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Effects of Ca^{2+} Channel Blockers on Ca^{2+} Translocation Across Synaptosomal Membranes

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Abstract: The binding of [^{3}H]nimodipine to purified synaptic plasma membranes (SPM) isolated from sheep brain cortex was characterized, and the effects of nimodipine, nifedipine, and (+)-verapamil on the [^{3}H]nimodipine binding were compared to the effects on $^{45}Ca^{2+}$ translocation under conditions that separate $^{45}Ca^{2+}$ fluxes through Ca^{2+} channels from $^{45}Ca^{2+}$ uptake via Na^+/Ca^{2+} exchange. [^{3}H]Nimodipine labels a single class of sites in SPM, with a K_D of 0.64 ± 0.1 nM, a B_{max} of 161 ± 27 fmol·mg^{-1} protein, and a Hill slope of 1.07, at 25°C. Competition of [^{3}H]nimodipine binding to purified SPM with unlabelled Ca^{2+} channel blockers shows that: (1) nifedipine and nimodipine are potent competitors, with IC_{50} values of 4.7 nM and 5.9 nM, respectively; (2) verapamil and (−)-D 600 are partial competitors, with biphasic competition behavior. Thus, (+)-verapamil shows an IC_{50} of 708 nM for the higher affinity component and the maximal inhibition is 50% of the specific binding, whereas for (−)-verapamil the IC_{50} is 120 nM, and the maximal inhibition is 30%; (−)-D 600 is even less potent than verapamil in inhibiting [^{3}H]nimodipine binding (IC_{50} = 430 nM). However, (+)-verapamil, nifedipine, and nimodipine are less potent in inhibiting depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes in the absence of Na^+/Ca^{2+} exchange than in competing for [^{3}H]nimodipine binding. Thus, (+)-verapamil inhibits Ca^{2+} influx by 50% at about 500 μM, whereas it inhibits 50% of the binding at concentrations 200-fold lower, and the discrepancy is even larger for the dihydropyridines. The Na^+/Ca^{2+} exchange and the ATP-dependent Ca^{2+} uptake by SPM vesicles are also inhibited by the Ca^{2+} channel blockers verapamil, nifedipine, and *d-cis*-diltiazem, with similar IC_{50} values and in the same concentration range (10^{-5}–10^{-3} M) at which they inhibit Ca^{2+} influx through Ca^{2+} channels. We conclude that high-affinity binding of the Ca^{2+} blockers by SPM is not correlated with inhibition of the Ca^{2+} fluxes through channels in synaptosomes under conditions of minimal Na^+/Ca^{2+} exchange. Furthermore, the relatively high concentrations of blockers required to block the channels also inhibit Ca^{2+} translocation through the Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger. In this study, clear differentiation is made of the effects of the Ca^{2+} channel blockers on these three mechanisms of moving Ca^{2+} across the synaptosomal membrane, and particular care is taken to separate the contribution of the Na^+/Ca^{2+} exchange from that of the Ca^{2+} channels under conditions of K^+ depolarization. **Key Words:** Synaptosomes—Synaptic membranes—Ca^{2+} channel blockers—[^{3}H]Nimodipine—Na^+/Ca^{2+} exchange—Ca^{2+} transport. **Carvalho C. A. M. et al.** Effects of Ca^{2+} channel blockers on Ca^{2+} translocation across synaptosomal membranes. *J. Neurochem.* **47**, 1774–1784 (1986).

The entry of Ca^{2+} into nerve terminals and axons is controlled mainly by voltage-sensitive Ca^{2+} channels (Baker, 1972; Llinás et al., 1972; Baker et al., 1973; Kostyuk, 1981). Membrane depolarization opens the Ca^{2+} channels and causes a transient increase in the $[Ca^{2+}]_i$ that, in turn, leads to neurotransmitter release (Katz and Miledi, 1970; Llinás et al., 1972; Hagiwara and Byerly, 1981).

The Ca^{2+} channel blockers potently block Ca^{2+} influx into smooth and cardiac muscle cells (Fleckenstein, 1977; Triggle, 1981), which possess high-affinity binding sites, as determined from studies in muscle membrane fractions using radiolabelled Ca^{2+} channel blockers (Bellemann et al., 1981, 1982; Ehlert et al., 1982; Glossmann et al., 1982; Bolger et al., 1983). High-affinity binding sites for Ca^{2+} channel blockers have also been found in homogenates presumably rich in brain membranes (Ferry and Glossmann, 1982; Glossmann et al., 1982; Gould et al., 1982; Murphy and Snyder, 1982; Marangos et al.,

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Abbreviations used: ChCl, choline chloride; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SPM, synaptic plasma membrane.

1982; Lee et al., 1984). However, in neuronal tissue the presence of voltage-operated Ca^{2+} channels, sensitive to 1,4-dihydropyridines, has been clearly demonstrated only in certain neuronal cell lines (Toll, 1982; Takahashi and Ogura, 1983; Albus et al., 1984; Freedman et al., 1984). In isolated synaptosomes, it was initially shown that depolarization-induced $^{45}Ca^{2+}$ influx is insensitive to dihydropyridines (Nachshen and Blaustein, 1979; Daniell et al., 1983). However, the results of a recent study (Turner and Goldin, 1985) indicate that under certain conditions organic calcium channel blockers also inhibit $^{45}Ca^{2+}$ influx into synaptosomes. Moreover, Middlemiss and Spedding (1985) in a recent report show that the agonist Bay K 8644 increases the Ca^{2+}-dependent neurotransmitter release in brain slices, and the effect is reversed by Ca^{2+} channel blockers. These various results indicate that the dihydropyridine binding site in brain may have a functional correlate, but that there is some difficulty in observing it consistently, especially in isolated brain functions.

It must be pointed out that Ca^{2+} may also enter the nerve cell by Na^+/Ca^{2+} exchange and that increasing the external K^+ in substitution for Na^+ during K^+ depolarization of synaptosomes increases the Ca^{2+} influx through Na^+/Ca^{2+} exchange (Coutinho et al., 1984). The Ca^{2+} entries through channels and through Na^+/Ca^{2+} exchange often are not separated, and this has added confusion to the field. There is at least one recent report showing that verapamil inhibits the Na^+/Ca^{2+} exchange in synaptic plasma membrane (SPM) vesicles (Erdreich et al., 1983), so that this adds further to the uncertainty regarding the action of Ca^{2+} channel blockers on Ca^{2+} fluxes studied in brain fractions.

