations of nitrendipine. It is apparent that inhibition of Ca^{2+} influx by nitrendipine results in diminution of the release of neurotransmitter from synaptosomes, which is the ultimate response to depolarization. However, a more quantitative study of the effects of dihydropyridines on neurotransmitter release is necessary to demonstrate that occupation of the high affinity receptor is related to inhibition of release. Such studies are currently under way in this laboratory.

Inhibition of Ca^{2+} uptake by other Ca^{2+} channel blockers. Three other Ca^{2+} channel blockers, verapamil, D600, and diltiazem, were tested for their ability to inhibit K^+ -stimulated Ca^{2+} uptake into synaptosomes. The log dose-response data for inhibition of Ca^{2+} uptake by these drugs in the presence and absence of external Na^+ are illustrated in Figure 7, A and B, respectively. Figure 8A shows an Eadie-Hofstee plot of the inhibition produced by these drugs in the presence of external Na^+ . In contrast to the data for the dihydropyridines (Figs. 4 and 5), the Eadie-Hofstee plots for these three drugs are curvilinear. Linear regression analysis of the high affinity portion of the curves gives K_{app} values of $310 \pm 100 \text{ nM}$ ($n = 3$) for verapamil, $150 \pm 60 \text{ nM}$ ($n = 3$) for D600, and $220 \pm 30 \text{ nM}$ ($n = 2$) for diltiazem. The I_{max} values obtained by extrapolation of this portion of the curve are 0.40 ± 0.06 , 0.47 ± 0.11 , and 0.28 ± 0.05 , respectively. Figure 8B is the Eadie-Hofstee plot of the inhibition in choline-substituted medium; it appears to be similar to the inhibition in the presence of external Na^+ except for the 10 to 30-fold increase in potency. The K_{app} values for the high affinity inhibition are $11.2 \pm 1.6 \text{ nM}$ ($n = 2$) for verapamil, $12.9 \pm 1.6 \text{ nM}$ ($n = 2$) for D600, and $12.7 \pm 1.4 \text{ nM}$ ($n = 2$) for diltiazem. The I_{max} values (the fraction of the net Ca^{2+} uptake that is sensitive to high potency inhibition) are 0.55 ± 0.05 , 0.41 ± 0.03 , and 0.45 ± 0.11 , respectively.

Discussion

The principal conclusion of this study is that rat brain synaptosomes contain functional voltage-sensitive Ca^{2+} channels with prop-

Figure 6. Inhibition of $[^3\text{H}]$ norepinephrine release. The time course of net K^+ -stimulated $[^3\text{H}]$ norepinephrine release from synaptosomes is shown in the presence (O) and absence (●) of $1 \mu\text{M}$ nitrendipine. Synaptosomes were loaded with $[^3\text{H}]$ norepinephrine, and release was observed in basal or depolarizing buffer that contained Na^+ . Release is defined as $[^3\text{H}]$ norepinephrine in the filtrate divided by the sum of the $[^3\text{H}]$ norepinephrine in the filtrate and on the filter. The data shown are representative of two experiments. Each data point was performed in triplicate; error bars denote SEM.

Figure 7. Dose-response for inhibition of Ca^{2+} uptake by other organic inhibitors. Inhibition of net K^{+} -stimulated Ca^{2+} uptake by verapamil (○), D600 (●), and diltiazem (▲) was determined in the presence (A) and absence (B) of external Na^{+} . The data shown are representative of several experiments with each drug. Each data point was performed in triplicate; error bars denote SEM.

