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Ca^{2+} Channel Inhibitors That Bind to Plant Cell Membranes Block Ca^{2+} Entry into Protoplasts†

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ABSTRACT: Ca^{2+} channel inhibitors of the phenylalkylamine and of the diphenylbutylpiperidine series, as well as other blockers such as bepridil, inhibit $^{45}Ca^{2+}$ influx into carrot protoplasts. The corresponding plasma membranes have a high density (120 pmol/mg of protein) of sites for the phenylalkylamine (−)-[^{3}H]-desmethoxyverapamil (K_d = 85 nM). For 10 different Ca^{2+} channel inhibitors, there was a good correlation between efficacy of blockade of $^{45}Ca^{2+}$ influx into protoplasts and efficacy of binding of the ^{3}H-ligand to membranes. Specific binding sites for the tritiated 1,4-dihydropyridine blocker (+)PN 200-110 could not be identified, and no blockade of Ca^{2+} influx was observed with several molecules in this series such as (+)PN 200-110, nifedipine, or nitrendipine.

Ca^{2+} is an important intracellular mediator for metabolic and developmental events in plants (Hepler & Wayne, 1985; Elliott, 1986). Ca^{2+}-mediated processes in plants include polarized growth, mitosis and cytokinesis, cytoplasmic streaming, physiological responses to red and blue lights, gravitropism, and physiological responses to plant growth substances.

Voltage-dependent Ca^{2+} channels have been shown to play a very important role in nerve, cardiac, and muscle cells to couple excitation to contraction and to secretion (Tsien, 1983; Reuter, 1983; Baker & Knight, 1984). On the other hand, Ca^{2+} channel blockers are a very important class of cardiovascular drugs (Henry, 1980; Nayler & Horowitz, 1983). They include chemically distinct series of molecules such as 1,4-dihydropyridines [nitrendipine, nifedipine, (+)PN

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200–110] and phenylalkylamines [verapamil, methoxyverapamil (D600), desmethoxyverapamil (D888)), diltiazem, and bepridil (Janis & Triggle, 1984; Miller & Freedman, 1984)]. New classes of drugs are emerging as Ca^{2+} channel inhibitors, such as diphenylbutylpiperidines (fluspirilene, pimozide) (Galizzi et al., 1986b) and ω-conotoxin (Kerr & Yoshikami, 1984). Some of these ligands have been very useful to affinity label and purify the putative Ca^{2+} channel protein(s).

In spite of the clear demonstration that Ca^{2+} influx systems are involved in plant physiology (Elliott, 1986), very little is known about Ca^{2+} channels in this type of cell. However, Ca^{2+} channel blockers are known to interfere with a variety of plant functions (Saunders & Hepler, 1982, 1983), and membrane receptors for Ca^{2+} channel blockers such as [^{3}H]verapamil have been recently identified (Hetherington & Trewavas, 1984; Andrejauskas et al., 1985, 1986).

The purpose of this paper is to extend previously published work (Andrejauskas et al., 1985) on the characterization of receptors for Ca^{2+} channel blockers and to analyze in parallel the relationship between binding studies and the action of these blockers on $^{45}Ca^{2+}$ influx into protoplasts that is demonstrated here for the first time.