In an attempt to clarify some of these problems in synaptosomes, we first studied the effects of various classes of Ca^{2+} channel blockers on the binding of [^{3}H]nimodipine to purified SPM since most previous studies have been performed with gross homogenates, and we then investigated the effects of the blockers on the Ca^{2+} influx in synaptosomes through the Ca^{2+} channels, under conditions of minimal Na^+/Ca^{2+} exchange. Furthermore, we investigated the effect of the Ca^{2+} blockers on the Na^+/Ca^{2+} exchange and on the ATP-dependent Ca^{2+} transport which regulate the Ca^{2+} concentration in the nerve cell (Baker, 1972; Blaustein, 1977; DiPolo and Beaugé, 1980; Gill et al., 1981; Coutinho et al., 1983, 1984). We conclude that under carefully controlled experimental conditions, which separate depolarization-induced $^{45}Ca^{2+}$ influx from Na^+/Ca^{2+} exchange in synaptosomes, voltage-sensitive Ca^{2+} influx is inhibited only by relatively high concentrations of Ca^{2+} blockers, and that these concentrations also inhibit the Na^+/Ca^{2+} exchange and the ATP-dependent Ca^{2+} transport. Therefore, the inherent difficulty in showing in isolated brain fractions an action of Ca^{2+} channel blockers on the Ca^{2+} channels at nanomolar concentrations of these drugs remains unresolved and recent results showing the contrary must be viewed with caution (Turner and Goldin, 1985).

MATERIALS AND METHODS

Isolation of subcellular fractions from sheep brain

Synaptosomes were isolated from sheep brain cortex homogenates according to the method described by Hajós (1975), with some modifications (Carvalho and Carvalho, 1979). The final synaptosomal pellets were washed by resuspension in 0.32 *M* sucrose buffered with 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Tris, pH 7.4, and centrifugation at 20,000 *g* for 30 min, and, finally, the synaptosomes were resuspended in the same medium. This synaptosomal suspension was immediately utilized for measuring $^{45}Ca^{2+}$ influx due to K^+ depolarization, or was submitted to a short preincubation in ionic medium, as described in a subsequent section.

Some synaptosomal pellets were submitted to hypotonic lysis, and SPM were partially purified from the lysate, as described previously by Coutinho et al. (1983). The SPM fractions used for enzymatic characterization were resuspended in 0.32 *M* sucrose buffered with 10 m*M* HEPES-Tris, pH 7.4, whereas those used for Ca^{2+} uptake (Na^+/Ca^{2+} exchange or ATP-dependent Ca^{2+} uptake) were washed and resuspended in ionic medium, containing 150 m*M* NaCl or KCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES-Tris, pH 7.4, at a protein concentration of about 20 mg/ml, as determined by the biuret method (Layne, 1957).

The microsomal fraction was prepared by centrifugation of the supernatant of the crude mitochondrial fraction at 40,000 *g* for 30 min, and the pellet was resuspended in 0.25 *M* sucrose buffered with 50 m*M* Tris-Cl, pH 7.4. This fraction, the crude microsomal pellet, was submitted to subfractionation in a sucrose density gradient, as follows: 5-ml portions were overlayed on a discontinuous sucrose gradient with the following composition: 8 ml each of 40%, 35%, 30%, and 25% (wt/wt) sucrose in buffer (50 m*M* Tris-Cl, pH 7.4). The gradients were centrifuged at 60,000 *g*, for 2 h, at 0–2°C, in the swinging bucket rotor of the International B-60 ultracentrifuge. Fractions F_1 through F_4 (see Fig. 1) were collected from the interfaces, diluted five times with ice-cold 50 m*M* Tris-Cl, pH 7.4, and centrifuged for 30 min at 40,000 *g*. The pellets used for enzymatic characterization were resuspended in 0.32 *M* sucrose plus 10 m*M* HEPES-Tris, pH 7.4, whereas the pellets used for [^{3}H]nimodipine binding were resuspended in 50 m*M* Tris-Cl, pH 7.4, divided into aliquots, frozen in liquid nitrogen, and kept at −60°C for further analysis. All the experiments were performed within 4 weeks after isolation, and the [^{3}H]nimodipine binding characteristics were preserved during this period of storage.

[^{3}H]Nimodipine binding

Prior to an experiment on [^{3}H]nimodipine binding, the purified membrane fractions were thawed at room temperature, and were homogenized at 0–4°C, by using a Polytron appartus (Kinematica GmbH), at setting 5, with two 10-s bursts at intervals of 15 s. The suspension was then diluted with buffer (50 m*M* Tris-Cl, pH 7.4), to obtain a protein concentration of 1 mg/ml, and was immediately utilized in the binding assay.

The [^{3}H]nimodipine binding assay was performed as follows: 0.6 mg of membrane protein were equilibrated in 1.8 ml of standard buffer (50 m*M* Tris-Cl, pH 7.4) containing

the indicated concentrations of [^{3}H]nimodipine in the presence (nonspecific binding) and in the absence (total binding) of 5 μM unlabelled nimodipine. The specific [^{3}H]nimodipine binding is defined as the difference in binding under the two conditions. For saturation kinetics [^{3}H]nimodipine was varied between 0.05 and 1.5 nM. The competition experiments were normally performed with 0.35 nM [^{3}H]nimodipine, in the presence of unlabelled drugs (8–10 different concentrations). In all cases 50-μl aliquots of the incubation mixture were taken for measurement of the concentration of tritiated drug present. Assays were normally performed in triplicate and under yellow dim light because of the light sensitivity of the dihydropyridines.

After the incubation period (45 min, normally), membrane-bound and free [^{3}H]nimodipine were separated by rapid vacuum filtration of 0.5-ml samples through Whatman GF/B glass fiber filters prewashed with 5 ml of ice-cold (0–4°C) buffer (50 mM Tris-Cl, pH 7.4). Filtration of the sample was followed by a 10-ml washing with the same medium. Filters, as well as samples of radioactive solutions, were placed in glass vials containing 8 ml of scintillation fluid {composition per liter of toluene: 7.3 g 2,5-diphenyloxazole (PPO), 176 mg *p*-bis-[2-(5-phenyloxazolyl)] benzene (POPOP), and 250 ml Triton X-100}, and the radioactivity was counted in a Packard TriCarb liquid scintillation spectrometer, model 460-CD. The quenching of the radioactivity in the samples was corrected automatically by using an efficiency correlation curve obtained for ^{3}H-quenched standards, by the external standardization method.

Calcium uptake studies

Calcium uptake due to K^+ depolarization. Sheep brain cortex synaptosomes isolated in 0.32 M sucrose buffered with 10 mM HEPES-Tris, pH 7.4, were diluted 20-fold (final protein concentration, 1 mg/ml) into Ca^{2+} uptake media containing 150 mM KCl (or 60 mM KCl, 73 mM NaCl), 1 mM $MgCl_2$, 10 mM glucose, 1 mM $CaCl_2$ supplemented with $^{45}CaCl_2$ (2.5 μCi/μmol), 10 mM HEPES-Tris, pH 7.4, and increasing concentrations of the calcium blockers. The reaction was conducted at 30°C, and was terminated by filtration of 0.5-ml samples through Watman GF/B filters prewashed with 10 ml of 0.32 M ice-cold (0–4°C) sucrose buffered with 10 mM Tris-Cl, pH 7.4. The samples retained on the filters were washed with 10 ml of buffered sucrose containing 1 mM $LaCl_3$, to remove the surface-bound $^{45}Ca^{2+}$. The filters were treated and radioactivity was counted as described above. The amount of Ca^{2+} retained by the synaptosomes in a control medium with the same composition as described above, except that 150 mM NaCl (or 128 mM NaCl, 5 mM KCl) was present instead of KCl, was determined for all the drug concentrations tested and was subtracted from total Ca^{2+} taken up to obtain the Ca^{2+} uptake due to K^+ depolarization (Fig. 4).