Figure 8. Eadie-Hofstee plots of inhibition of Ca^{2+} uptake by other organic inhibitors. Eadie-Hofstee plots of the inhibition of net K^{+} -stimulated Ca^{2+} uptake into synaptosomes by verapamil (○), D600 (●) and diltiazem (▲), in the presence (A) and absence (B) of external Na^{+} are shown. The data are representative of several experiments for each drug; the data points are mean values of triplicate samples. The K_{app} values for the high affinity portion of the curve (right) were determined by linear regression analysis.

erties similar to those of Ca^{2+} channels found in smooth muscle and cardiac tissue. A rapidly terminating, depolarization-stimulated Ca^{2+} uptake occurs in the absence of external Na^{+} . The uptake is inactivated by prior depolarization of the synaptosomes, consistent with electrophysiological studies in other systems which demonstrate Ca^{2+} channel inactivation by depolarization (Lee and Tsien, 1983). The Ca^{2+} uptake is unaccompanied by depolarization-stimulated Na^{+} efflux. These results rule out $\text{Na}^{+}/\text{Ca}^{2+}$ exchange as a mediator of this component of Ca^{2+} uptake. A substantial component (30 to 50%) of the Ca^{2+} uptake is blocked with high potency by representatives of the three major classes of organic Ca^{2+} channel blockers: dihydropyridines (nifedipine and nitrendipine), phenylalkylamines (D600 and verapamil), and benzothiazepines (diltiazem).

The relationship between synaptosomal calcium channel blockade and the high affinity dihydropyridine binding site. This study

reveals a class of synaptosomal [^3H]nitrendipine binding sites that are homogeneous with respect to affinity for nitrendipine and nifedipine. The nitrendipine dissociation constant (0.35 nM) and the binding site density (40 fmol/mg of protein) are comparable, within a factor of 3, to values for these parameters for nitrendipine binding to rat brain membranes (Murphy and Snyder, 1982; Curtis and Catterall, 1983). The nitrendipine binding affinity is also in similar agreement with that measured for rat pheochromocytoma cells (Toll, 1982), canine myocardium (Williams and Jones, 1983), cultured chick ventricular cells (Marsh et al., 1983), and guinea pig smooth muscle (Bolger et al., 1983).

We find that a substantial fraction of depolarization-stimulated synaptosomal Ca^{2+} uptake is blocked with high potency by dihydropyridines. The potency of these drugs is highest when measured on a short time scale and in the absence of external Na^{+} . The apparent

potency (K_{app}) for nitrendipine blockade of this Ca^{2+} uptake in the absence of external Na^+ is 1.7 nM, which is 4.5-fold higher than the K_d for nitrendipine binding measured under the same conditions. The lack of complete quantitative agreement between the K_{app} and the K_d is not inconsistent with the hypothesis that the occupancy of the synaptosomal nitrendipine binding site mediates blockade of the synaptosomal Ca^{2+} channel. A probable explanation for this discrepancy is that, although we can accurately determine an equilibrium value for nitrendipine binding, we have not determined an accurate value for the fraction of synaptosomal Ca^{2+} channels blocked by nitrendipine. The minimum time scale on which Ca^{2+} uptake was measured (1 sec) is too slow to determine the initial rate of Ca^{2+} uptake. Determination of an initial rate is important, because it has been observed that elevation of internal Ca^{2+} concentrations as a result of Ca^{2+} influx may be involved in the inactivation of Ca^{2+} channels in nerve terminals (Tillotson, 1979; Linden and Brooker, 1980; Tsien, 1983). Thus, one would expect that 50% inhibition of the synaptosomal Ca^{2+} uptake would require blockade of more than 50% of the Ca^{2+} channels that mediate this uptake. This is because blockade of a certain fraction of the Ca^{2+} channels by nitrendipine may reduce the rate of channel inactivation by reducing the rate of Ca^{2+} influx and may partially offset the effect of blockade of the Ca^{2+} channel by increasing mean channel open time. Based on the K_d for nitrendipine, 82% of the nitrendipine binding sites would be occupied when 50% of the total nitrendipine-sensitive uptake measured at 1 sec is blocked, which is entirely consistent with our expectations based on the kinetics of the Ca^{2+} uptake. In order to obtain an accurate value for the inhibition constant of nitrendipine blockade of Ca^{2+} uptake, the Ca^{2+} fluxes would have to be measured on a more rapid time scale using rapid mixing techniques, as has been done for ion fluxes mediated by the nicotinic receptor (Neubig and Cohen, 1980) and the voltage-sensitive Na^+ channel (Tanaka et al., 1983). Such studies are currently in progress.