Experimental Procedures

Cell Culture and Protoplast Preparation. Friable carrot cell aggregates were grown on solid medium as previously described (Wildholm, 1977). In a typical experiment, 1 g of 7-day-old cells was transferred into a 8-cm-diameter Petri dish containing 20 mL of 700 mM mannitol, 2% caylase 345, 0.1% pectolyase Y23, 1 mM iodoacetamide, 0.1 mM phenylmethanesulfonyl fluoride, and 0.01 mM pepstatin A in 25 mM 2-(*N*-morpholino)ethanesulfonic acid–tris(hydroxymethyl)-aminomethane (Mes–Tris) buffer at pH 5.5. The suspension was incubated at 36 °C with gentle shaking (44 oscillations/min) for 90 min. Then the suspension was filtered through 25-μm nylon mesh, and the eluate was centrifuged at 500*g* for 3 min. The pellet containing crude protoplasts was resuspended in 2 mL of buffer made up of 700 mM mannitol, 20% Ficoll 400, and 25 mM Mes–Tris at pH 6.7 (buffer A). On top of the protoplast suspension, a Ficoll gradient was prepared by layering successively 2 mL of buffer A in 10% Ficoll 400 and 1 mL of buffer A without Ficoll 400. This discontinuous gradient was centrifuged at 500*g* for 30 min at 4 °C. The purified protoplasts were obtained at the interface 0–10% Ficoll and washed by centrifugation at 500*g* for 3 min with 4 mL of buffer A without Ficoll. The pellet was resuspended in buffer A without Ficoll at a concentration of 3–4 × 10^6 protoplasts/mL. The protoplast suspension was stored in ice for at least 3 h before use.

Ca^{2+} Uptake. Protoplasts (10^6/mL) were preincubated in a buffer containing 700 mM mannitol, 5 mM KCl, and 25 mM Mes–Tris buffer at pH 6.7 for 60 min at 20 °C in the presence of adequate Ca^{2+} channel blocker drugs. Then Ca^{2+} uptake was initiated by adding 0.1 mM $CaCl_2$ and 0.7 μCi/mL $^{45}CaCl_2$. Times of uptakes are indicated in the figure legends. At the end of the chosen period, 300-μL aliquots of the incubation medium were filtered under reduced pressure through HAWP Millipore filters and rapidly washed 3 times with 2 mL of 100 mM $MgCl_2$, 500 mM mannitol, and 20 mM Tris-HCl buffer at pH 7.5 and 4 °C. The radioactivity remaining on filters was counted in a liquid scintillation spectrometer. Experiments were done in duplicate.

Cell Microsome Preparation. In a routine preparation, 25 g of 7-day-old friable carrot aggregates were ground with 2.5 g of poly(vinylpyrrolidone) and 2.5 g of acid-washed sand. Cell membranes were extracted with 3 × 100 mL of 300 mM sorbitol, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM iodoacetamide, 0.1 mM phenylmethanesulfonyl fluoride, and 0.01 mM pepstatin A in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/NaOH buffer at pH 7.5 and 4 °C. The extract was squeezed through 60-μm nylon mesh. The eluate was centrifuged first at 4000*g* for 15 min. The pellet was discarded, and the supernatant was centrifuged at 10000*g* for 15 min. The new pellet was discarded, and the supernatant was centrifuged at 80000*g* for 40 min. The pellet (8 mg of microsome proteins) was resuspended in 20 mM Hepes/NaOH buffer and stored in aliquots at −70 °C until use.

Standard (−)[^{3}H]D888 and (+)[^{3}H]PN 200–110 Equilibrium Binding Assays. In routine assays, microsomes (20 μg/mL) were incubated at 20 °C in 1 mL of a solution containing either 20 mM Hepes/NaOH buffer at pH 7.5 or 5 mM KCl, 0.1 mM $CaCl_2$, 700 mM mannitol, and 20 mM Hepes/NaOH at pH 7.5 (flux buffer), with the required concentrations of (−)[^{3}H]D888 or (+)[^{3}H]PN 200–110. Nonspecific binding was measured in the presence of 50 μM of (−)D888 or (+)PN 200–110. After 60 min of incubation, the reaction was stopped by rapid filtration of 400-μL aliquots of the incubation mixture through Whatman GF/C filters under reduced pressure. The filters were washed 3 times with 8 mL of a cold solution made of 100 mM Tris-HCl buffer at pH 7.5.

Protein concentration was determined by the method of Hartree (1972) using bovine serum albumin as a standard.