In the experiments shown in Figs. 3 and 5, the synaptosomal suspension was preincubated in a Na-rich medium (128 mM NaCl; 5 mM KCl; 1.2 mM $MgCl_2$; 10 mM glucose; 10 mM HEPES-Tris, pH 7.4; 1 mM Na_2HPO_4) for 15 min, at 30°C, prior to the Ca^{2+} uptake assays. After the incubation, the suspension was centrifuged for 15 min at 20,000 g, and the pellet was resuspended in ionic medium containing 128 mM choline chloride (ChCl); 5 mM KCl; 1 mM $MgCl_2$; 10 mM glucose; 10 mM HEPES-Tris, pH 7.4, at 20 mg of protein/ml and was utilized for studying $^{45}Ca^{2+}$ uptake in the next 1–2 h.

Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} uptake. The effects of calcium blockers (verapamil, nifedipine, and *d-cis*-diltiazem) on Ca^{2+} uptake by SPM under conditions that permit Na^+/Ca^{2+} exchange were studied as described previously by Coutinho et al. (1983), by diluting membrane vesicles preloaded with Na^+ overnight (150 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES-Tris, pH 7.4) into Ca^{2+} uptake media [150 mM KCl or 150 mM NaCl; 10 mM glucose; 10 mM HEPES-Tris, pH 7.4; and 20 μM $CaCl_2$ + ^{45}Ca (2.5 μCi/μmol)] at a final protein concentration of 0.5 mg/ml. The reaction media also contained increasing concentrations of either nifedipine, verapamil, or *d-cis*-diltiazem. The reaction proceeded at 30°C and, after 3 min, was terminated by filtering 0.5-ml samples, as described above.

To study the effects of calcium blockers on the ATP-dependent Ca^{2+} uptake by SPM, the membrane vesicles isolated in KCl medium, as described above, were diluted 20-fold into a reaction medium of the following composition: 150 mM KCl; 1 mM $MgCl_2$; 10 mM glucose; 10 mM HEPES-Tris, pH 7.4; 20 μM $CaCl_2$ + ^{45}Ca (2.5 μCi/μmol) at a final protein concentration of 0.5 mg/ml, and supplemented with various concentrations of the various blockers. Ca^{2+} uptake in the absence and in the presence of 1 mM ATP-Mg was determined, and the reaction was followed at 30°C, for 3 min, after which duplicate samples were filtered, as described above.

Materials

All chemicals used were of highest purity available commercially. The radiolabelled 1,4-dihydropyridine utilized in this study, [^{3}H]nimodipine (150.2 Ci/mmol), and unlabelled nimodipine were provided by Prof. Hoffmeister, from Bayer AG, Wuppertal, F.R.G. Nifedipine and *d-cis*-diltiazem were provided by Prof. T. Macedo, Faculty of Medicine, Coimbra, Portugal. Verapamil (racemic mixture) was obtained from Knoll Lusitana, Portugal. Both (−)- and (+)-verapamil and (−)-D 600 were provided by Prof. H. Glossmann, Rudolf-Bucheim-Institut für Pharmakologie, Giessen, F.R.G. The 1,4-dihydropyridines were first dissolved in absolute ethanol at 1 mM and then were diluted to the appropriate concentrations with buffer (50 mM Tris-HCl, pH 7.4). Due to the extreme lability of the 1,4-dihydropyridines to light, all solutions were stored light-protected at −20°C.

RESULTS

Distribution of [^{3}H]nimodipine binding sites in sheep brain membrane fractions

High-affinity binding sites for [^{3}H]nimodipine have been reported previously in brain (Ferry and Glossmann, 1982, 1983; Bellemann et al., 1982; Glossmann et al., 1983). However, most binding studies were performed with total membrane preparations (homogenates), and there is some uncertainty about subcellular membrane localization of the 1,4-dihydropyridine binding sites in brain tissue (Bellemann et al., 1982). To clarify this issue, we studied the binding of [^{3}H]nimodipine to purified sheep brain membrane subfractions obtained from synaptosomal and microsomal preparations, and correlated the binding with the Na^+,K^+-ATPase activity in the various fractions (Fig. 1).

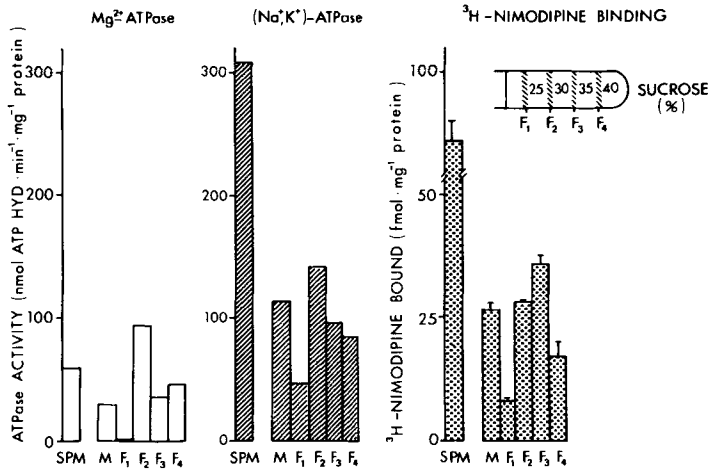

FIG. 1. Distribution of ATPase activities and [^{3}H]nimodipine specific binding in sheep brain membrane fractions. The Mg^{2+}-ATPase is the ATPase activity not inhibited by 1.0 m*M* ouabain and the Na^+,K^+-ATPase was taken as the activity inhibited by 1.0 m*M* ouabain. [^{3}H]Nimodipine binding was determined at a free [^{3}H]nimodipine concentration of 0.25 n*M*, in 50 m*M* Tris-Cl, pH 7.4, in the absence (total binding) or in the presence (nonspecific) of 5 μM unlabelled nimodipine. The specific binding is taken as the difference between total and nonspecific binding. Values for the ATPase activities are means of the values obtained in two different experiments, and agreed within 10%. Data for [^{3}H]nimodipine binding are means ± SD from three different experiments, each performed in triplicate.

As shown in Fig. 1, the SPM obtained after osmotic lysis of synaptosomes followed by partial purification (Coutinho et al., 1983) are enriched in [^{3}H]nimodipine binding sites (about 70 fmol [^{3}H]nimodipine bound/mg protein) and in Na^+,K^+-ATPase activity (about 300 nmol ATP hydrolyzed/min/mg protein). Therefore, it is assumed that the 1,4-dihydropyridine binding sites are localized predominantly at the plasma membrane of nerve terminals. Figure 1 also shows that the brain microsomal fraction has some binding sites for [^{3}H]nimodipine, but there is no further significant purification of these binding sites when the crude microsomal fraction (M) is subfractionated into four fractions (F_1 through F_4) by sucrose gradient centrifugation. Therefore, the SPM fraction was preferentially utilized in subsequent studies to characterize the [^{3}H]nimodipine binding sites and to study the effects of various Ca^{2+} channel blockers on Ca^{2+} translocation in brain.

