

1,4-Dihydropyridine Binding Sites in Moss Plasma Membranes

PROPERTIES OF RECEPTORS FOR A CALCIUM CHANNEL ANTAGONIST*

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An increase in cytoplasmic calcium is an early event in hormone (cytokinin)-induced vegetative bud formation in the moss *Physcomitrella patens*. Whole cell and calcium transport studies have implicated 1,4-dihydropyridine-sensitive calcium channels in this increase in cellular calcium. To understand the molecular nature of the dihydropyridine-sensitive calcium channel, we have established conditions for the binding of the arylazide 1,4-dihydropyridine, [³H]azidopine, to its receptor in moss plasma membranes. [³H]Azidopine bound specifically in a saturable and reversible manner. The K_D for [³H]azidopine binding was 5.2 nM and the B_{max} was 35.6 pmol/mg of protein. Association and dissociation of the receptor and [³H]azidopine were temperature-dependent, and association varied as a function of pH. Binding was inhibited by dihydropyridine, phenylalkylamine, and benzothiazepine calcium channel blockers, bepridil, lanthanum, and *N*-ethylmaleimide. [³H]Azidopine binding was stimulated by cations including calcium, strontium, manganese, and barium. [³H]Azidopine binding was also stimulated by cytokinin with a K_m value for kinetin of 0.13 nM. These studies utilize a simple plant system to provide a biochemical framework for understanding calcium regulation during development and have implications for understanding mechanisms of signal transduction in plants.

Controlled changes in cellular calcium concentrations have been identified as important components of signal transduction pathways in plants. Cytoplasmic calcium concentrations are highly regulated; levels are modulated by coordinating passive fluxes and active transport across organellar and plasma membranes (1–4). Cytoplasmic calcium levels have been shown to increase in response to a variety of stimuli including light (5, 6) and hormones (7, 8), and small fluctuations in cellular calcium may modulate processes as diverse as secretory activity in the barley aleurone (7), pollen tube growth (9), and phase transition in mitosis (10). Studies examining the effect of calcium channel inhibitors on physiological processes (11–14) have suggested a role for calcium channels in stimulus-induced increases in cytoplasmic calcium levels; however, little is known about the biochemical or molecular properties of these transport systems.

Calcium acts as an intracellular messenger in hormone (cytokinin)-induced vegetative bud formation during the development of the filamentous protonemata (the young gametophore)

in the moss *Physcomitrella patens* (15–18). Formation of vegetative buds is an integral part of the moss life cycle leading to the development of the mature gametophore which is essential for subsequent sexual reproduction. Cytokinin applied to moss cells causes profuse premature bud formation (19). Localized increases in calcium take place after addition of cytokinin but precede the cytokinin-induced cell division (15, 18). In moss cells not stimulated by cytokinin, cytoplasmic calcium levels (250 nM) are three orders of magnitude lower than levels in the external medium (0.1–1.0 mM) (18). After addition of cytokinin, cytoplasmic calcium levels increase to 750 nM (18). Whole plant studies indicate that cytokinin-modulated calcium entry takes place via dihydropyridine (DHP)¹-sensitive channels (20). In moss protonemata, application of DHP calcium channel agonists in the absence of cytokinin stimulates bud initial formation, whereas DHP calcium channel antagonists block cytokinin-induced bud formation (20). We have previously characterized calcium influx into isolated moss protoplasts and have established that the transport activity of the moss calcium channel shares common characteristics with L-type calcium channels in animal cells. Calcium transport in moss is voltage-dependent, stimulated by DHP agonists, and inhibited by DHP antagonists, phenylalkylamines, and benzothiazepines (21). A novel feature of the transport activity of this channel is hormonal modulation by cytokinin (21).

Cytokinin-induced bud formation in moss is a simple, highly ordered developmental process and is one of few plant responses that allows direct study of stimulus-response coupling at the biochemical and molecular levels. In view of the importance of this DHP-sensitive, hormone-stimulated calcium channel in early events in bud formation, it is of considerable interest to determine the molecular properties of this channel. In this study, conditions are established to investigate the interaction of DHPs with moss membranes. We demonstrate the presence of a DHP binding activity, determine its localization in purified plasma membranes, show an absolute requirement for physiological concentrations of calcium for binding, and also provide evidence for regulation of DHP binding by cytokinin.

EXPERIMENTAL PROCEDURES

Plant Material—*P. patens* (Hedw.) Br. Eur. was cultured and grown aseptically using a modified Knop's medium solidified with 1.5% (w/v) agar (basal medium) (22). Plants were grown at 25 °C under continuous white light (45–50 $\mu\text{E}/\text{m}^2\cdot\text{s}^{-1}$) supplied by fluorescent tubes. Petri dishes containing appropriately supplemented basal medium overlaid with sterile cellophane were inoculated with spore suspensions. To prepare spore suspensions, mature sporophytes were harvested, and 50 capsules were sterilized by soaking in 70% ethanol (5 ml) for 2 min followed by 5.25% (w/v) sodium hypochlorite, 0.1% Triton X-100 (10 ml) for 10 min with occasional swirling. The capsules were washed four times