Chemicals. (−)[^{3}H]D888 at 67 Ci/mmol and (+)[^{3}H]PN 200–110 at 80 Ci/mmol were from Amersham. $^{45}CaCl_2$ was from New England Nuclear, and (−)D888, (+)D888, (+)-D600, (−)D600, (+)-verapamil, and (−)-verapamil were from Knoll AG, FRG. *d-cis*-Diltiazem and *l-cis*-diltiazem were from Synthelabo, Paris, France. (+)-Bepridil and (−)-bepridil were from CERM, Riom, France. Fluspirilene was from Janssen, Belgium. Abscisic acid, auxin, gibberellin (GA3), and cytokinin were gifts from Dr. M. T. Le Page-Degivry, Nice, France. Caylase 345 (a protease poor cellulase) was from Société Caylase, Toulouse, France. Pectolyase Y23 was from Seishin Pharmaceutical Co., Tokyo, Japan. Other products were from standard sources.

Results

Ca^{2+} Uptake into Carrot Protoplasts and Inhibition of Ca^{2+} Influx by Ca^{2+} Channel Inhibitors. Figure 1A illustrates the time course of Ca^{2+} uptake by carrot protoplasts incubated in the presence of a 5 mM K^+ buffer. The Ca^{2+} channel inhibitor (−)D888 eliminates part of the Ca^{2+} influx. The same type of result was obtained with different external K^+ concentrations between 5 and 100 mM K^+ instead of 5 mM K^+ as used in Figure 1A. The (−)D888-sensitive Ca^{2+} uptake component remained the same in all these different conditions of external K^+ (not shown).

Figure 1B shows the inhibition of Ca^{2+} influx into protoplasts by (−)D888 and a series of Ca^{2+} channel inhibitors including neuroleptics of the fluspirilene series (Galizzi et al., 1986b). Half-maximum inhibition ($K_{0.5}$) by the different Ca^{2+} channel inhibitors varies between 0.5 and 15 μM (Table I). The rank order of potency is R 66204 > (−)-bepridil > (−)-verapamil, (−)D888, and fluspirilene > (+)-bepridil > (−)D600 > (+)D888, (+)D600, and (+)-verapamil. R 66204, the best inhibitor of $^{45}Ca^{2+}$ influx, is a new molecule belonging to the fluspirilene series. The classical and potent 1,4-dihydropyridine Ca^{2+} channel inhibitors nifedipine, nitrendipine, and (+)PN 200–110 and the diltiazem enantiomers (*d*-cis and