Affinities of several Ca^{2+} blockers for [^{3}H]nimodipine binding sites in purified SPM

The purified SPM fraction isolated from sheep brain contains high-affinity binding sites for [^{3}H]nimodipine, as shown in Fig. 2, which shows the results of saturation experiments. [^{3}H]Nimodipine labels a single class of sites in SPM, with an apparent K_D of 0.64 ± 0.1 n*M* and a B_{max} of 161 ± 27 fmol·mg^{-1} protein, as determined from Scatchard analysis of specific binding (Fig. 2B), measured at 25°C. The Hill slope is 1.07 (Fig. 2C), which implies that one homogeneous group of binding sites is present.

The specific binding of [^{3}H]nimodipine was defined as that displaced by an excess of unlabelled nimodipine (Fig. 2A), as described previously by other investigators for gross membrane homogenates (Bellemann et al., 1982; Ferry and Glossmann, 1982; Glossmann et al., 1983). In the present experiments, specific binding accounted for 50–80% of the total

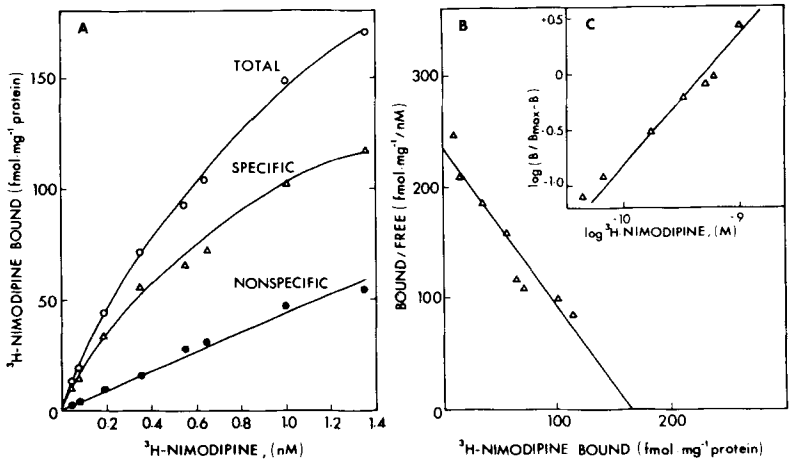

FIG. 2. Equilibrium binding of [^{3}H]nimodipine by SPM fraction as a function of increasing concentrations [^{3}H]nimodipine. **A:** The membranes were incubated, as described in Materials and Methods, with concentrations of [^{3}H]nimodipine varying between 0.05 and 1.4 n*M*. Nonspecific binding was measured in the presence of 5 μM unlabelled nimodipine. **B:** Scatchard plot of the specifically bound [^{3}H]nimodipine. The data are means of three experiments, each performed in triplicate, and K_D and B_{max} values obtained by linear regression analysis are 0.64 ± 0.1 n*M* and 161 ± 27 fmol·mg^{-1} protein, respectively. **C:** Hill transformation of the specific binding data (slope = 1.07).

binding, and it decreased with increasing concentration of radioligand. At 0.6 n*M* [^{3}H]nimodipine, which is about the K_D value, the specific binding is 73% of the total binding.

We also determined the relative affinities of various Ca^{2+} channel blockers for the [^{3}H]nimodipine binding sites in SPM by performing competition studies in which the membranes were labelled with [^{3}H]nimodipine and then increasing concentrations of various unlabelled Ca^{2+} blockers were added to displace the bound [^{3}H]nimodipine. The results, summarized in Table I, show that: (1) nimodipine and nifedipine are potent competitors for the [^{3}H]nimodipine binding sites, with IC_{50} values of 4.7 and 5.8 n*M*, respectively, as determined by Hill transformation of the inhibition-binding data. The Hill slopes for these compounds are 0.93 and 0.83, respectively, indicating competition for an homogeneous class of [^{3}H]nimodipine binding sites. (2) On the other hand, (+)- and (−)-verapamil and (−)-D 600 are weak competitors, and display a biphasic competition behavior as described previously by Ferry and Glossmann (1982) and Glossmann et al. (1983); thus, for (+)-verapamil, the IC_{50} for the higher affinity component is 708 n*M*, the Hill slope is 0.73, and the maximal inhibition is 50% of the specific binding, whereas for (−)-verapamil the IC_{50} is lower (120 n*M*), the Hill slope is 0.57, but the maximal inhibition is only 30% of the specific binding, at concentrations of 3×10^{-5} *M* of unlabelled drug (Table I).

These results indicate that there is some stereoselectivity in the interaction of phenylalkylamines with the [^{3}H]nimodipine binding sites in SPM, as shown previously in other systems (Ferry and Glossmann, 1982; Glossmann et al., 1983). The stereoselectivity for the 1,4-dihydropyridines in inhibiting binding has also been shown previously by Bellemann et al. (1981) and by Glossmann et al. (1982).

TABLE 1. *Binding-inhibition constants of [^{3}H]nimodipine binding to SPM by various Ca^{2+} channel blockers*

Drug	IC_{50} (n*M*)	n_H	Maximal inhibition (% of total binding)
Nimodipine	4.7 ± 0.50	0.93 ± 0.60	70
Nifedipine	5.8 ± 0.45	0.83 ± 0.07	70
(+)-Verapamil	708[a]	0.73	50[b]
(−)-Verapamil	120[a]	0.57	30[b]
(−)-D 600	430[a]	—	40[b]

IC_{50} is the concentration of drug causing 50% inhibition of specific [^{3}H]nimodipine binding, as calculated from linear regression analysis of data transformed into the Hill equation. n_H is the Hill slope. Binding-inhibition curves were performed with a fixed [^{3}H]nimodipine concentration (0.37 n*M*), and increasing concentrations of the competing unlabelled drug. The data are means of three experiments, each performed in triplicate. Examples of binding-inhibition experiments are shown in Fig. 4.

[a] Higher affinity component.

[b] At concentrations = 3×10^{-5} *M*.

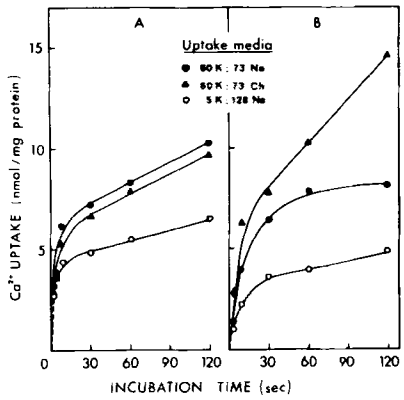

FIG. 3. Time course of $^{45}Ca^{2+}$ uptake by synaptosomes preincubated in two different media: (**A**) the ion-free medium, which contained 0.32 *M* sucrose and 10 m*M* HEPES-Tris, pH 7.4 or (**B**) The Na^+-rich medium, containing (in m*M*): 128 NaCl, 5 KCl, 10 glucose, 1.2 $MgCl_2$, 10 HEPES-Tris, pH 7.4. $^{45}Ca^{2+}$ uptake was studied in basal (○, 128 Na; 5 K) and depolarizing media in the presence of external Na^+ (●, 60 K; 73 Na), or in the absence of external Na^+ (▲, 60 K; 73 Ch), all containing 1 m*M* $^{45}CaCl_2$. See Materials and Methods for experimental details.

