Multiple Calcium Channels in Synaptosomes: Voltage Dependence of 1,4-Dihydropyridine Binding and Effects on Function†

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Received December 22, 1987; Revised Manuscript Received March 25, 1988

ABSTRACT: The voltage dependence of binding of the calcium channel antagonist, (+)-[^{3}H]PN200-110, to rat brain synaptosomes and the effects of dihydropyridines on $^{45}Ca^{2+}$ uptake have been investigated. Under nondepolarizing conditions (+)-[^{3}H]PN200-110 binds to a single class of sites with a K_d of 0.07 nM and a binding capacity of 182 fmol/mg of protein. When the synaptosomal membrane potential was dissipated either by osmotic lysis of the synaptosomes or by depolarization induced by raising the external K^+ concentration, there was a decrease in affinity (approximately 7-fold) with no change in the number of sites. The effects of calcium channel ligands on $^{45}Ca^{2+}$ uptake by synaptosomes have been measured as a function of external potassium concentration, i.e., membrane potential. Depolarization led to a rapid influx of $^{45}Ca^{2+}$ whose magnitude was voltage-dependent. Verapamil (100 μM) almost completely inhibited calcium uptake at all potassium concentrations studied. In contrast, the effects of dihydropyridines (2 μM) appear to be voltage-sensitive. At relatively low levels of depolarization (10–25 mM K^+) nitrendipine and PN200-110 completely inhibited $^{45}Ca^{2+}$ influx, whereas the agonist Bay K8644 slightly potentiated the response. At higher K^+ concentrations an additional dihydropyridine-insensitive component of calcium uptake was observed. These results provide evidence for the presence of dihydropyridine-sensitive calcium channels in synaptosomes which may be activated under conditions of partial depolarization.

In excitable cells the influx of calcium ions through voltage-sensitive calcium channels plays a crucial role in many cellular functions including muscle contraction and neurotransmitter release (Hagiwara & Byerly, 1981; Tsien, 1983). Recently, the study of the molecular properties of calcium channels was given great impetus by the finding that organic drugs which modulate calcium channel activity in heart and smooth muscle bind tightly and specifically to membrane-bound proteins in a variety of tissues. These drugs, particularly the 1,4-dihydropyridine (DHP)[1] derivatives, are proving to be useful probes in the study of the structure and function of calcium channel proteins.

Voltage-dependent calcium channels in different tissues can be distinguished by their biophysical properties and their sensitivity to drugs [reviewed in Tsien et al. (1987)]. Although DHP antagonists have been shown to block functional responses in smooth and cardiac muscle, their role in modulating calcium channels in skeletal muscle and brain is less well established. In these latter tissues there is therefore some controversy as to whether DHP binding proteins represent functional calcium channels.

High-affinity binding sites for DHPs have been demonstrated to exist in the brain [reviewed in Glossmann et al. (1985) and Triggle and Janis (1987)]. The functional significance of these sites has been questioned since in many early studies it appeared that neuronal calcium channels were insensitive to DHP antagonists (Miller & Freedman, 1984). More recently, calcium channels in several neuronal cell lines have been shown to be modulated with appropriate pharmacological specificity by DHP antagonists and agonists [reviewed in Miller (1987)]. Some insight into the molecular basis for the observed anomalies has come from the electrophysiological characterization of multiple types of calcium channels in neurons and other excitable cells [reviewed in Tsien et al. (1987)]. Nowycky et al. (1985) identified three types of calcium channels in dorsal root ganglion (DRG) neurons which they designated as L, T, and N on the basis of their different biophysical properties. Only the L-type ("long-lasting") channel appeared to be sensitive to DHPs. The relative contributions of DHP-sensitive and DHP-insensitive calcium channels to calcium uptake and neurotransmitter release are unclear.

Synaptosomes have been widely used to study the properties of voltage-dependent calcium channels in nerve terminals (Nachsen & Blaustein, 1980). In most studies no inhibition of calcium uptake by DHP antagonists has been observed (Nachsen & Blaustein, 1979; Daniell et al., 1983; Nachsen, 1985; Suszkiw et al., 1986; Reynolds et al., 1986; Rivier et al., 1987) despite the presence of high-affinity DHP binding sites in the same preparations (Suszkiw et al., 1986). In some recent reports, however, DHP antagonists have been shown to partially inhibit both $^{45}Ca^{2+}$ uptake by synaptosomes (Turner & Goldin, 1985; White & Bradford, 1986; Nordstrom et al., 1986) and neurotransmitter release (Turner & Goldin, 1985). The relationship between DHP binding and their effects on calcium channel function therefore remains controversial. Clearly, biochemical characterization of the different types of calcium channel in synaptosomes is an important step in elucidating their roles in neuronal function.

In the present study I have examined the effects of depolarization on dihydropyridine binding and ^{45}Ca uptake by

† This research was supported by grants from the G. Harold and Leila Y. Mathers Charitable Foundation and the Pharmaceutical Manufacturers Association Foundation.