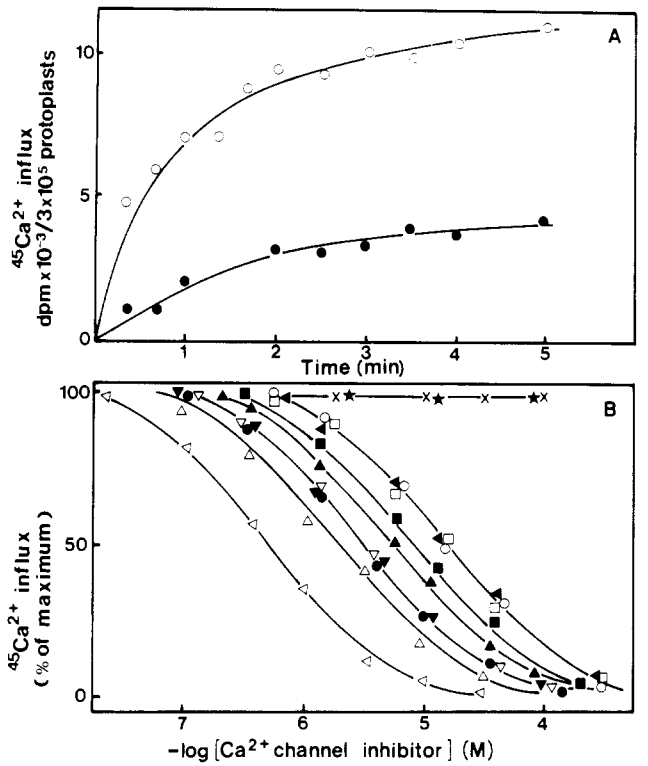

FIGURE 1: Time course of $^{45}Ca^{2+}$ influx into carrot protoplasts and effects of organic calcium channel inhibitors. (A) Time course of $^{45}Ca^{2+}$ influx in the absence (○) or in the presence of (●) of 50 μM (−)D888. (B) Inhibition of $^{45}Ca^{2+}$ influx by increasing concentrations of R 66204 (◁), (−)-bepridil (△), (−)-verapamil (●), (−)D888 (▽), fluspirilene (▼), (+)-bepridil (▲), (−)D600 (■), (+)D888 (○), (+)D600 (□), (+)-verapamil (◀), *d-cis*- or *l-cis*-diltiazem (×), and nifedipine, nitrendipine, or (+)PN 200–110 (★). Time of $^{45}Ca^{2+}$ influx was 45 s. $^{45}Ca^{2+}$ counts remaining on the filters without protoplasts were already subtracted (less than 10% of total counts).

Table I: $K_{0.5}$ Values of Ca^{2+} Channel Inhibitors for Half-Inhibition of Specific (−)[^{3}H]D888 to Carrot Microsomes (and to T-Tubule Membranes) in Comparison to $K_{0.5}$ Values for Half-Inhibition of $^{45}Ca^{2+}$ Influx into Carrot Protoplasts

	$K_{0.5}$ (nM)			
Ca^{2+} channel inhibitor	binding to carrot microsomes[a]	binding to carrot microsomes[b]	$^{45}Ca^{2+}$ flux into protoplasts	binding to T-tubules[c]
(−)D888	90	500	3000	2
(+)D888	80	2000	15000	3
(−)-verapamil	40	200	3000	40
(+)-verapamil	300	4000	15000	10
(−)D600	66	900	8000	20
(+)D600	165	3000	15000	40
(−)-bepridil	60	300	2000	20
(+)-bepridil	130	900	5000	15
d-cis-diltiazem	400	*d*	*d*	60
l-cis-diltiazem	400	*d*	*d*	900
fluspirilene	900	900	3000	0.4
R 66204	100	100	500	

[a] Binding carried out in 20 mM Hepes/NaOH, pH 7.5. [b] Binding carried out in 0.1 mM $CaCl_2$, 5 mM KCl, 700 mM mannitol, and 20 mM Hepes/NaOH buffer at pH 7.5. [c] Rabbit skeletal muscle [taken from Galizzi et al. (1986a,b) and from J. P. Galizzi, M. Fosset, and M. Lazdunski, unpublished experiments]. [d] No detectable effect. Assay up to 100 μM diltiazem.

l-cis) were without effect when they were used at concentrations up to 100 μM. Plant growth substances including auxin, gibberellin (GA3), cytokinins (6-benzylaminopurine, 6-furfurylaminopurine), and abscisic acid were also without effect when assayed at 1 μM.