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¹ The abbreviations used are: DHP, 1,4-dihydropyridine; Bay K8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate; BTP, bis-tris propane or 1,3-bis-[tris(hydroxymethyl)methylamino]propane.

with sterile distilled water (10 ml each) and resuspended in 10 ml of sterile distilled water. Capsules were opened with sterile forceps, and plates were inoculated with 1 ml of spore suspension each (approximately 4×10^3 viable spores). After incubation at room temperature for 2 weeks, protonemal tissue was harvested and ground in sterile water (1 g/6 ml) with a tissue homogenizer (Tissue Tearor, Biospec Products Inc., Bartlesville, OK) for 2 min at 11,000 rpm. Ground tissue (0.25 g/1.5 ml) was transferred to sterile flasks containing 50 ml of Gottwald's medium (23). Flasks were incubated at room temperature under fluorescent lights (as above) on a rotary shaker at 100 rpm for 3 weeks. The tissue was then subcultured by transferring 9-ml aliquots of culture into flasks containing 50 ml of Gottwald's medium. Cultures were incubated for an additional 2 weeks, and the tissue was harvested, weighed (yield: approximately 1 g tissue/flask), and used for membrane isolation.

Plasma Membrane Isolation—All procedures were conducted at 4 °C. Moss vegetative tissue (35–60 g) was homogenized by mortar and pestle in a medium containing 250 mM sorbitol, 3 mM EGTA, 25 mM Hepes-BTP (pH 7.4), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 0.01 mM pepstatin A, 0.2% bovine serum albumin (fatty acid free), and 0.25 g/g of fresh weight polyvinylpyrrolidone at a medium-to-tissue ratio of 1.5 ml/g of fresh weight. After filtration through cheesecloth, the debris was homogenized again in 1 ml of homogenization medium/g of the original tissue weight, washed in 0.5 ml/g of the same medium, and filtered. The homogenate was centrifuged for 15 min at $13,000 \times g$; the resulting supernatant was centrifuged for 30 min at $60,000 \times g$ (Beckman SW 28 rotor, r_{max}). The resulting pellet (crude microsomal fraction) was resuspended in 250 mM sorbitol, 2.5 mM Hepes-BTP (pH 7.2), and 1 mM dithiothreitol (resuspension buffer). The suspension (1.1 ml) was layered over a two-step (6 and 12%, w/w) dextran gradient (5 ml each) prepared in resuspension buffer. After centrifugation for 2 h at $70,000 \times g$ (Beckman SW 28.1 rotor, r_{max}), a turbid band at the 6–12% dextran interface was collected and is referred to as plasma membrane-enriched vesicles.

To fractionate microsomal membranes, the following modifications to the above procedures were used. The crude microsomal pellet was resuspended, and 12 ml were layered over a three-step (4, 6, and 12%) dextran gradient (8 ml of each). After centrifugation for 2 h at $70,000 \times g$ (Beckman SW 28 rotor, r_{max}), membranes at the 0–4, 4–6, and 6–12% interfaces were collected and used for localization studies.

Protein Determination and Enzyme Assays—For all ATPase assays, reaction mixtures (0.5 ml) contained 10–30 μ g of protein, 4.5 units of pyruvate kinase, 5 mM $MgSO_4$, 5 mM phosphoenol pyruvate, 0.012% Triton X-100, with 30 mM Hepes-BTP and 5 mM ATP-BTP buffered to the appropriate pH. Reactions were initiated by addition of ATP, and incubation was at 37 °C for 30 min. P_i was determined by the method of Fiske and SubbaRow (24). For measurement of vanadate-sensitive ATPase activity (plasma membrane), Hepes and ATP were buffered to pH 6.7 with BTP; 50 mM KCl, 0.1 μ M bafilomycin A_1 , and 5 mM sodium azide (NaN_3) were included in all assays. Vanadate-sensitive activity was calculated as the difference in activity in the absence and presence of 0.1 mM sodium vanadate (Na_3VO_4). For measurement of bafilomycin A_1 -sensitive ATPase activity (vacuolar membrane), Hepes and ATP were buffered to pH 7.5 with BTP; 50 mM BTP-Cl, 0.1 mM vanadate, and 5 mM azide were included in all assays. Bafilomycin A_1 at a concentration of 0.1 μ M was used to determine bafilomycin A_1 -sensitive activity.

UDPase activity (Golgi membrane) was determined in the presence of Triton X-100 (25). The final concentrations of reaction components were 30 mM Hepes-BTP (pH 7.0), 3 mM $MgSO_4$, 3 mM UDP- Na_2 , 0.02% Triton in a volume of 0.5 ml. Reactions were initiated by addition of UDP and incubated at 35 °C for 30–40 min. Reactions were terminated with 1% ammonium molybdate ($(NH_4)_6Mo_7 \cdot 24 \cdot 4H_2O$) in 2 N H_2SO_4 plus 1% SDS at room temperature.

Antimycin A-insensitive NADH cytochrome *c* reductase activity (endoplasmic reticulum) was assayed by monitoring the reduction of cytochrome *c* spectrophotometrically at 550 nm (26).