[1] Abbreviations: DHP, 1,4 dihydropyridine; diSC$_3$(5), 3,3′-dipropylthiodicarbocyanine iodide; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane.

0006-2960/88/0427-5275$01.50/0

synaptosomes. It is demonstrated that DHP-sensitive calcium channels are present in synaptosome preparations and that both DHP binding and their effects on functional responses are voltage-sensitive.

Materials and Methods

Synaptosomes were prepared from the cerebral cortices of male Sprague-Dawley rats (Biolabs, 150–200 g) essentially as described by Ray et al. (1978) except that all sucrose solutions contained 5 mM potassium phosphate, pH 7.4, and homogenizations were carried out by using a Potter S glass–glass homogenizer (B. Braun Instruments) operating at 600 rpm. After sucrose density gradient centrifugation, synaptosomes were collected from the 1.0 M layer and the 1.0/1.2 M interface. The sucrose concentration was reduced to 0.32 M by dropwise addition of ice-cold 5 mM potassium phosphate, pH 7.4, with constant stirring over a 20-min period. The synaptosomes were collected by centrifugation at $12000g_{max}$ for 45 min and were resuspended in 10 mM Hepes–Tris, pH 7.4, 145 mM choline chloride, 5 mM KCl, 5.5 mM glucose, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$ ("basal" buffer). The preparations were stored on ice, and all experiments were carried out within 4 h of preparation. Protein was measured by the method of Lowry et al. (1951), and buffers used for depolarization had KCl isoosmotically substituted for choline chloride. Where indicated, the basal buffer contained 145 mM NaCl in place of choline chloride. Prior to all experiments the synaptosomes were allowed to warm to room temperature (approximately 23 °C) for 10–15 min.

The binding of (+)-[^{3}H]PN200-110 (New England Nuclear) was measured in filtration assays which were carried out under subdued lighting to minimize ligand photolysis. Aliquots of synaptosomes (100 μL) were added to different concentrations of (+)-[^{3}H]PN200-110 (0.01–4 nM) prepared in basal or depolarizing buffers to give a final protein concentration of 0.5 mg/mL in a total volume of 0.8 mL. After incubation in the dark for 120 min at room temperature, 0.5 mL of each sample was rapidly diluted into 3 mL of ice-cold basal buffer and immediately filtered under vacuum through GF/C filters (Whatman) by using a Hoefer filtration apparatus. The filters were rapidly washed with two 4-mL volumes of ice-cold buffer, dried, and extracted overnight in 5 mL of 3a70 scintillation fluid (Research Products International) before counting for ^{3}H. Duplicate 50-μL samples of the incubation mixtures were also counted directly for estimations of total ligand. Nonspecific binding was estimated from measurements of binding in the presence of 1 μM nitrendipine. It has previously been shown that filtration assays may lead to an underestimation of the true amount of nonspecific binding during the incubation period (Weiland & Oswald, 1985). Centrifugation assays were therefore used to investigate this possibility and showed that this did not significantly affect the results. Where indicated, synaptosomes were first lysed by dilution in a large volume of ice-cold distilled water, collected by centrifugation, and resuspended in basal buffer containing 0.2 mg/mL saponin (Sigma Chemical Co.). [^{3}H]PN200-110 binding was measured as described above except that incubation buffers also contained 0.2 mg/mL saponin.

Measurements of membrane potential changes were carried out with the fluorescent voltage-sensitive dye $diSC_3(5)$ (Molecular Probes). A 5-μL aliquot of dye (0.5 mM in ethanol) was added to 2.45 mL of the appropriate buffer in a quartz cuvette. The fluorescence of the dye was measured by using a Perkin-Elmer MPF44A fluorometer thermostated at 25 °C. Excitation and emission wavelengths were 622 and 670 nm, respectively. After stabilization of the fluorescence (approximately 2 min) 50 μL of synaptosomes in basal buffer was added to give a final protein concentration of about 0.1 mg/mL and the fluorescence was recorded.

For measurements of calcium uptake, synaptosomes in basal buffer were incubated with the desired concentration of drug for 5 min at room temperature. The drugs were prepared as stock solutions in DMSO, and in order to eliminate solvent artifacts all samples, including controls, contained DMSO at a final concentration of 0.18%. Uptake of $^{45}Ca^{2+}$ (ICN) was initiated by diluting 100 μL of synaptosomes into 900 μL of appropriate buffer containing drugs at the same concentration as in the preincubation and 1–2 μCi of $^{45}Ca^{2+}$. After various time periods uptake was halted by dilution of 200-μL aliquots into 3 mL of ice-cold basal buffer. The samples were immediately filtered under vacuum through GF/C filters and rapidly washed with two 4-mL volumes of ice-cold buffer. Radioactivity trapped on the filters was determined by liquid scintillation counting for ^{45}Ca as described above.

(±)-Nitrendipine and (±)-Bay K8644 were provided by Dr. A. Scriabine (Miles Laboratories, Inc., New Haven, CT), and (±)-PN200-110 was provided by Dr. D. Römer (Sandoz Ltd., Basel). (±)-Verapamil hydrochloride was from Sigma Chemical Co.