Effects of Ca^{2+} blockers on K^+ depolarization-induced Ca^{2+} influx in sheep brain synaptosomes

In this study, we investigated the correlation between the effect of various Ca^{2+} blockers on the binding of [^{3}H]nimodipine and the effect on Ca^{2+} influx in synaptosomes. Thus, we first determined the conditions for studying Ca^{2+} uptake under which the Ca^{2+} channels are supposed to open (that is, when synaptosomes are depolarized by addition of K^+ to the external medium) and a minimum of Na^+/Ca^{2+} exchange takes place (Coutinho et al., 1984). Thus, Fig. 3 shows the time course of Ca^{2+} uptake in synaptosomes preincubated in two different media: sucrose medium (Fig. 3A) or Na^+-rich medium (Fig. 3B). The uptake was then studied in depolarizing medium in the presence of external Na^+ (60 m*M* K^+; 73 m*M* Na^+), or in the absence of external Na^+ (60 m*M* K^+; 73 m*M* Ch). The results show that in synaptosomes preincubated in sucrose, most of the Ca^{2+} influx is due to K^+ depolarization (Fig. 3A), since no significant difference is observed in the level of Ca^{2+} uptake when Na^+ is absent from external medium, indicating that Ca^{2+} uptake by Na^+/Ca^{2+} exchange is minimal. However, in synaptosomes preincubated in Na^+-rich medium prior to the Ca^{2+} uptake, there is an increased Ca^{2+} uptake when Na^+ is absent from uptake medium (Fig. 3B), indicating that, under these conditions, there is a large contribution of the Na^+/Ca^{2+} exchange to total Ca^{2+} uptake, especially at the end of 120 s (Fig. 3B).

We studied the effect of Ca^{2+} channel blockers on Ca^{2+} uptake by synaptosomes under the condition described in Fig. 3A, which ensures that most Ca^{2+} enters through the Ca^{2+} channels, rather than through Na^+/Ca^{2+} exchange. The results show that (+)-vera-

pamil, nifedipine, and nimodipine are much less potent in inhibiting Ca^{2+} influx than in inhibiting the binding of [^{3}H]nimodipine to the purified membranes (Fig. 4). Thus, (+)-verapamil inhibits Ca^{2+} influx induced by K^+ depolarization by 50% at about 500 μM, whereas it inhibits 50% of the [^{3}H]nimodipine binding at concentrations 200-fold lower. The discrepancy is even larger for nimodipine and nifedipine (Fig. 4). Therefore, the results show that, under our experimental conditions, there is no close correlation between the affinities of the Ca^{2+} blockers for the SPM binding sites and their potency for blocking Ca^{2+} influx due to K^+ depolarization in synaptosomes under conditions of minimal Na^+/Ca^{2+} exchange. These results, obtained in the absence of Na^+/Ca^{2+} exchange, are at variance with those recently reported by Turner and Goldin (1985). See later for discussion.

We further examined this problem by studying the effect of verapamil on the total Ca^{2+} that enters the synaptosomes both by Ca^{2+} influx due to K^+ depolarization and by Na^+/Ca^{2+} exchange. As indicated above (Fig. 3), Ca^{2+} influx by both mechanisms occurs in synaptosomes that have been preincubated in a Na^+-rich medium prior to utilization in studies of Ca^{2+} influx, as described also in the legend to Fig. 5. This preincubation in Na^+-rich medium has been normal practice by other investigators who, nevertheless, assumed they were studying only Ca^{2+} influx due

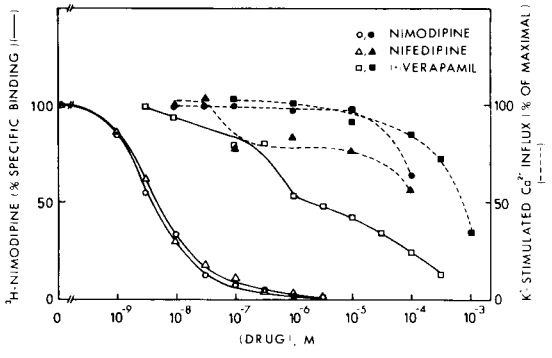

FIG. 4. Relationship of the relative inhibition of the specific binding of [^{3}H]nimodipine by nifedipine (○), nimodipine (△), and (+)-verapamil (■) with their inhibition of $^{45}Ca^{2+}$ influx into sheep brain synaptosomes. [^{3}H]Nimodipine binding was performed at a fixed [^{3}H]nimodipine concentration (0.37 nM), in the presence of increasing concentrations of each of the competing unlabelled drug, and maximal specific binding is defined as that displaced by 5 μM unlabelled nimodipine. The $^{45}Ca^{2+}$ uptake experiments were performed by transferring synaptosomes isolated in 0.32 M sucrose, 10 mM HEPES-Tris, pH 7.4, to depolarizing media containing 150 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES-Tris, pH 7.4, 100 μM $CaCl_2$ supplemented with $^{45}CaCl_2$ (2.5 μCi/μmol), and increasing concentrations of nimodipine, nifedipine, or (+)-verapamil. The amount of Ca^{2+} retained in control media, containing 150 mM NaCl instead of KCl, was determined and was subtracted for all drug concentrations tested. The binding data are means of three independent experiments performed in triplicate. The Ca^{2+} influx data are means of two experiments performed in duplicate.

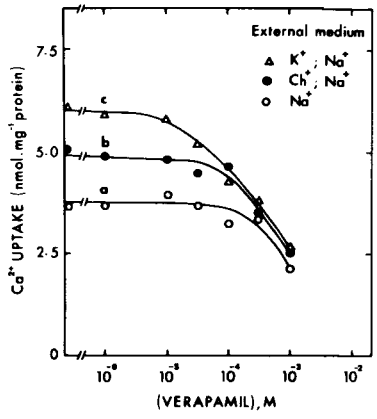

FIG. 5. Effect of verapamil on Ca^{2+} uptake induced by K^+ (curve c − curve b), or by Na^+/Ca^{2+} exchange (curve b − curve a), in sheep brain synaptosomes. Synaptosomes were preincubated for 15 min, at 30°C, in Na^+-rich medium, as described in the Materials and Methods, and the Ca^{2+} uptake was initiated by transferring preincubated synaptosomes (0.5 mg/ml, final protein concentration) to three different media: 128 mM NaCl and 5 mM KCl (○); 60 mM ChCl and 73 mM NaCl (●); 60 mM KCl and 73 mM NaCl (△), all containing, additionally, 1 mM $MgCl_2$, 10 mM glucose, 1 mM Na_2HPO_4, 10 mM HEPES-Tris, pH 7.4, 1 mM $CaCl_2$ supplemented with $^{45}CaCl_2$ (2.5 μCi/μmol), and increasing concentrations of verapamil. Ca^{2+} uptake was terminated by filtration after 2 min of reaction at 30°C.

to K^+ depolarization (Blaustein, 1977; Nachshen and Blaustein, 1979, 1980, 1982). The preincubation is usually for a period of 15 min, at 30°C, followed by addition of K^+ to the external medium, to obtain membrane depolarization and opening of the Ca^{2+} channels. The addition of K^+ is accompanied with an equimolar reduction in the Na^+ concentration in the outside medium, to maintain the osmolarity constant. In our experience, this procedure leads to Ca^{2+} influx not only due to K^+ depolarization but also by Na^+/Ca^{2+} exchange (Coutinho et al., 1984), since there is a favorable Na^+ gradient from inside to the outside, which drives Ca^{2+} uptake by Na^+/Ca^{2+} exchange (Fig. 3B).