Protein was determined by the method of Lowry with bovine serum albumin as the standard (27).

$[^3H]$ Azidopine Binding Assays—Membranes were incubated at 20 °C in a solution containing 20 mM Hepes-NaOH (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride, 5 mM KCl, 1 mM $CaCl_2$, and 10 nM $[^3H]$ azidopine at a final protein concentration of 0.066 mg/ml. Nonspecific binding, measured by preincubation of membranes with 100 μ M of the DHP antagonist, nifedipine, on ice for 60 min, was <10% of total binding at a 10 nM concentration of ligand. Nonspecific binding was subtracted from total binding to yield specific binding. Variations of these conditions for individual experiments are indicated in the appropriate figures and tables. Experiments were initiated by addition of membrane

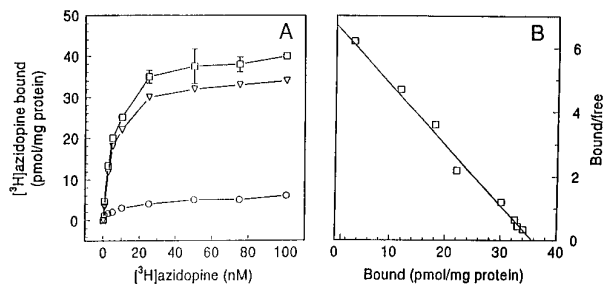


FIG. 1. Equilibrium binding of $[^3H]$ azidopine to moss membranes. Binding was measured in reaction mixtures as described under "Experimental Procedures" with increasing concentrations of $[^3H]$ azidopine (0–100 nM) at 20 °C for 60 min. *A*, binding to moss membranes in the absence (\square , total binding) or presence (\circ , nonspecific binding) of 100 μ M unlabeled nifedipine. Nonspecific binding was subtracted from total binding to give specific binding (∇). Points represent the means \pm S.E. of three experiments. *B*, Scatchard plot of specific binding ($y = 6.871 - 0.193x$, $R^2 = 0.992$). The specific binding component resulted in an equilibrium dissociation constant (K_D) of 5.2 nM and a maximum binding capacity (B_{max}) of 35.6 pmol/mg of protein.

vesicles to reactions; 60 min incubations were used in standard equilibrium binding experiments. Glass fiber filters (Whatman GF/C) used for filtration were presoaked for at least 1 h in 0.3% polyethylenimine. Under these conditions, $[^3H]$ azidopine bound to filters in the absence of membranes and could be displaced by nonradioactive ligand. Control experiments were performed without vesicles for each experiment and the binding data was corrected for nonspecific filter interactions. Incubations were stopped by rapid filtration of reaction aliquots (usually 450 μ l) under reduced pressure. The filters were immediately washed twice with 5 ml of an ice cold solution containing 100 mM Tris-HCl (pH 7.5) and 0.1% bovine serum albumin. Duplicate experiments were systematically carried out using at least three different membrane preparations. Ten- μ l samples of the incubation mixture were taken for measurement of the total amount of $[^3H]$ azidopine present; radioactivity was measured by scintillation counting. All experiments were carried out under dim light due to the light sensitivity of the dihydropyridines. Solvent controls were included in all assays where necessary. In the experiments to determine sensitivity of $[^3H]$ azidopine binding to sulfhydryl reagents, membranes were preincubated with the reagents for 30 min at 37 °C before addition of $[^3H]$ azidopine. Inhibition of $[^3H]$ azidopine binding by different drugs was measured under equilibrium conditions as described above in the presence of 10 nM $[^3H]$ azidopine.

For determination of association rate constants (k_1), the amount of labeled ligand-receptor complex in aliquots taken at various times between initiation of the reaction and the time to equilibrium was determined, and the data were analyzed as previously described (28). Dissociation rate constants (k_{-1}) were determined by measuring the amount of labeled ligand-receptor complex remaining at various times after the addition of excess (100 μ M) unlabeled ligand.

Materials— $[^3H]$ Azidopine (70–90 Ci/mmol) was purchased from Dupont NEN. Unlabeled nifedipine, bepridil, diltiazem, and Bay K8644 were purchased from Calbiochem. Verapamil was purchased from Sigma. Bafilomycin A_1 was obtained from Dr. Karl Heinz Altendorf, University of Osnabrück, Germany. All other chemicals were of the highest quality available from commercial sources.

RESULTS

Specific Binding of $[^3H]$ Azidopine to Sites in Moss Membranes—To understand the interaction of the moss channel with the dihydropyridine ligand, equilibrium binding of $[^3H]$ azidopine to membranes was carried out in the presence and absence of 100 μ M of the DHP antagonist, nifedipine (Fig. 1*A*). The specific binding, defined as the difference between total and nonspecific binding, was a saturable function of $[^3H]$ azidopine concentration. Specific binding of $[^3H]$ azidopine varied linearly with increasing concentrations of moss membranes in the range of 10–130 μ g of membrane protein/ml (data not shown); all binding studies were carried out in the linear range. In typical experiments using 0.066 mg of protein/ml, total binding in the presence of 10 nM $[^3H]$ azidopine was approximately 6500 cpm, while nonspecific binding in the presence of 100 μ M

TABLE I
Subcellular distribution of specific [³H]azidopine binding to moss membranes

The total activity of each marker enzyme in the three dextran interfaces was set to 100% and all enzyme activities expressed as percent of the total. Equilibrium binding was determined using concentrations of [³H]azidopine ranging from 0 to 100 nM. The dissociation constants (K_D) and the maximal binding capacities (B_{max}) were estimated from Scatchard plots. All results represent the means of at least three experiments.