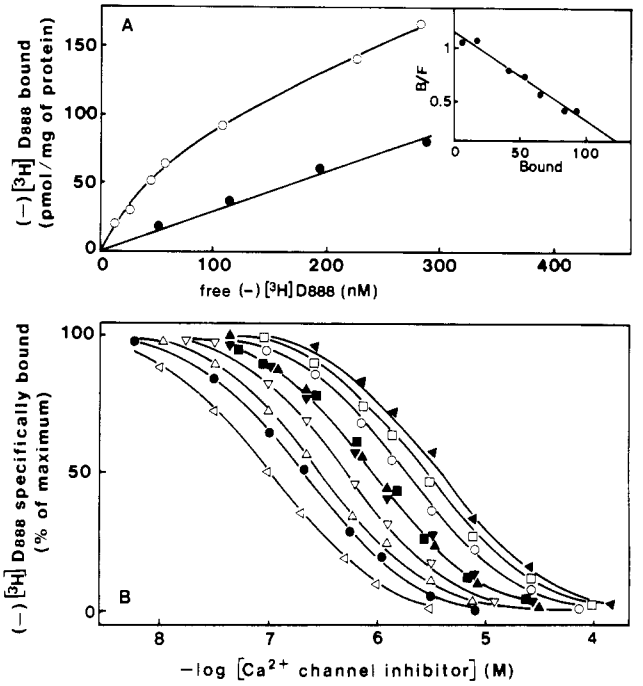

FIGURE 2: Equilibrium binding of (−)[^{3}H]D888 to carrot microsomes and inhibition of binding by various Ca^{2+} channel inhibitors. (A) Equilibrium binding was measured by using increasing concentrations of (−)[^{3}D]D888 (at 6–7 Ci/mmol) and 20 μg/mL of microsomes in 20 mM Hepes/NaOH buffer at pH 7.5 and 20 °C. (Main panel) Binding of (−)[^{3}H]D888 to microsomes in the absence (○) or in the presence of (●) of 50 μM (−)D888. (Inset) Scatchard plot for the specific (−)[^{3}H]D888 binding component. Bound is in pmol/mg of protein; B/F is in mL mg^{-1} (B, bound; F, free). (B) Inhibition by Ca^{2+} channel inhibitors of (−)[^{3}H]D888 binding to carrot microsomes at equilibrium. Binding of (−)[^{3}H]D888 (1 nM) to microsomes (20 μg/mL) was measured in a 1-mL volume of flux buffer at pH 7.5 in the presence of increasing concentrations of R 66204 (◁), (−)-verapamil (●), (−)-bepridil (△), (−)D888 (▽), fluspirilene (▼), (+)-bepridil (▲), (−)D600 (■), (+)D888 (○), (+)D600 (□), and (+)-verapamil (◀). Nonspecific binding represented 10% of total binding (not shown).

Equilibrium Binding of (−)[^{3}*H*]*D888 to Carrot Microsomes and Inhibition by* Ca^{2+} *Channel Inhibitors.* (−)[^{3}H]D888 has been shown to be a useful ligand for the identification of the phenylalkylamine receptor of the Ca^{2+} channel in mammalian tissues (Galizzi et al., 1986a; Ruth et al., 1986; Glossmann et al., 1987). Figure 2A shows equilibrium binding of (−)[^{3}H]D888 to carrot microsomes in Hepes/NaOH buffer. The specific binding component is large enough as compared to the nonspecific binding component. The Scatchard plot of the specific binding component shows a single type of binding site with an equilibrium dissociation constant K_d = 85 nM and a high maximal binding capacity B_{max} = 120 pmol/mg of protein (Figure 2A, inset).

Specific (−)[^{3}H]D888 binding to carrot microsomes was inhibited by increasing concentrations of unlabeled (−)D888 and of other Ca^{2+} channel inhibitors, either in Hepes/NaOH buffer alone (Table I) or in conditions used previously in flux studies (Figure 2B and Table I). The rank order of potency of the different Ca^{2+} channel inhibitors in preventing (−)[^{3}H]D888 binding to carrot microsomes in the flux buffer conditions was the same as that previously found for inhibition of Ca^{2+} influx into carrot protoplasts (Table I). Differences of affinities found for (−)[^{3}H]D888 and analogues in the Hepes/NaOH buffer in the absence of K^+ and Ca^{2+}, on one hand, and in flux buffer conditions, on the other hand, are consistent with the previously observed inhibitory effect of both monovalent and divalent cations on tritiated phenylalkylamine

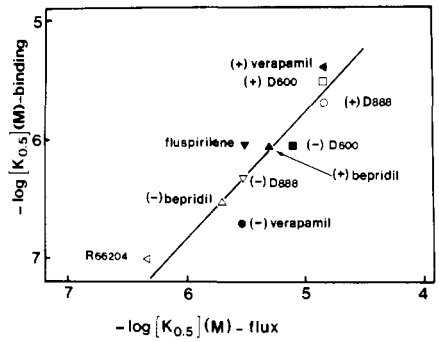

FIGURE 3: Correlation between inhibition by various Ca^{2+} channel inhibitors of specific (-)[^{3}H]D888 binding and of $^{45}Ca^{2+}$ influx. $K_{0.5}$ (half-inhibition of specific (-)[^{3}H]D888 binding by Ca^{2+} channel inhibitors in flux buffer conditions) was plotted versus $K_{0.5}$ (half-inhibition of $^{45}Ca^{2+}$ influx). Symbols are similar to those in Figure 2B.