As shown in Fig. 5, when synaptosomes preincubated for 15 min, at 30°C, in a Na^+-rich medium (128 mM NaCl; 5 mM KCl), were transferred to a Ca^{2+} uptake medium of the same ionic composition, there is a basal level of Ca^{2+} binding (about 3.5 nmol Ca^{2+}/mg protein; Fig. 5, curve a). However, when the external medium contains part of the NaCl substituted for ChCl (60 mM ChCl; 73 mM NaCl; 5 mM KCl), a Na^+ gradient from inside to outside is created, Ca^{2+} uptake in exchange for Na^+ is induced, and it is observed that 5 nmol of Ca^{2+} are taken up per milligram of protein (Fig. 5, curve b). Furthermore, if part of the NaCl in the external medium is substituted for KCl (60 mM KCl; 73 mM NaCl), we simultaneously depolarize the membrane (by increasing K^+ outside) and create a Na^+ gradient, which results in Ca^{2+} uptake simultaneously through Ca^{2+} channels and by Na^+/Ca^{2+} ex-

change. Consequently, total Ca^{2+} uptake increases in this situation, to about 6.5 nmol Ca^{2+}/mg protein (Fig. 5, curve c). These results indicate that the conditions reported in the literature for K^+ depolarization also induce some Na^+/Ca^{2+} exchange, and most published data must be reviewed, if care was not taken to distinguish Ca^{2+} entry through the channels from Ca^{2+} entry through Na^+/Ca^{2+} exchange (Nachshen and Blaustein, 1980, 1982; Daniell et al., 1983; Rampe et al., 1984).

When the effect of verapamil on Ca^{2+} uptake by synaptosomes by these mechanisms (Fig. 5) was determined, it was observed that this Ca^{2+} blocker inhibits the Ca^{2+} uptake due both to K^+ depolarization and to Na^+/Ca^{2+} exchange, for concentrations higher than 10^{-5} *M* of the drug, and that the potency for inhibiting either Ca^{2+} uptake system is similar. Therefore, in the concentration range studied there is no selective effect of verapamil on Ca^{2+} channels. We observed similar results for the inhibition of Ca^{2+} uptake in synaptosomes by other Ca^{2+} blockers, such as *d-cis*-diltiazem and nifedipine (results not shown), i.e., the Ca^{2+} blockers inhibit not only the K^+ depolarization-induced Ca^{2+} uptake, but also the Ca^{2+} uptake that occurs through Na^+/Ca^{2+} exchange. This was further studied in SPM, and the results are presented in the next section.

Comparative effect of Ca^{2+} blockers on the Ca^{2+} transport systems in SPM

We further studied the effects of various Ca^{2+} channel blockers on the Ca^{2+}transport in membrane vesicles derived from SPM, in which the ionic gradients can be controlled. Thus, SPM vesicles were preloaded with NaCl or KCl medium, as described in Materials and Methods, and Ca^{2+} uptake by Na^+/Ca^{2+} exchange (in the NaCl-loaded vesicles) or ATP-dependent Ca^{2+} uptake (in the KCl-loaded vesicles) was studied in the presence of increasing concentrations of the Ca^{2+} blockers (Figs. 6 and 7).

The results in Fig. 6 show that the three types of Ca^{2+} blockers studied (verapamil, nifedipine, and *d-cis*-diltiazem) inhibit the Na^+/Ca^{2+} exchanger in SPM. The concentrations required to inhibit 50% of the Ca^{2+} uptake by this mechanism are 4.6×10^{-4} *M*, 3×10^{-4} *M*, and 10^{-3} *M* for verapamil, nifedipine, and *d-cis*-diltiazem, respectively. The values for Ca^{2+} uptake shown in Fig. 6 are taken after 3 min of reaction, and therefore represent total Ca^{2+} uptake. However, we also determined the effects of the Ca^{2+} blockers on the initial rate of Na^+/Ca^{2+} exchange, and the same percent effect is observed in both the rate and total Ca^{2+} uptake (results not shown).

The ATP-dependent Ca^{2+} uptake in SPM isolated from sheep brain is also inhibited by high concentrations of the Ca^{2+} blockers, as shown in Fig. 7. The concentrations of the drugs required to inhibit 50% of the Ca^{2+} uptake are 8.7×10^{-4} *M*, 8.7×10^{-4} *M*, and 10^{-3} *M* verapamil, nifedipine, and *d-cis*-diltiazem, respectively. The significance of the effects of the Ca^{2+} blockers on the Ca^{2+} transport systems in the brain is referred to in the Discussion.

DISCUSSION

In the present study, we investigated the affinities of the Ca^{2+} channel blockers for their binding sites in purified brain membranes and also the effects of the blockers on the Ca^{2+} channels and the Ca^{2+} transport systems of the plasma membrane, namely the Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} transport. To study the affinity of the Ca^{2+} channel blockers for the binding sites, we utilized mostly the SPM fraction, enriched in Na^+,K^+-ATPase activity (Coutinho et al., 1983) and in [^{3}H]nimodipine binding sites (Fig. 1). The value of the dissociation constant for [^{3}H]nimodipine binding to SMP, of 0.6 n*M* (Fig. 2), indicates a high-affinity binding, and the competition of the various classes of Ca^{2+} channel blockers for the [^{3}H]nimodipine binding (Table I, Fig. 4) is similar to that

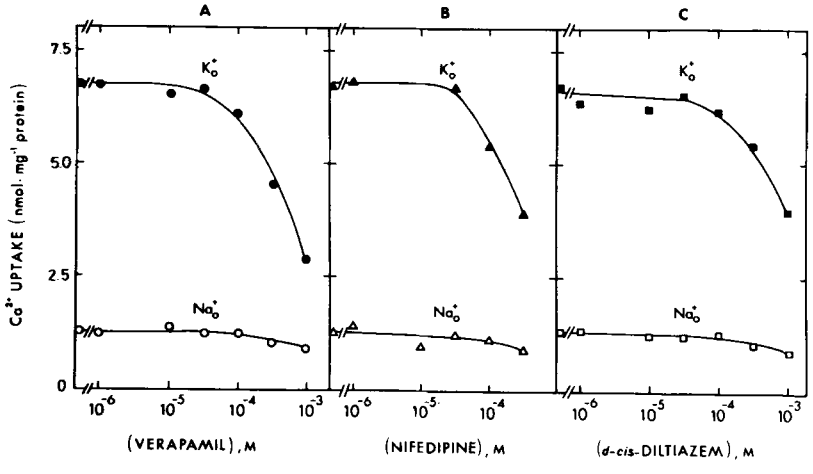

FIG. 6. Effects of verapamil, nifedipine, and *d-cis*-diltiazem on Na^+/Ca^{2+} exchange in SPM. The SPM were loaded overnight at 0–4°C in medium containing 150 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES-Tris, pH 7.4, to load the vesicles with Na^+. The Na^+/Ca^{2+} exchange was assayed by transferring samples of the SPM suspension into media containing 150 m*M* NaCl (Na_o) or 150 m*M* KCl (K_o) and 20 μ*M* $CaCl_2$, supplemented with $^{45}CaCl_2$ (2.5 μCi/μmol) and the various drug concentrations tested. The reaction was conducted for 3 min, at 30°C, and was terminated by filtration of duplicate samples (0.5 ml, containing 0.25 mg protein), as described in Materials and Methods. Data are means of three independent experiments.