Results

Fluorescence Measurements of Synaptosomal Membrane Potential. Synaptosomes were prepared in basal buffer containing high concentrations of choline rather than Na^+ in order to minimize the contribution from electrogenic Na^+/Ca^{2+} exchange (Blaustein, 1977) which may obscure measurements of calcium uptake through voltage-dependent calcium channels. From measurements of intrasynaptosomal concentrations of cations in a similar buffer at 30 °C, Suszkiw et al. (1986) have estimated that such preparations retain a resting potential of approximately −80 mV. It may be expected that substitution of choline by K^+ will depolarize the synaptosomes in a manner approximated by the potassium equilibrium potential:

$$E_m \sim E_K = (2.3RT/F) \log ([K^+]_o/[K^+]_i)$$

The ability of the synaptosomes to retain a membrane potential and be depolarized by increasing $[K]_o$ has been verified by using the potential-sensitive dye $diSC_3(5)$. In basal buffer containing 5 mM $[K]_o$ the fluorescence of the dye was markedly quenched upon addition of synaptosomes, as expected if the synaptosomes had retained their resting, inside negative potential. With increasing $[K]_o$ the magnitude of the quench became progressively smaller and the final fluorescence level increased linearly with log $[K]_o$ as predicted from the above equation. These results demonstrate that changes in extravesicular $[K^+]$ can be directly correlated to changes in synaptosomal membrane potential. Similar fluorescence experiments have also been carried out to show that the synaptosomes retain their membrane potential during the prolonged incubation at room temperature required for the equilibrium binding experiments described below. The magnitude of the quench upon addition of synaptosomes to a solution of $diSC_3(5)$ in basal buffer did not significantly change after incubation of the synaptosomes at room temperature for 120 min.

Effects of Depolarization on the Binding of (+)-[^{3}*H*]-*PN200-110.* In basal buffer in which the synaptosomes retain their resting membrane potential the binding of (+)-[^{3}H]-PN200-110 appeared to be to a homogeneous class of sites as

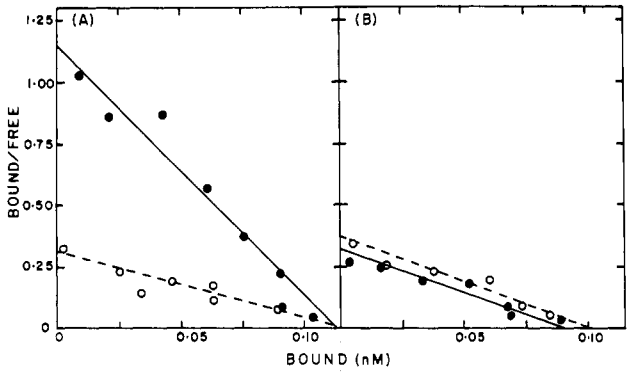

FIGURE 1: Scatchard plots of (+)-[^{3}H]PN200-110 binding to synaptosome preparations. (A) Binding to intact synaptosomes in basal buffer (●) containing 5 mM K^+ or depolarizing buffer (○) with 150 mM K^+. Linear least-squares fits gave K_d = 99 pM and R_0 = 0.113 nM in basal buffer and K_d = 374 pM and R_0 = 0.115 nM in depolarizing buffer. (B) Binding to lysed synaptosomes in the presence of 0.2 mg/mL saponin by use of the same buffers as in (A) with low K^+ and high choline (●) or high K^+ (○). Values obtained were K_d = 292 pM and R_0 = 0.092 nM in choline and K_d = 282 pM and R_0 = 0.105 nM in potassium. In all experiments the protein concentration was 0.5 mg/mL.

Table I: Parameters for Binding of (+)-[^{3}H]PN200-110 to Synaptosomes

exptl conditions	K_d (nM)	B_{max} (pmol/mg)
intact synaptosomes		
5 mM K^+ (polarized, n = 6)	0.07 ± 0.02	0.18 ± 0.03
150 mM K^+ (depolarized, n = 3)	0.44 ± 0.08	0.21 ± 0.01
lysed synaptosomes		
5 mM K^+ + saponin (n = 8)	0.36 ± 0.21	0.26 ± 0.04
150 mM K^+ + saponin (n = 6)	0.44 ± 0.21	0.27 ± 0.08