Dextran interface	K_D^a	B_{max}^b	Relative total enzyme activities of subcellular fractions			
			Tonoplast ^c	Endoplasmic reticulum ^d	Golgi ^e	Plasma membrane ^f
%						
0–4	5.5	0.6	78	36	7	2
4–6	6.2	1.4	19	55	73	12
6–12	5.2	35.6	3	9	20	86

^a Nifedipine-sensitive activity (nM).

^b Nifedipine-sensitive activity (pmol/mg of protein).

^c Bafilomycin A₁-sensitive ATPase activity (100% = 3.21 μmol of P_i/h).

^d Antimycin A-insensitive NADH cytochrome *c* reductase activity (100% = 156 nmol of cytochrome *c* reduced/min).

^e UDPase activity in the presence of Triton (100% = 5.88 μmol of P_i/h).

^f Vanadate-sensitive ATPase activity (100% = 6.34 μmol of P_i/h).

nifedipine was approximately 700 cpm (data not shown). The linearity of the Scatchard plot shown in Fig. 1B indicates that the results are consistent with specific binding to a single class of sites and gives a value of 35.6 pmol/mg of protein for the maximum number of [³H]azidopine sites (B_{max}) and 5.2 nM for the equilibrium dissociation constant (K_D) of the azidopine-receptor complex.

Localization of [³H]Azidopine Binding Sites—To determine the cellular location of the [³H]azidopine binding sites, a crude microsomal suspension was separated with a three-step dextran gradient. The 0–4% interface was enriched in tonoplast vesicles as shown by the presence of bafilomycin A₁-sensitive (vanadate- and azide-insensitive) ATPase activity (Table I). UDPase and antimycin A-insensitive NADH cytochrome *c* reductase, markers for the Golgi and endoplasmic reticulum, respectively, were found predominantly in the 4–6% interface. Vanadate-sensitive (bafilomycin A₁- and azide-insensitive) ATPase activity, a marker for the plasma membrane, was highly enriched in the 6–12% interface. Some nifedipine-sensitive [³H]azidopine binding was found in all fractions as indicated by the B_{max} values shown in Table I. However, levels were significantly higher in the membranes recovered from the 6–12% interface, suggesting that [³H]azidopine binding is primarily localized in plasma membranes. The K_D values calculated for the binding reactions from the 0–4 and 4–6% interfaces (5.5 and 6.2 nM, respectively) were very similar to the value calculated for binding in the 6–12% fraction (5.2 nM).

Temperature Dependence and Reversibility of [³H]Azidopine Binding—The temperature dependence of the interaction of the dihydropyridine with its receptor in moss plasma membranes was determined by direct binding experiments at 4 and 20 °C. [³H]Azidopine binding was temperature-dependent; 60-min incubation periods indicated that specific [³H]azidopine binding was maximum at 20 °C, with approximately half as much binding at 4 °C (data not shown). Kinetic studies indicated a single phase for the association and dissociation of [³H]azidopine. The association reaction of [³H]azidopine at pH 7.5 and 20 °C rapidly reached a steady state within 20 min when 10 nM ligand and 0.066 mg/ml membrane protein were used (Fig. 2A). Kinetic data for association were plotted semi-logarithmically (Fig. 2B) and show a linear relationship as would be expected for a pseudo-first order reaction. Using the first-order rate equation, this representation gives a k_1 of $9.6 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ ($n = 3$).

The dissociation of [³H]azidopine from its receptor in moss plasma membranes was examined by incubating [³H]azidopine with membranes for 60 min, adding 100 μM nifedipine, and examining the residual binding at different time points (Fig. 2C). The time course of dissociation followed first-order kinetics