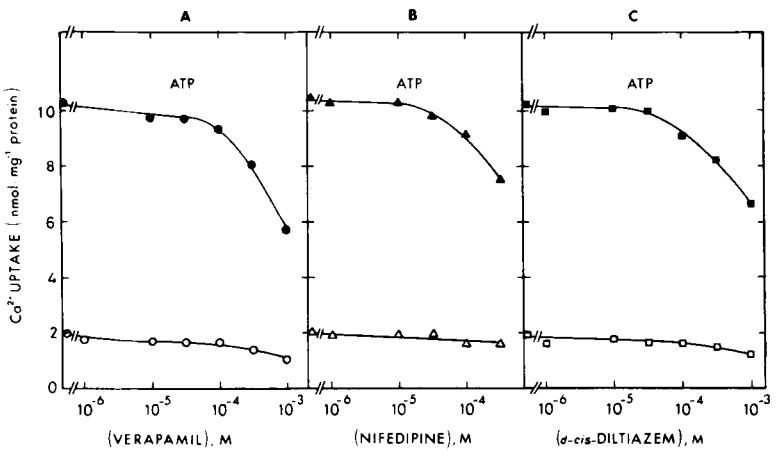

FIG. 7. Effects of verapamil, nifedipine, and *d-cis*-diltiazem of the ATP-dependent Ca^{2+} uptake by SPM. The membrane vesicles were incubated overnight at 0–4°C in medium containing 150 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES-Tris, pH 7.4. ATP-dependent Ca^{2+} uptake was assayed by transferring samples of SPM suspension to a similar KCl medium containing, additionally, 20 μM $CaCl_2$ supplemented with $^{45}CaCl_2$ (2.5 μCi/μmol), 0.1 m*M* dinitrophenol, 0.1 m*M* azide, 1.0 μg/ml oligomycin, and the various drug concentrations tested. The reaction was initiated by addition of 1 m*M* ATP-Mg, was conducted for 3 min at 30°C, and was terminated by filtration. The effects of the various drug concentrations on the retention of Ca^{2+} by SPM in a control medium without ATP (open symbols) were also tested. The data are means of three independent experiments.

reported in previous studies carried out with whole brain homogenates (Bellemann et al., 1982; Ferry and Glossmann, 1982; Glossmann et al., 1983), indicating drug specificity for the binding sites.

However, a clear selective effect of the Ca^{2+} channel blockers on the Ca^{2+} fluxes through Ca^{2+} channels at the concentrations that saturate the binding sites of SPM has not yet been clearly demonstrated by previous studies in synaptosomes. In fact, studies on the effect of Ca^{2+} channel blockers on the depolarization-induced Ca^{2+} fluxes in brain synaptosomes (Nachshen and Blaustein, 1979; Akerman and Nicholls, 1981) show that verapamil and D 600 only partially inhibit Ca^{2+} fluxes, even at concentrations of the order of 10^{-5}–10^{-4} *M*. Furthermore, nifedipine (10^{-5} *M*) was also shown to be ineffective (Nachshen and Blaustein, 1979), and other dihydropyridines, such as nitrendripine, nimodipine, and nisoldipine, at concentrations up to 10^{-5} *M*, did not alter Ca^{2+} influx into brain synaptosomes (Daniell et al., 1983). More recently, it was also shown that BAY K 8644, a 1,4-dihydropyridine Ca^{2+} channel activator in smooth and cardiac muscles, is also without effect on Ca^{2+} uptake in brain synaptosomes (Rampe et al., 1984), but Middlemiss and Spedding (1985) reported that BAY K 8644 can augment the K^+-stimulated release of 5-hydroxytryptamine (serotonin) from rat cortex slices and that this effect can be antagonized by Ca^{2+} antagonists.

There is only a recent study (Turner and Goldin, 1985) showing partial inhibition of depolarization-stimulated Ca^{2+} uptake in synaptosomes by nitrendipine, nifedipine, verapamil, D 600, and diltiazem in the nanomolar concentration range of these drugs. These workers tried to eliminate the Na^+/Ca^{2+} exchange component in their experiments, working in choline medium in the absence of Na^+, but we have shown, previously, under similar conditions, that K^+ stimulates the Na^+/Ca^{2+} exchange in SPM (Coutinho et al., 1983). Thus, some of the effect of the Ca^{2+} blockers on Ca^{2+} fluxes in synaptosomes reported by Turner and Goldin (1985) may reflect the effect of the blockers in the K^+-dependent Na^+/Ca^{2+} exchange (Coutinho et al., 1983). The fact that Turner and Goldin (1985) did not find a corresponding $^{22}Na^+$ efflux may reflect the inherent difficulty of carrying out this type of experiment with ^{22}Na (K. Phillipson, personal communication).

In most studies previously designed to determine the effects of Ca^{2+} channel blockers on Ca^{2+} uptake, synaptosomes were submitted to a preincubation in a Na^+-rich medium, which allows some accumulation of Na^+ (Coutinho et al., 1984) and subsequently, when the synaptosomes were transferred to the K^+-rich medium containing ^{45}Ca, there was ^{45}Ca entry through Na^+/Ca^{2+} exchange that is superimposed on the Ca^{2+} entry through the Ca^{2+} channels. Thus, it is important to differentiate between the effects of Ca^{2+} channel blockers on the two Ca^{2+} entry mechanisms.

In this study we utilized synaptosomes isolated in buffered sucrose, not exposed to Na^+, to study the effect of nimodipine, nifedipine, and (+)-verapamil on the Ca^{2+} influx when membrane depolarization by high K^+ was induced (Fig. 4). It is observed that, under these experimental conditions, in which there is minimal Na^+/Ca^{2+} exchange and the Ca^{2+} influx takes place through Ca^{2+} channels, there is inhibition of Ca^{2+} entry only for very high concentrations of the drugs (10^{-4}–10^{-3} *M*). This does not correlate with the effect of the Ca^{2+} channel blockers on their inhibition of the binding of [^{3}H]nimodipine as shown in Fig. 4. Under conditions in which Ca^{2+} influx occurs both through the Ca^{2+} channels and by Na^+/Ca^{2+}, there is a similar sensitivity of both mechanisms to inhibition by verapamil (Fig. 5). Previous studies have also demonstrated inhibition by Ca^{2+} channel blockers of Na^+/

Ca^{2+} exchange in heart and brain mitochondria (Vághy et al., 1982; Matlib and Schwartz, 1983), as well as in brain microsomes (Liron et al., 1985), and in SPM (Erdreich et al., 1983), and in all cases relatively high concentrations of the blockers are necessary.