shown by the linear Scatchard plot in Figure 1A. Depolarization, by raising $[K^+]_o$ to 150 mM, resulted in an approximately 4-fold decrease in affinity with no change in the number of binding sites. These results were somewhat surprising since in other preparations it appears that DHPs bind more tightly when the membranes are depolarized (see Discussion). Control experiments have been carried out to verify that the observed decrease in affinity was a specific effect of depolarization and was not due to a direct inhibition of binding by the elevated potassium concentration. The binding of [^{3}H]PN200-110 to synaptosomes that had been lysed by osmotic shock has been measured in the presence of the detergent saponin. This latter precaution was taken to ensure that the synaptic membranes could not redevelop a membrane potential upon resealing. The results illustrated in Figure 1B show that under these conditions the affinity for the radioligand is identical in buffers enriched in either potassium or choline and is similar to that measured in intact, depolarized synaptosomes. Estimates of K_d's and binding capacities from several experiments are summarized in Table I. Similar results were obtained with synaptosomes prepared in buffer containing Na^+ rather than choline (data not shown). The binding of (+)-[^{3}H]PN200-110 is therefore voltage-dependent and becomes weaker when the synaptosomes are depolarized. This appears to be a characteristic of the whole synaptosome preparation since no evidence for binding site heterogeneity has been obtained. The results shown in Figure 2 demonstrate that the decrease in affinity occurs also under conditions of partial depolarization since at subsaturating concentrations of (+)-[^{3}H]PN200-110 the amount of ligand bound decreased linearly with log $[K^+]_o$, i.e., membrane potential.

Effects of Depolarization on $^{45}Ca^{2+}$ Uptake. When synaptosomes prepared in basal buffer were diluted into the same

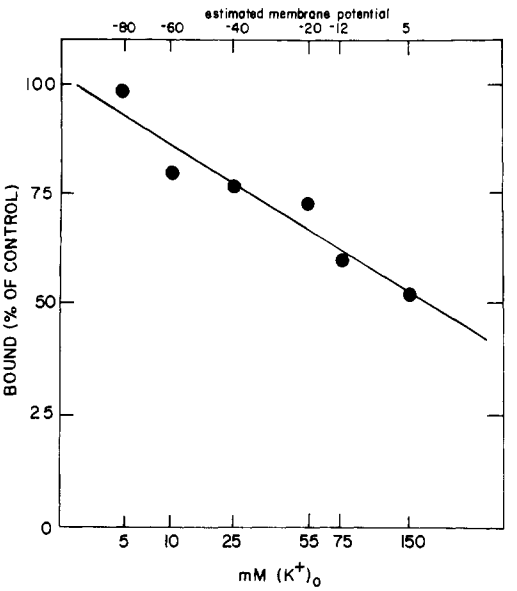

FIGURE 2: Effects of external potassium concentration on the binding of (+)-[^{3}H]PN200-110 to synaptosomes. Synaptosomes, at a protein concentration of 0.5 mg/mL, were incubated with 0.1 nM (+)-[^{3}H]PN200-110 in buffers containing various concentrations of KCl. Bound ligand was measured as described in the text, and binding in basal buffer (5 mM KCl) was used as a control (100% binding). In the figure estimates of the membrane potential obtained from the equation in the text are given for each external potassium concentration used.

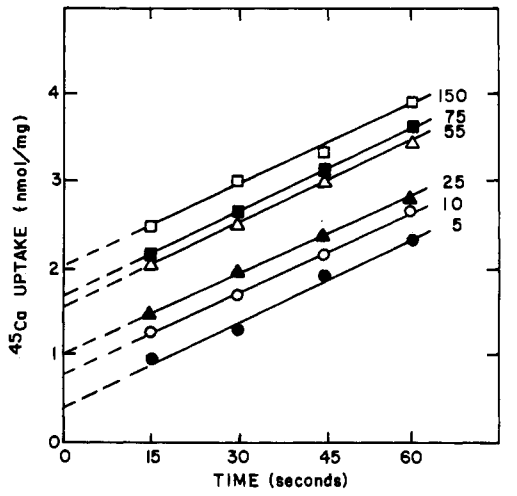

FIGURE 3: Uptake of $^{45}Ca^{2+}$ after dilution of the synaptosomes into nondepolarizing (5 mM KCl) or depolarizing buffers containing varying concentrations of KCl as indicated in the figure. Influx was halted at the times indicated, and the amount of $^{45}Ca^{2+}$ uptake was measured as described in the text. Final protein concentration was 0.3 mg/mL, and the solid lines are linear least-squares fits of the data.

buffer containing $^{45}Ca^{2+}$, there was a slow uptake of $^{45}Ca^{2+}$ which was linear with time between 15 and 60 s (Figure 3). Dilution into depolarizing buffers induced a rapid influx which was complete by the first measured time point (15 s). This was followed by a slow uptake whose rate was indistiguishable from the basal rate. As shown in Figure 3, the magnitude of the rapid $^{45}Ca^{2+}$ influx increased with increasing $[K^+]_o$, but in no case was there any change in rate of the linear component. It appears therefore that depolarization stimulates a voltage-dependent influx of Ca^{2+} whose initial rate is too rapid to be resolved by using the present techniques.