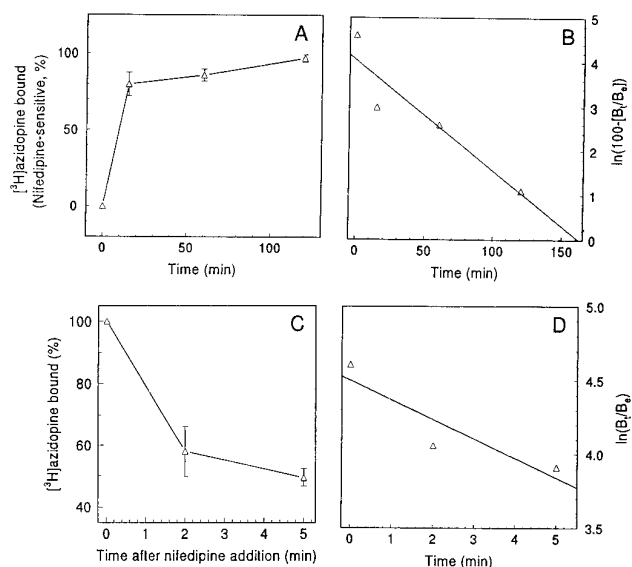


FIG. 2. Kinetics of formation and dissociation of the [³H]azidopine-moss receptor complex. A, the concentration of the [³H]azidopine-receptor complex formed was measured by sampling the reaction mixture (containing 10 nM [³H]azidopine) after the indicated times of incubation at 20 °C. Data are the means \pm S.E. of three experiments. B, pseudo-first order representation of the data ($y = 4.049 - 0.025x$, $R^2 = 0.876$). B_e represents the equilibrium specific binding of [³H]azidopine and B_t is specifically bound [³H]azidopine at assay time t . The association rate constant (k_1) was $9.6 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$. C, after equilibrium was reached (60 min), the rate of dissociation of the complex was monitored following addition of 100 μM nifedipine. Points represent the means \pm S.E. of three experiments. D, first order representation of specifically bound [³H]azidopine ($y = 4.503 - 0.132x$, $R^2 = 0.823$). The dissociation rate constant (k_{-1}) was $5.1 \times 10^{-3} \text{ s}^{-1}$.

(Fig. 2D) producing a rate constant of dissociation (k_{-1}) of $5.1 \times 10^{-3} \text{ s}^{-1}$ ($n = 3$) and a half-life of dissociation ($t_{1/2}$) of 2.26 min. The K_D value determined from the ratio k_{-1}/k_1 is 5.3 nM which agrees well with results from equilibrium experiments (Fig. 1B).

pH Dependence of [³H]Azidopine Binding—Specific binding of [³H]azidopine to moss plasma membrane receptors increased with increasing pH between pH 5 and 7.5 and remained constant above pH 7.5 (Fig. 3). The relationship between specific binding of [³H]azidopine and pH resembled a simple titration curve, which indicated that there is one ionizable group with a pK_a value near 7.1 which is important for the association of [³H]azidopine with its receptor.

Interaction of Calcium Channel Antagonists, Sulfhydryl Reagents, and Reducing Agents with the Moss Azidopine Receptor—In addition to DHP analogs, L-type calcium channels in

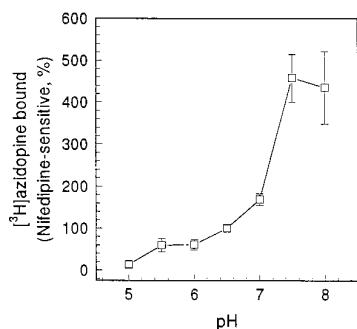


FIG. 3. pH dependence of [³H]azidopine binding to moss plasma membrane receptors. Hepes buffer (20 mM final concentration) was adjusted with NaOH to the desired pH at 20 °C. Points represent the means \pm S.E. of at least three experiments.

animal cells are modulated by distinct chemical classes of calcium antagonists including phenylalkylamines and benzothiazepines. These drugs exert their effect by binding to distinct allosterically coupled drug receptors residing on the α_1 subunit of the calcium channel and inhibiting binding of DHPs (29). To determine whether these agents inhibit azidopine binding to receptors in moss plasma membranes, a number of agents including unlabeled dihydropyridines, verapamil, diltiazem, and bepridil were examined (Figs. 4 and 5). These titration curves were performed in the presence of 10 nM [³H]azidopine. Increasing concentrations of most of the molecules (in the range of picomolar to micromolar) gradually inhibited [³H]azidopine binding to moss plasma membranes. Mean I_{50} values (concentration of inhibitors producing half-maximal inhibition of [³H]azidopine binding) and the maximal inhibition (percent) observed at the highest inhibitor concentrations are given in the legends to the figures. Bound [³H]azidopine was displaced with the following rank order of compounds: Bay K8644 > verapamil > nifedipine > bepridil > diltiazem. Due to limited solubilities, total displacement of [³H]azidopine binding by verapamil, diltiazem, or bepridil did not occur, so molar affinities of these drugs for the binding sites were not estimated.

[³H]Azidopine binding was sensitive to sulfhydryl alkylating agents and reducing agents (Fig. 6). The sulfhydryl blocking reagent, *N*-ethylmaleimide, inhibited binding, suggesting that free thiol groups are essential for receptor-ligand interaction. The moss azidopine receptor showed reduced sensitivity to iodoacetamide (Fig. 6). This lack of inhibition is similar to the effect of iodoacetamide on skeletal muscle T-tubule azidopine receptors and suggests that the essential thiol group may be located in a hydrophobic domain (43). Reducing agents like dithiothreitol (Fig. 6) and β -mercaptoethanol (data not shown) inhibited [³H]azidopine binding at high concentrations implying that intact, but not easily cleaved, disulfide bridges are required for the channel to bind [³H]azidopine with high affinity (30).