binding to skeletal muscle membranes (Galizzi et al., 1984, 1985). The exact values of dissociation constants observed from binding experiments in the flux buffer conditions and from $^{45}Ca^{2+}$ uptake measurements are not exactly the same, but it should be remembered that in $^{45}Ca^{2+}$ flux experiments with protoplasts the external and internal ionic conditions are different, whereas in binding experiments both sides of the isolated membrane are in contact with the flux buffer, i.e., the external solution in $^{45}Ca^{2+}$ uptake measurements. A stereoisomeric effect (3–20-fold) between enantiomeric pairs of Ca^{2+} channel inhibitors has been observed (Table I, flux buffer conditions). Drugs that were without effect on $^{45}Ca^{2+}$ uptake by protoplasts were also without effect on (-)[^{3}H]D888 binding to carrot microsomes. We did not detect any component of specific binding to these carrot microsomes for the 1,4-dihydropyridine type of Ca^{2+} channel inhibitor assayed with (+)[^{3}H]PN 200–110 (not shown).

Correlation between Potency of Different Ca^{2+} Channels Inhibitors To Inhibit $^{45}Ca^{2+}$ Influx into Carrot Protoplasts ($K_{0.5}$ Values) and Binding of (-)[^{3}H]D888 to Microsomes ($K_{0.5}$ Values). A good correlation (slope = 0.82; r = 0.90) between potency to inhibit $^{45}Ca^{2+}$ influx to carrot protoplasts and binding for a receptor site measured with (-)[^{3}H]D888 (flux buffer conditions) is shown in Figure 3 for several Ca^{2+} channel inhibitors belonging to the phenylalkylamine and the phenylbutylpiperidine series.

Discussion

The cell wall of plants contains a large number of negatively charged compounds that may trap divalent cations. For these reasons plant cells are not very suitable for measurements of Ca^{2+} influx. Therefore, protoplasts derived from carrot cell cultures have been used in this work to carry out $^{45}Ca^{2+}$ influx experiments. The Ca^{2+} entry system revealed by $^{45}Ca^{2+}$ flux measurements was insensitive to variation of external K^+ concentration that could have changed protoplasts polarization. However, protoplasts might have already been fully depolarized even at the smaller external K^+ concentrations (Cornel et al., 1983). $^{45}Ca^{2+}$ uptake by protoplasts was sensitive to phenylalkylamines (verapamil, D888, D600), to bepridil, and to neuroleptics acting as Ca^{2+} channel blockers such as fluspirilene (Galizzi et al., 1986b) [$K_{0.5}$ values between 0.5 and 15 μM (Table I)]. There was some stereospecificity between enantiomeric pairs (2–3-fold).

Verapamil and D600 have been previously reported to inhibit cytokinin, and exogenous Ca^{2+} stimulated bud formation in the moss *Funaria* (Saunders & Hepler, 1982, 1983) with ED_{50} values of 15–40 μM. These ED_{50} values are similar to $K_{0.5}$ values ($K_{0.5}$ = 3–15 μM, Table I) found in this work for the inhibition of $^{45}Ca^{2+}$ influx into carrot protoplasts.

Binding studies with (-)[^{3}H]D888 have revealed one family of binding sites for (-)D888 (K_d = 85 nM) in carrot membranes and a high density of phenylalkylamine receptors (B_{max} = 120 pmol/mg of protein). These results are similar to those previously reported for [^{3}H]-verapamil binding to zucchini membranes (Andrejauskas et al., 1985). High densities (B_{max} = 50–75 pmol/mg of protein) of phenylalkylamine receptor sites have been previously found in transverse tubule (T-tubule) membranes of skeletal muscle (Galizzi et al., 1986a). However, the affinity of binding sites in T-tubule membranes for (-)[^{3}H]D888 was much higher (K_d = 2 nM) (Table I). Affinities for verapamil and D600 in the two systems were more similar. High densities of phenylalkylamine receptor sites have also been found in sea urchin spermatozoids (Kazazoglou et al., 1985).