Effects of Calcium Channel Ligands on $^{45}Ca^{2+}$ Influx. The experiments described above demonstrate the presence of both high-affinity DHP binding sites and voltage-dependent calcium

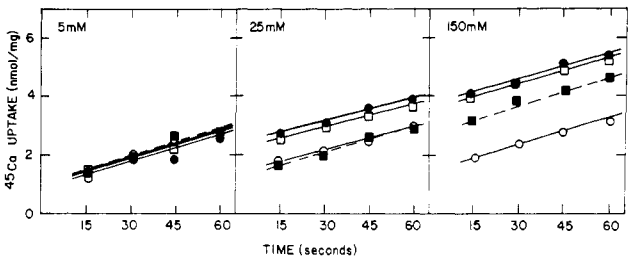

FIGURE 4: Effects of no drugs (□), Bay K8644 (●), nitrendipine (■), and verapamil (○) on the influx of $^{45}Ca^{2+}$ in nondepolarizing buffer (5 mM KCl) and depolarizing buffers containing 25 and 150 mM KCl. The concentrations of Bay K8644 and nitrendipine were 2 μM, and the concentration of verapamil was 100 μM. Solid lines are linear least-squares fits of the data.

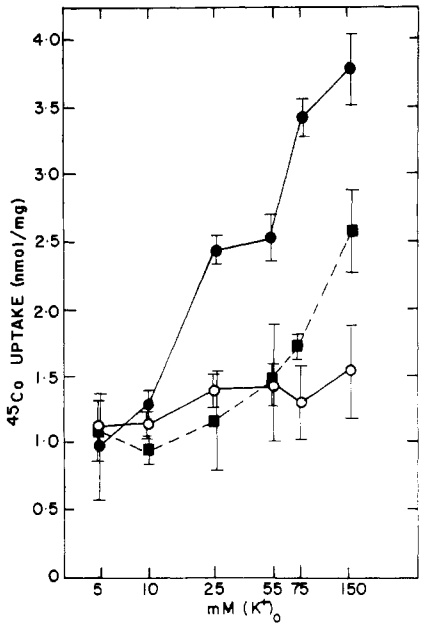

FIGURE 5: Rapid influx of $^{45}Ca^{2+}$ as a function of external K^+ in the presence of 2 μM Bay K8644 (●), 2 μM nitrendipine (■), or 100 μM verapamil (○). Data were obtained from experiments as shown in the figure where uptake was measured after 15, 30, 45, and 60 s. The amount of rapid $^{45}Ca^{2+}$ uptake was estimated from the intercepts at time = 0 from linear least-squares fits of the data. Error bars represent the standard deviation of this parameter. In this experiment ^{45}Ca uptake by control synaptosomes in the absence of drugs was only slightly less (approximately 10%) than that observed in the presence of Bay K8644, and these data are omitted for clarity.

channels in synaptosomes. It was therefore important to establish whether the observed calcium flux was sensitive to modulation by DHPs. Since the binding of (+)-[^{3}H]-PN200-110 had been found to be voltage-sensitive, particular attention was paid to the possibility that the effects of ligands on calcium channel function are also voltage-dependent. The effects of ligands on calcium influx have therefore been measured as a function of changes in $[K^+]_o$. In basal buffer (5 mM K^+) neither verapamil nor the dihydropyridines, nitrendipine and Bay K8644, had any effect on calcium uptake (Figure 4). However, when $[K^+]_o$ was raised to 25 mM, which should depolarize the synaptosomes by about 40 mV (Blaustein & Goldring, 1975), the stimulated influx was inhibited to basal levels by both verapamil and nitrendipine but was slightly potentiated (about 10%) by Bay K8644. Complete depolarization of the synaptosomes by raising $[K^+]_o$ to 150 mM resulted in an increase in the magnitude of the stimulated uptake which was again slightly enhanced by Bay K8644. Verapamil completely inhibited this response but nitrendipine caused only a partial inhibition (Figure 4).

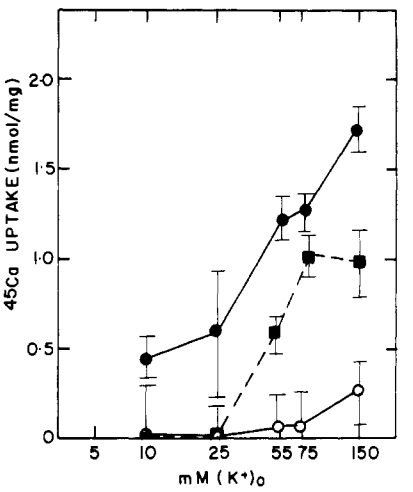

FIGURE 6: Stimulated influx of $^{45}Ca^{2+}$ as a function of external K^+ in the presence of no drugs (●), 2 μM PN200-110 (■), or 100 μM verapamil (○). Data were obtained from experiments as shown in Figure 5, and stimulated uptake was estimated from the intercept at time = 0 and subtraction of the amount of uptake in nondepolarizing buffer (5 mM KCl). Error bars represent the standard deviation of this estimate obtained from [(SE of stimulated uptake)^2 + (SE of basal uptake)^2]$^{1/2}$.

The effects of $[K^+]_o$ on stimulated $^{45}Ca^{2+}$ influx and the effects of drugs are illustrated in Figures 5 and 6. Verapamil almost completely inhibited calcium uptake at all $[K^+]_o$ examined. In these experiments a relatively high concentration of verapamil (100 μM) was used, and the effects of lower concentrations have not been investigated. Since high concentrations of verapamil are thought to have effects on other proteins such as the voltage-dependent sodium channel (Nachsen & Blaustein, 1979), it remains to be established whether the observed inhibition of flux is due to a specific effect of verapamil on the calcium channel protein.