Effects of Divalent Cations on [³H]Azidopine Binding—To determine if DHPs bind sites linked to calcium channels, the effect of calcium on [³H]azidopine binding to moss plasma membranes was evaluated. While substantial amounts of calcium may be bound to membranes, moss plasma membranes were isolated in the presence of 3 mM EGTA which should remove most bound calcium. Binding was dependent on the presence of calcium ions (Fig. 7A); as little as 10 nM calcium increased the binding and maximum stimulation of binding occurred at 1 mM calcium. [³H]Azidopine binding was stimulated by $302 \pm 30\%$ ($n = 4$) at 1 mM calcium (Table II), and half-maximal stimulation occurred at a concentration of 16 μ M ($n = 3$) free calcium (Fig. 7B).

The specific binding of [³H]azidopine was modulated by a

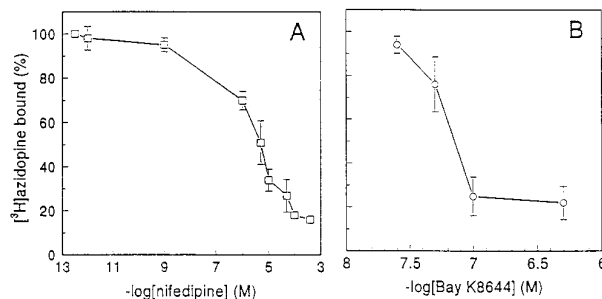


FIG. 4. Inhibition of [³H]azidopine binding to moss plasma membrane receptors by dihydropyridines. Binding of [³H]azidopine was measured with the addition of increasing concentrations of nifedipine (A) or Bay K8644 (B). Control [³H]azidopine binding was 38 pmol/mg of protein. Points represent the means \pm S.E. of three independent experiments. Mean I_{50} values (μ M) and the maximal inhibition (%) observed at the highest drug concentrations are 5.0 and 86 (nifedipine) and 0.01 and 78 (Bay K8644), respectively.

number of divalent metal ions (Table II). Some stimulation of binding was observed with low concentrations of most of the cations examined; however, the patterns varied considerably. Calcium, strontium, and manganese provided the greatest stimulation of [³H]azidopine binding; however, maximal stimulation by strontium and manganese were only two-thirds and one-half as great as with calcium, respectively (Table II). Cobalt had both agonist and antagonist effects as low levels of cobalt stimulated [³H]azidopine binding while inhibitory effects were seen at higher concentrations. Ions such as lanthanum, which block the transport of calcium, inhibited [³H]azidopine binding at all concentrations tested.

Effects of Hormones on [³H]Azidopine Binding—Cytokinins, which provide the primary stimulus for bud formation in mosses (33), were tested for their ability to stimulate or inhibit [³H]azidopine binding to receptors in moss plasma membranes. Kinetin stimulated [³H]azidopine binding in a concentration-dependent manner (Fig. 8A) with half-maximal stimulation at 0.13 nM (Fig. 8B). Other cytokinins also stimulated [³H]azidopine binding with the rank order of kinetin > *trans*-zeatin > *cis*-zeatin (Table III). The hormonally inactive but chemically related adenine and the hormones indoleacetic acid and gibberellic acid did not stimulate [³H]azidopine binding.

DISCUSSION

Plasma membranes from the moss *P. patens* contain a single class of binding sites for the calcium channel blocker [³H]azidopine (Fig. 1). [³H]Azidopine binding is saturable (Fig. 1A) and reversible (Figs. 2C and 4). The maximum binding capacity is high (Fig. 1B), similar to values reported for DHP receptor densities found in T-tubule membranes from rabbit muscle (31, 32) and significantly higher than values for receptors in brain, heart, and smooth muscle microsomes (0.1–1 pmol/mg of protein) (33, 34). The equilibrium dissociation constant (K_D) for the azidopine-membrane complex suggests that [³H]azidopine binds with high affinity to membranes in this plant. Specific binding of azidopine was eliminated by pretreatment of the membranes with trypsin or chymotrypsin at concentrations of 0.1 mg/ml (data not shown). This protease sensitivity suggests that the interaction of azidopine with moss membranes is protein-mediated, and we have used the term receptor to describe this protease-sensitive binding activity. Prior to the present study, only preliminary evidence existed for binding of DHPs to plant membranes; Hetherington and Trewavas (35) showed that microsomal membranes isolated from etiolated pea shoots exhibited a low level of binding of the DHP antagonist, nitrendipine.

Kinetic parameters indicate that association of [³H]azidop-

FIG. 5. **Inhibition of [³H]azidopine binding to moss plasma membrane receptors by bepridil, diltiazem, and verapamil.** Binding of [³H]azidopine was measured with the addition of increasing concentrations of bepridil (A), diltiazem (B), or verapamil (C). Points represent the means \pm S.E. of three independent experiments. Mean I_{50} values (μ M) and the maximal inhibition (%) observed at the highest drug concentration are 6.3 and 60 (bepridil), 20.0 and 71 (diltiazem), and 2.0 and 80 (verapamil), respectively.