As for T-tubule membranes (Galizzi et al., 1986a,b) and for other mammalian tissues containing Ca^{2+} channels (Ptasienski et al., 1985; Reynolds et al., 1986), binding of (-)[^{3}H]D888 was antagonized by Ca^{2+} channel blockers belonging to chemically distinct series such as bepridil, diltiazem, and diphenylbutylpiperidine (fluspirilene and R 66204) (Table I). However, again, the affinity for the diphenylbutylpiperidine class of Ca^{2+} channel inhibitors represented by fluspirilene was higher for T-tubules than for plant membranes.

One important problem was of course to know whether occupation of binding sites for all these Ca^{2+} channel blockers was at all related to blockade of a Ca^{2+} transport system. The correlation between binding and flux data that is presented in Figure 3 suggests that for the series of phenylalkylamines (verapamil, D600, D888) as well as for diphenylbutylpiperidines (fluspirilene, R 66204) and for bepridil this seems to be the case. It is strongly believed from work with skeletal (Galizzi et al., 1986a) and cardiac muscle (Cooper et al., 1987) that the L-type Ca^{2+} channel contains receptors not only for all the chemical classes of drugs listed in Table I but also for the most typical class of Ca^{2+} channel blockers, that of the 1,4-dihydropyridines, which includes molecules such as nitrendipine, nifedipine, or (+)PN 200–110.

An interesting property of plant membranes is that they are apparently devoid of 1,4-dihydropyridine receptors (or else that these receptors have such a low affinity that they have not been detected). Our observations on this point with carrot microsomes are in agreement with previous observations with zucchini and corn microsomes (Andrejauskas et al., 1985) but disagree with reports indicating low levels (a few fmol/mg of protein) of [^{3}H]nitrendipine binding sites in pea shoot membranes (Hetherington & Trewavas, 1984). This absence of receptors for (+)PN 200–110 and nitrendipine in plant membranes is consistent with the absence of effect of these Ca^{2+} channel blockers on $^{45}Ca^{2+}$ entry.

d-cis and *l-cis*-diltiazem have been shown to bind to plant microsomes (Table I) but in conditions of low ionic concentrations. At the high ionic concentrations used in flux experiments, no detectable binding has been found, and diltiazem enantiomers were apparently without effect on $^{45}Ca^{2+}$ entry.

In conclusion, this paper strongly suggests that phenylalkylamine receptors identified in this work with (-)[^{3}H]D888 are related to a transmembrane Ca^{2+} transport structure. It would not be surprising if this Ca^{2+} transport system is a voltage-dependent Ca^{2+} channel since voltage-dependent Ca^{2+} currents have been previously identified in a giant algae (Hayama et al., 1979; Williamson & Ashley, 1982; Kikuyama & Tazawa, 1983; Lanevsky et al., 1983).

It will be interesting in the future to know whether the polypeptide composition of the receptor for Ca^{2+} channel blockers in plant membranes is similar to that previously identified in skeletal muscle, cardiac muscle, and neuronal membranes (Borsotto et al., 1985; Barhanin et al., 1987; Schmid et al., 1986a; Curtis & Catterall, 1984; Cooper et al., 1987; Galizzi et al., 1986a; Ferry et al., 1985). However, it is already clear at present that protein structures of receptors of Ca^{2+} channel blockers in plant and animal membranes have enough differences to prevent polyclonal (Schmid et al., 1986a,b) or monoclonal antibodies (Vandaele et al., 1987; Cooper et al., 1987) recognizing the Ca^{2+} channel protein in animal membranes to cross-react with plant membranes.

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