The effects of Bay K8644 have been found to be somewhat erratic. In all experiments Bay K8644 was seen to act as an agonist, but the magnitude of its effects was found to be variable. In some preparations a marked stimulation by up to 2-fold of $^{45}Ca^{2+}$ influx was observed at intermediate membrane potentials (25–55 mM $[K^+]_o$), but in most cases the stimulation was much less pronounced as illustrated in Figure 4. This observed variability may be a reflection of the racemic nature of the (±)-Bay K8644 used in these studies since only one of the isomers is an agonist [see Triggle and Janis (1987)] and the effects of this isomer may be obscured by the antagonist form.

The effects of the DHP antagonists, nitrendipine and PN200-110, appeared to be voltage-dependent. At low $[K^+]_o$ these ligands were found to completely inhibit the depolarization-induced $^{45}Ca^{2+}$ influx, but at higher $[K^+]_o$ only partial inhibition was observed. The magnitude of the stimulated uptake that is inhibited by DHPs appears to be fairly constant at all levels of depolarization (Figures 5 and 6). Similar results have been obtained with five different synaptosome preparations, and the average extent of inhibition by nitrendipine was 90 ± 14% of the stimulated uptake at 25 mM external K^+ and 27 ± 11% when the K^+ was raised to 150 mM. Corresponding values for PN200-110 (three preparations) were 86 ± 16% and 24 ± 18%. However, given the errors inherent in these experiments and the lack of time resolution of the filtration assays used (see Discussion), more quantitative data will be required to definitively describe the different components of calcium flux. These data do, however, suggest that there may be at least two types of voltage-gated calcium channels in

synaptosome preparations, one of which is opened at low extents of depolarization and is modulated by dihydropyridines, and the other of which is opened at higher $[K^+]_o$ and is DHP-insensitive.

Discussion

The results described above demonstrate the presence of dihydropyridine-sensitive calcium channels in synaptosomes. Under conditions of partial depolarization, the stimulated influx of $^{45}Ca^{2+}$ was completely inhibited by DHP antagonists. This suggests that at relatively negative membrane potentials there is a specific activation of DHP-sensitive calcium channels. When the synaptosomes were completely depolarized, an additional component of DHP-insensitive calcium uptake was observed. A possible explanation for these observations is that multiple types of calcium channels are present in synaptosomes and that these channels differ in their sensitivity to DHPs and in their voltage dependence of activation. However, since the binding of the DHP antagonist (+)-[^{3}H]PN200-110 has also been shown to be voltage-dependent, it cannot be excluded that the component of calcium influx that is not modulated by DHPs is due, not to the presence of discrete calcium channels, but to voltage-dependent changes in the mode of interaction of DHPs with their receptors.

Synaptosomes were prepared in a low K^+ (5 mM) buffer containing choline chloride. In this buffer it is expected that the synaptosomes maintain a resting potential of approximately −80 mV, similar to that found in the nerve terminal (Suszkiw et al., 1986). Fluorescence changes of a potential sensitive dye have demonstrated that these preparations may be depolarized by substituting K^+ for choline and that the extent of depolarization is dependent on extravesicular K^+. This method has therefore been used to investigate the effects of membrane potential on both DHP binding and $^{45}Ca^2$ uptake.

Calcium channels, like other voltage-gated ion channels, must undergo voltage-dependent conformational transitions between open and closed states. Since DHPs are thought to interact with calcium channels, voltage-dependent changes in ligand affinity may reflect transitions between functionally important states of the channel. In previous studies of the voltage dependence of DHP binding to cardiac muscle cells (Green et al., 1985; Reuter et al., 1987) and cardiac sarcolemma preparations (Schilling & Drewe, 1986; Kamp & Miller, 1987) it has been found that depolarization results in an *increase* in affinity for DHPs (Schilling & Drewe, 1986; Reuter et al., 1987) or an increase in the number of binding sites (Green et al., 1985; Kamp & Miller, 1987). The effects of DHPs on calcium channel function in heart cells have also been shown to be voltage-dependent (Bean, 1984; Sanguinetti & Kass, 1984; Burges et al., 1987), and they appear to block channel function more potently in depolarized tissues. It has therefore been suggested that DHPs bind more tightly to an inactivated state of the calcium channel that is favored by depolarization (Bean, 1984). Further support for this suggestion has been obtained from observations that depolarization results in tighter binding of (+)-[^{3}H]PN200-110 to both arterial smooth muscle preparations (Morel & Godfraind, 1987) and PC12 cells (Greenberg et al., 1986).