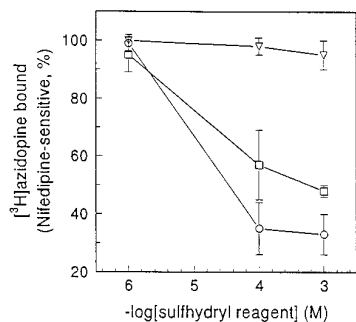
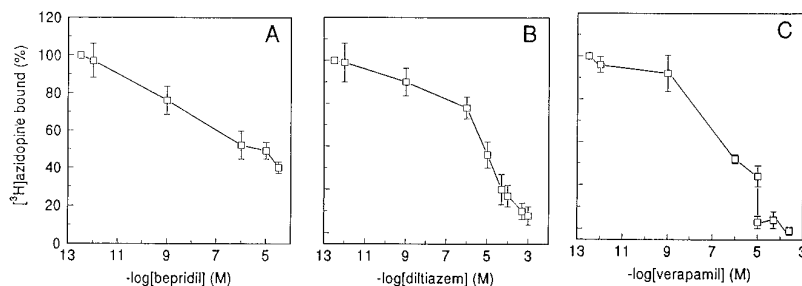


FIG. 6. **Effect of sulfhydryl reagents and reducing agents on [³H]azidopine binding to moss plasma membrane receptors.** Membranes (0.066 mg of protein/ml) were preincubated with *N*-ethylmaleimide (○), dithiothreitol (□) or iodoacetamide (▽) at the indicated concentrations for 30 min at 37 °C. Membranes were then added to binding reactions, total and nonspecific binding was determined, and specific binding was normalized with respect to controls. Points represent the means \pm S.E. of three experiments. Mean I_{50} values (μ M) and the maximal inhibition (%) observed at the highest drug concentration are 38.9 and 66 (*N*-ethylmaleimide) and 603.0 and 52 (dithiothreitol), respectively.

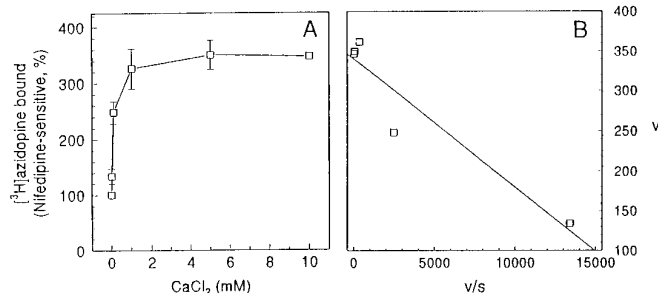


FIG. 7. **Effect of calcium on [³H]azidopine binding to moss plasma membrane receptors.** A, calcium regulation of [³H]azidopine binding to moss plasma membranes. Total and nonspecific [³H]azidopine binding were determined without KCl and with calcium concentrations varying from 0–10 mM. Points represent the means \pm S.E. of three experiments. B, Eadie-Hofstee plot of the data ($y = 340.8655 - (0.0161)x$, $R^2 = 0.901$) resulted in a K_m for calcium of 16.1 μ M.

ine with its receptor is slower than would be expected for a diffusion controlled process ($10^8 \text{ M}^{-1} \text{ s}^{-1} < k_1 < 10^9 \text{ M}^{-1} \text{ s}^{-1}$). This rate of association suggests that a conformational change of the ligand (L)-receptor (R) complex occurs following the association of [³H]azidopine, as has been observed in inhibitor binding to skeletal muscle T-tubule membranes (36). The interaction to form the complex ($L + R \rightleftharpoons LR^* \rightleftharpoons LR$) likely takes place in at least two steps: via a rapid second order absorption of the ligand to the receptor ($L + R \rightleftharpoons LR^*$) followed by a slow first order rearrangement of the complex ($LR^* \rightleftharpoons LR$) (37).

Membrane fractionation studies indicate that [³H]azidopine binding sites are located in the plasma membrane. Maximum receptor densities were found in membranes isolated from the 6–12% dextran interface, the fraction that was highly enriched in the plasma membrane marker, vanadate-sensitive ATPase activity (Table I). Similar levels of vanadate-sensitive ATPase

TABLE II
Effect of cations on binding of [³H]azidopine to moss plasma membranes

Binding was measured under equilibrium conditions without KCl and with cations at the concentrations indicated. Control [³H]azidopine binding was 0.18 ± 0.003 pmol/mg of protein. Data are the means \pm S.E. of three or four experiments.