Our studies with isolated SPM also show that the uptake of Ca^{2+} by Na^+/Ca^{2+} exchange and by the Ca^{2+}-ATPase systems is inhibited by verapamil, nifedipine, and *d-cis*-diltiazem at concentrations above 10^{-4} *M* (Figs. 6 and 7).

In our attempt to dissociate the depolarization-dependent Ca^{2+} entry into synaptosomes from the Na^+/Ca^{2+} exchange we resorted to using synaptosomes not exposed previously to Na^+ and to carry out the K^+ depolarization in the absence of choline because in both instances there occurs Na^+/Ca^{2+} exchange. However, our results are at variance with those reported by Turner and Goldin (1985), since under conditions that would ensure $^{45}Ca^{2+}$ influx through only the Ca^{2+} channels the Ca^{2+} blockers tested are not effective at concentrations at which they saturate the membrane binding sites (Figs. 2 and 4).

The Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} uptake are important mechanisms in regulating intracellular Ca^{2+} concentration in nerve (Baker, 1972; Blaustein and Nelson, 1982; Gill et al., 1981; Coutinho et al., 1983) and muscle cells (Hurwitz et al., 1983; van Breemen et al., 1979; Ozaki and Urakawa, 1979; Morel et al., 1981; Verbist et al., 1984), but the finding that Ca^{2+} channel blockers can inhibit these transport systems at relatively high concentrations ($>10^{-4}$ *M*) (Figs. 6 and 7) probably is of little significance under normal conditions. However, when high doses of the Ca^{2+} channel blockers are administered to patients with cardiovascular diseases some of these drugs may reach concentrations in membranes that are much higher than their extracellular level, because of their high lipid solubility (Pang and Sperelakis, 1984). We are currently determining the partition coefficients of various Ca^{2+} channel blockers in membranes isolated from brain, heart, and smooth muscle to estimate the maximal concentration of these drugs in the lipid phase of the membranes.

It is of interest that the binding affinities and the inhibition of Ca^{2+} fluxes by Ca^{2+} channel blockers are correlated in certain neuronal cell lines. In fact, in PC12 cells the Ca^{2+} currents and the Ca^{2+} uptake through the channels are inhibited by low concentrations of Ca^{2+} channel blockers (Toll, 1982); also, neurotransmitter release and K^+-induced Ca^{2+} uptake in some neuronal cell lines are sensitive to low (nanomolar) concentrations of Ca^{2+} channel blockers (Takahashi and Ogura, 1983; Albus et al., 1984; Freedman et al., 1984). Thus, since neuronal cells in culture are sensitive to Ca^{2+} channel blockers, it was suggested (Spedding and Middlemiss, 1985) that, under culture conditions, it is possible that the cells lose a factor protecting from blockade by Ca^{2+} channel blockers in brain tissue. The search for such a factor is of great interest for the elucidation of the discrepancies observed between the behavior of cells in culture and homogenated fractions of brain tissue.

Furthermore, a recent study (Middlemiss and Spedding, 1985) shows that, under certain specific conditions, Ca^{2+} channel blockers can directly affect neurotransmitter release in brain slices. In these studies, the agonist Bay K 8644 increased neurotransmitter release, in a Ca^{2+}-dependent manner, and the effect was, in turn, inhibited by Ca^{2+} antagonists at low (1 μM) concentrations. Therefore, the conclusion from these observations is that Ca^{2+} channel blockers can have effects on neuronal Ca^{2+} channels, but only subsequent to certain forms of activation of the channels. Thus although there are indications of a functional correlate of the 1,4-dihydropyridine binding sites in brain tissue, it is not easy to demonstrate this correlation, especially in isolated brain fractions. For instance, in isolated brain synaptosomes, Ca^{2+} uptake due to K^+ depolarization is not sensitive to Bay K 8644 (Rampe et al., 1984), which is not the case in brain slices (Middlemiss and Spedding, 1985). As referred to earlier we cannot explain the recent report by Turner and Goldin (1985) that nitrendipine inhibits depolarization-stimulated Ca^{2+} uptake and [^{3}H]norepinephrine release from synaptosomes at low nitrendipine concentrations (K_{app} of 56 n*M* in the presence of Na^+ and K_{app} of 1.7 n*M* in the absence of Na^+).

In another development Bean (1984) and Sanguinetti and Kass (1984) reported that the binding of the 1,4-dihydropyridines is voltage-dependent. Thus, Bean (1984) showed that nitrendipine blocks cardiac Ca^{2+} currents very potently (K_D, 0.36 n*M*) at holding potentials at which most Ca^{2+} channels are inactivated and, at more negative holding potentials, the block is less potent by a factor of >1,000. Thus, it is suggested that nitrendipine binds tightly to the inactivated state of the Ca^{2+} channel and much more weakly to the resting state. These electrophysiological studies were complemented by radioligand binding (Reuter et al., 1985), and it was shown that the binding of [^{3}H]nimodipine to living cardiac cells is voltage-dependent. Schwartz et al. (1985) also showed that, in skeletal muscle fibers, depolarization increases the number of binding sites in the high-affinity state. The authors (Schwartz et al., 1985) also compare the binding results with the voltage-clamp measurements of Ca^{2+} channel blockade and conclude that less than a few percent of the binding sites in skeletal muscle represent functional Ca^{2+} channels. Similar voltage dependence binding studies of Ca^{2+} channel blockers in brain cells are not available to our knowledge. This type of experiment should also be feasible with synaptosomes and we are currently exploring this possibility. Our preliminary results show a slight difference in the amount of nitrendipine bound to synaptosomal membranes in the presence of K^+ or Na^+, indicating that the membrane potential may in fact be an impor-

tant factor in determining the availability of the binding sites.

In summary, the results reported in this study show that, although purified SPM are enriched in high-affinity binding sites for [^{3}H]nimodipine (K_D in the nanomolar range), the various types of Ca^{2+} channel blockers (verapamil, nifedipine, and *d-cis*-diltiazen) inhibit Ca^{2+} influx through Ca^{2+} channels, the Na^+/Ca^{2+} exchange, and the ATP-dependent Ca^{2+} uptake, in a range of concentrations (10^{-5}–10^{-4} *M*) that are much higher than the concentrations that saturate the 1,4-dihydropyridine binding sites in the brain membranes. In this study clear differentiation is made of the effects of the Ca^{2+} channel blockers on these three mechanisms of moving Ca^{2+} across the synaptosomal membrane, and particular care is taken to separate the contribution of the Na^+/Ca^{2+} exchange from that of the influx through Ca^{2+} channels under conditions of K^+ depolarization. Under carefully defined conditions, we confirm the inherent difficulty in showing in isolated brain fractions an action of Ca^{2+} channel blockers on the Ca^{2+} channels at the nanomolar concentrations required for the drugs to saturate the membrane binding sites, and recent results claiming the contrary must be viewed with caution (Turner and Goldin, 1985).

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