Comparison of the potency of organic calcium channel blockers in synaptosomes with their effects on intact cells and tissues. Nifedipine and nitrendipine blockade of ventricular muscle Ca^{2+} channels has been analyzed previously (Hachisu and Pappano, 1983). The concentrations of these dihydropyridines required for half-maximal inhibition of Ca^{2+} -dependent action potential amplitude (ID_{50}) range between 100 and 600 nM. Our observed potencies for nifedipine and nitrendipine blockade of a component of depolarization-stimulated synaptosomal Ca^{2+} uptake at physiological Na^+ levels are 63 nM and 56 nM, respectively. Although our observed potencies are somewhat higher than the reported ID_{50} values obtained for suppression of ventricular action potentials, it should be borne in mind that, based on the electrophysiological properties of the Ca^{2+} channel (Tsien, 1983), the reduction of the ventricular action potential amplitude is not proportional to the fraction of Ca^{2+} channels that are blocked, but would be lower than the latter quantity. Thus, the agreement between our findings and those of the electrophysiological studies is probably even closer when viewed in the same terms. Direct electrophysiological measurements of the Ca^{2+} channel in synaptosomes using patch clamp methods would be useful for more quantitative dose-response comparison.

Nitrendipine inhibits guinea pig smooth muscle phasic contraction at ID_{50} levels of 68 nM (Bolger et al., 1983). Neutralization of K^+ -induced contraction of pig vascular smooth muscle occurred at ID_{50} values of 8 nM (Fleckenstein et al., 1976). Although the former value is in excellent agreement with our findings for synaptosomal Ca^{2+} channels at physiological Na^+ levels, the latter value approaches our K_{app} value for inhibition in the absence of external Na^+ . Again, in these systems the relationship between Ca^{2+} channel blockade and muscle contraction is unclear but is unlikely to be linear. It should be noted that K_d values for dihydropyridine binding sites in smooth and cardiac muscle have been reported in the range between 0.15 and 0.26 nM, similar to our values of 0.35 nM for synaptosomes. An additional lower affinity dihydropyridine binding site ($K_d = 19$ nM)

was recently proposed to exist in cultured chick ventricular muscle cells (Marsh et al., 1983).

Verapamil and diltiazem block depolarization-induced guinea pig smooth muscle contraction with ID_{50} values of 200 nM (Fleckenstein et al., 1976). Verapamil inhibits K^+ -induced $^{45}\text{Ca}^{2+}$ influx in rabbit aorta smooth muscle cells with an ID_{50} of 170 nM (Terai et al., 1981). Half-maximal inhibition of the amplitude of skeletal muscle slow Ca^{2+} current in Na^+ -free medium by verapamil occurs at 20 nM (Kerr and Sperelakis, 1982). Submicromolar levels of D600 and verapamil, and 5 μM diltiazem, substantially but not completely inhibit ventricular Ca^{2+} channels (Lee and Tsien, 1983). These findings are in accord with the effects of verapamil on the rate of rise of ventricular muscle Ca^{2+} action potentials (Hachisu and Pappano, 1983). We observe inhibition of a substantial fraction of depolarization-induced Ca^{2+} uptake at K_{app} values of 150 to 300 nM for verapamil, D600, and diltiazem in the presence of physiological external Na^+ concentrations. Elimination of external Na^+ reduces the K_{app} values for these drugs to 10 to 15 nM. Again, these results are comparable with the studies on intact muscle cells and tissue. It should be noted that the highest potency reported for verapamil blockade of skeletal muscle Ca^{2+} channels occurred under conditions employing Na^+ -free external solutions (Kerr and Sperelakis, 1982), conditions that maximize the potency of the organic Ca^{2+} channel blockers in our hands with respect to the synaptosomal Ca^{2+} channels.