In the present study it is shown that depolarization of synaptosomes leads to a *decrease* in affinity for (+)-[^{3}H]-PN200-110 (Figure 1) with no change in the number of binding sites. This voltage dependence is the opposite to that which would be expected from the results described above. This may reflect fundamental differences in the conformational states of brain and cardiac muscle receptors favored at different membrane potentials which, in turn, may have important implications for the effects of DHPs on calcium channel function in the two tissues. The decrease in affinity of synaptosomes upon depolarization was only 7-fold (Table I), and therefore PN200-110 still binds tightly to depolarized membranes (K_d 0.44 nM). This is consistent with previous reports of high-affinity binding of DHPs to brain membranes in the absence of a membrane potential [reviewed in Triggle and Janis (1987)].

Depolarization of the synaptosomes led to a rapid uptake of $^{45}Ca^{2+}$ whose magnitude was voltage-dependent. The stimulated uptake was completely blocked by nitrendipine, PN200-110, and verapamil at the fairly low levels of depolarization induced by raising the external K^+ to 10 and 25 mM. These conditions are expected to produce depolarizations of approximately 15 and 40 mV. At these relatively negative membrane potentials calcium influx appears to be mediated specifically by DHP-sensitive calcium channels. It is intriguing to compare the present results with previous studies in which it has been demonstrated that the DHP agonist Bay K8644 augments both the depolarization-induced uptake of $^{45}Ca^{2+}$ by synaptosomes (White & Bradford, 1986) and the release of neurotransmitter from rat frontal cortex slices (Middlemiss & Spedding, 1985) *only* under conditions of partial depolarization (25 mM external K^+). In these previous studies the effects of Bay K8644 were therefore found to have a similar voltage dependence to the results obtained with DHP antagonists in this report, i.e., preferential effects at low levels of depolarization.

Nowycky et al. (1985) have recently identified multiple types of calcium channel in DRG neurons and other cells. One of the characteristics of the DHP-sensitive L-type calcium channel is that in most cells strong depolarizations (to 0 mV) are required for activation (Tsien et al., 1987). This property does not, however, appear to be shared by all DHP-sensitive calcium channels. Transverse tubule membranes from skeletal muscle are the richest known source of binding proteins for DHPs (Fosset et al., 1983). Although these membranes are not accessible to conventional electrophysiological techniques, calcium channel activity has been recorded after incorporation of the membranes into planar bilayers (Affolter & Coronado, 1985). These channels, which were sensitive to activation by Bay K8644, could open at strongly negative potentials. The present findings show that DHP-sensitive calcium channels in synaptosomes can also be activated at negative potentials, suggesting that there is considerable diversity in the functional properties of DHP-sensitive calcium channels in different tissues.

A component of calcium influx in synaptosomes that was not inhibited by DHP antagonists was observed as the external K^+ concentration was raised above 25 mM (i.e., at a predicted E_m of >-40 mV). Under these conditions the affinity for [^{3}H]PN200-110 is lower than in the resting state. However, at the concentrations of DHPs used in the flux experiments (2 μM) all of the available high-affinity sites (K_d 0.44 nM) would be occupied. The lack of inhibition of calcium uptake by DHPs cannot therefore be simply explained by the voltage-dependent decrease in affinity of these binding sites. Perhaps the simplest explanation is that the two components of calcium uptake, i.e., DHP sensitive and DHP insensitive, are mediated by different types of voltage-dependent calcium channel. However, there are frequently serious quantitative discrepancies between the concentrations of ligands required to saturate the high-affinity binding sites in vitro and the much higher concentrations necessary to modulate functional responses. The above results do not exclude the possibility that

the observed lack of inhibition of a component of calcium flux is due to a voltage-dependent change in channel–ligand interaction.

Clearly detailed studies of the effects of ligand concentration on flux responses occurring under different levels of depolarization are essential to distinguish among the various possibilities. The lack of time resolution of the calcium flux assays used in the present study has precluded such quantitative analysis. In filtration assays in which the flux response can be measured only on slow (second) time scales, information on the magnitude of calcium uptake but *not* on the initial rate of uptake can be obtained. Under these conditions it is not possible to relate the concentration dependence of effects on function to occupancy of ligand binding sites. As previously discussed by Turner and Goldin (1985), it is has been observed that inactivation of voltage-dependent calcium channels in nerve endings results from the elevation of intracellular Ca^{2+} concentrations [reviewed by Tsien (1983)]. When manual filtration assays are used, it is unlikely that occupancy of 50% of the available binding sites for antagonists will be reflected in a 50% block of $^{45}Ca^{2+}$ influx since a decrease in the number of open channels will be offset by an increase in the average open time of the remaining unoccupied, functional channels. It is necessary, therefore, to develop methods to measure the initial rates of calcium uptake in order to quantitate the effects of antagonists on flux and to relate these to ligand binding phenomena. Such kinetic experiments are currently in progress.