Previous reports have suggested that dihydropyridine Ca^{2+} channel blockers had no effect on synaptosomal depolarization-induced $^{45}\text{Ca}^{2+}$ uptake (Nachsen and Blaustein, 1979) or on catecholamine release from mammalian brain synaptosomes (Ebstein and Daly, 1982). We do not fully understand why these investigators saw no effect, although the fact that their observations were made in external Na^+ on a minute time scale rather than in seconds, as in our study, may account in part for the discrepancy. Ca^{2+} uptake mediated by $\text{Na}^+/\text{Ca}^{2+}$ exchange could complicate the resolution of the effects of Ca^{2+} channel blockers over longer incubation times. Nachsen and Blaustein (1979) found that 100 μM verapamil was required for substantial inhibition of K^+ -stimulated $^{45}\text{Ca}^{2+}$ uptake observed on a 2-min time scale. Verapamil at these elevated concentrations has been shown to block $\text{Na}^+/\text{Ca}^{2+}$ exchange in brain synaptic plasma membrane vesicles (Erdeich et al., 1983).

We have observed that approximately half of the depolarization-stimulated Ca^{2+} uptake is much less sensitive to blockade by organic Ca^{2+} channel blockers. A question of interest is whether this second rapidly terminating component represents a distinct class of Ca^{2+} channels. Electrophysiological evidence for multiple classes of Ca^{2+} channels in dorsal root ganglion cells (Nowicky et al., 1984) and *Aplysia* neurons (Chad et al., 1984) based on different time constants for inactivation has been reported.

Estimate of single-channel flux. One can estimate the single-channel flux for the synaptosomal Ca channel, based on the assumption that the [^3H]nitrendipine binding site is responsible for mediating the Ca^{2+} blocking effect of dihydropyridine drugs. By using the K_{app} values for inhibition of Ca^{2+} flux and the equilibrium dissociation constant, K_d , one can calculate the fraction of channels that are occupied by nitrendipine at the concentration that elicits a half-maximal inhibitory response. In the presence of external Na^+ , nitrendipine occupies 99.4% of its binding sites, leaving 0.6% of the total number of sites unoccupied at half-maximal inhibitory drug levels. The total number of unoccupied sites can be calculated as the product of the fraction of unoccupied sites and the total site density, B_{max} . The amount of nitrendipine-sensitive Ca^{2+} uptake measured during 1 sec of incubation that remains unblocked at a nitrendipine concentration equal to its K_{app} is 0.31 nmol/mg of protein. By dividing this uptake by the density of unoccupied nitrendipine receptors, one arrives at a single-channel flux of 1.4×10^6 ions/channel-sec, which is in reasonable agreement with values of 0.08 to 1.2×10^6 ions/sec obtained in electrophysiological experiments (Tsien, 1983). Our estimate is a minimum value for single-channel flux because it assumes that the synaptosomal Ca^{2+} chan-

nel remains open throughout the 1-sec incubation period, whereas they probably are open for only a fraction of that time. The calculation also assumes that all nitrendipine binding sites represent activatable Ca^{2+} channels, and if any of the binding sites were not active, the estimated value for single-channel flux would increase.

The conclusion that is well supported by the above experimental evidence is that synaptosomes contain functional Ca^{2+} channels. These channels are blocked by dihydropyridine, phenylethylalkylamine, and benzothiazepine derivatives with potencies that, to a first approximation, correlate with their effects on Ca^{2+} channels in smooth muscle and cardiac tissue. This has been the only report to date of the existence of such Ca^{2+} channels in an *in vitro* system other than intact cells. A practical consequence of these findings is that synaptosomes are a viable starting material for the isolation of functional Ca^{2+} channels. The unknown factors that produce loss of Ca^{2+} channel function in other *in vitro* preparations appear not to be encountered in this synaptosome preparation.

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