Verapamil at a concentration of 100 μM completely inhibited calcium influx at all K^+ concentrations studied. A similar lack of dependence of verapamil block on the extent of depolarization was previously reported by Nachsen and Blaustein (1979) although in this earlier report only about 50% inhibition was observed. As discussed above, further characterization of the relative contributions of DHP-sensitive and DHP-insensitive components to $^{45}Ca^{2+}$ flux will require detailed studies of the effects of ligand concentration on functional responses. Additional studies are also necessary to determine whether the two components of flux arise from the existence of distinct channels. In this respect, characterization of the block of calcium uptake by the peptide neurotoxin ω–conotoxin GVIA (Olivera et al., 1985, 1987) is likely to be important since this has been shown to be a specific blocker of neuronal L- and N-type calcium channels (McCleskey et al., 1986; Rivier et al., 1987).

In most previous studies, voltage-dependent calcium flux in synaptosomes has been found to be insensitive to DHPs (Nachsen & Blaustein, 1979; Daniell et al., 1983; Suszkiw et al., 1986; Reynolds et al., 1986; Rivier et al., 1987). The present results are, however, consistent with those of Turner and Goldin (1985), who showed that approximately half of the $^{45}Ca^{2+}$ uptake induced by 57.5 mM K^+ was sensitive to blockade by nitrendipine. The reasons for the observed discrepancies remain to be established. Some complications may arise from Na^+–Ca^{2+} exchange occurring in cases where synaptosomes have been prepared in high Na^+ buffer (Coutinho et al., 1984). Also, the voltage dependence of the effects of DHPs may complicate the interpretation of the results. As shown in Figures 4–6, the inhibition of $^{45}Ca^{2+}$ uptake is more readily measured at low levels of depolarization due to the greater percentage inhibition than at higher K^+ concentrations where only partial inhibition is observed. This problem is compounded by the difficulty in directly measuring membrane potentials in synaptosome preparations. In addition, discrepancies inevitably arise from the lack of time resolution in these experiments, which may result in specific channel-mediated responses being obscured by slower transport events and inactivation.

The demonstration that DHP-sensitive calcium channels are present in synaptosome preparations has important implications for the usefulness of DHPs as probes for the identification and purification of voltage-dependent calcium channels from different tissues. Since transverse tubule membranes have the highest known density of DHP binding proteins (Fosset et al., 1983), these membranes have been the preferred starting material for purification of the putative calcium channel protein (Glossmann & Ferry, 1983; Curtis & Catterall, 1984; Borsotto et al., 1985; Flockerzi et al., 1986; Leung et al., 1987). Antibodies raised against the purified receptor subunits cross-reacted with peptides of similar molecular weight in brain (Cooper et al., 1987; Takahashi & Catterall, 1987), suggesting that the proteins share some degree of structural homology.

ACKNOWLEDGMENTS

I thank Colleen Martin for expert technical assistance. I also thank Rick Shelman and Mike Agey for help with synaptosome preparations.

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Solute Partitioning into Lipid Bilayer Membranes†

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Received December 3, 1987; Revised Manuscript Received March 18, 1988

ABSTRACT: We have measured the membrane/water partition coefficients of benzene into lipid bilayers as a function of the surface density of the phospholipid chains. A simple ^2H NMR method was used for the measurement of surface densities; it is shown to give results similar to those obtained from more demanding X-ray diffraction measurements. We observe that benzene partitioning into the bilayer is dependent not only on the partitioning chemistry, characterized by the oil/water partition coefficient, but also on the surface density of the bilayer chains. Increasing surface density leads to solute exclusion: benzene partitioning decreases by an order of magnitude as the surface density increases from 50% to 90% of its maximum value, a range readily accessible in bilayers and biomembranes under physiological conditions. This effect is independent of the nature of the agent used to alter surface density: temperature, cholesterol, and phospholipid chain length were tested here. These observations support the recent statistical thermodynamic theory of solute partitioning into chain molecule interphases, which predicts that the expulsion of solute is due to entropic effects of the orientational ordering among the phospholipid chains. We conclude that the partitioning of solutes into bilayer membranes, which are interfacial phases, is of a fundamentally different nature than partitioning into bulk oil and octanol phases.

The partitioning of solute molecules into lipid bilayers and biological membranes is the basis for drug and metabolite uptake and passive transport across membranes and may be involved in the molecular mechanism of anesthetic drug action. In related interfacial phases, where short chains are likewise confined at high density to an interface, solute partitioning processes underly micellar stability and catalysis and selectivity and retention in reverse-phase liquid chromatography (Dill, 1987).

This partitioning process has often been characterized with bulk thermodynamic models as though bilayer membranes were identical with bulk phases. Lipid bilayer membranes, however, have high surface to volume ratios, they are interfacial phases of matter. In interfacial phases physical properties vary with distance from the interface. In contrast, in bulk phases physical properties are uniform throughout. For example, there is a gradient of chain disorder in the hydrocarbon core of the bilayer: the surfactant chains are most highly aligned near the headgroups, and the order diminishes with distance toward midbilayer (Hubbell & McConnell, 1971; Seelig, 1977; Dill & Flory, 1980). Moreover, the chain ordering of the bilayer phospholipids increases with surface

† This work was supported by grants from the PEW Foundation and the NIH.
‡ Supported by NIH Training Grant GM07175.

0006-2960/88/0427-5281$01.50/0