Cation	[³ H]Azidopine bound (nifedipine-sensitive)	
	0.1 mM	1 mM
	%	
None (control)	100	100
Ca ²⁺	250 \pm 6	302 \pm 30
Ba ²⁺	154 \pm 88	154 \pm 0.5
Sr ²⁺	261 \pm 23	269 \pm 73
Co ²⁺	147 \pm 15	130 \pm 63
Mg ²⁺	153 \pm 37	163 \pm 18
Mn ²⁺	237 \pm 9	278 \pm 21
La ³⁺	87 \pm 3	55 \pm 2

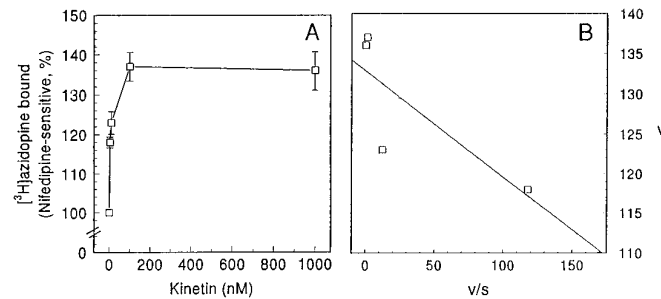


FIG. 8. **Effect of hormones on [³H]azidopine binding to moss plasma membrane receptors.** A, kinetin regulation of [³H]azidopine binding to moss plasma membranes. Total and nonspecific [³H]azidopine binding were determined without KCl or CaCl₂ and with kinetin concentrations varying from 0–1 μ M. Points represent the means \pm S.E. of three experiments. B, Eadie-Hofstee plot of the data ($y = 132.8804 - (0.1329)x$, $R^2 = 0.639$) resulted in a K_m for kinetin of 0.13 nM.

activity were seen when plasma membranes were isolated using aqueous two-phase partitioning (38) (data not shown); however, yields of purified plasma membranes were so low as to make this method impractical for subsequent binding studies. Recent electrophysiological studies have identified calcium channels on the vacuolar membranes of broad bean guard cells (39) and sugar beet cell suspension cultures (40, 41) that share a number of properties with animal L-type channels. No information is available, however, about the abundance of these vacuolar channels or their binding affinity for DHPs. While [³H]azidopine binding to moss membranes isolated from the 0–4% dextran interface may represent binding to receptors in the vacuolar membrane, two lines of evidence suggest that binding to this fraction (and to membranes isolated from the 4–6% interface) represents binding to contaminating plasma membranes: (i) The K_D values calculated for binding in these fractions were very similar to the K_D calculated for binding in the 6–12% interface, and (ii) there was a low level of vanadate-sensitive ATPase activity in these fractions.

TABLE III
Effect of hormones on binding of [³H]azidopine to moss plasma membranes

Binding was measured under equilibrium conditions without KCl or CaCl₂ and with adenine or hormones at the concentrations indicated. Control [³H]azidopine binding was 34 ± 3.9 pmol/mg of protein. Data are the means ± S.E. of four or five experiments.

Hormone	³ H]Azidopine bound (nifedipine-sensitive)	
	0.001 μM	0.1 μM
	%	
None (control)	100	100
Adenine	69 ± 7.6	63 ± 14.2
Cytokinins		
Kinetin	119 ± 0.9	146 ± 2.2
<i>trans</i> -Zeatin	112 ± 2.9	128 ± 1.9
<i>cis</i> -Zeatin	106 ± 4.4	115 ± 5.8
Gibberellic acid	96 ± 11.1	89 ± 0.8
Indoleacetic acid	102 ± 5.3	91 ± 6.3

There is strong evidence for three calcium antagonist receptor sites on the L-type voltage-dependent calcium channels in animal cells: one for DHPs, one for phenylalkylamines, and one for benzothiazepines (42). We investigated the ability of these molecules to compete for [³H]azidopine binding sites in moss plasma membranes. All molecules reduced [³H]azidopine binding in a manner qualitatively similar to their ability to inhibit calcium influx into moss protoplasts (21). Inhibition is most likely due to a direct competition for the azidopine-binding site by the DHPs and due to an allosteric inhibition of azidopine binding by verapamil and diltiazem (30–33). While it has been possible to show involvement of phenylalkylamine-sensitive calcium channels in processes in higher (vascular) plants (43–47), it has been difficult to show a role for DHP-sensitive calcium channels. Recent experiments examining the ability of nifedipine to inhibit hormone-induced tracheary element differentiation in *Zinnia elegans* suggest that DHP-sensitive channels are present in higher plants but may be in low abundance, and that their expression may be temporal and highly tissue specific (48). The DHP effect seen in the *Zinnia* studies required the use of a simplified culture system (suspension cultures) rather than the whole plant. The ability to demonstrate DHP-sensitivity in moss membranes may be due to the high density of high affinity DHP binding sites and suggests that the simplicity of the moss system makes it particularly well suited for understanding calcium channel involvement in plant processes.

In the present study, increasing concentrations of KCl added to the binding buffer stimulated [³H]azidopine binding to moss membranes. Maximum stimulation of binding (250%) was seen with 5 mM KCl (data not shown). This K⁺ stimulation of binding may reflect a direct stimulation of azidopine binding to its receptor. Alternatively, because the membranes used in this study were isolated using dextran gradients that enrich for sealed vesicles (49), K⁺ stimulation of binding may be due to an altered conformation of the channel caused by a change in the potential across the vesicle membrane. The membranes used likely represent a mixture of right-side out and inside-out vesicles. K⁺ depolarization of the plasma membrane in inside-out vesicles, leading to increased [³H]azidopine binding, would be equivalent to a hyperpolarization of the plasma membrane potential in the cell. However, we have shown previously that conditions that depolarize the plasma membrane in moss protoplasts (K⁺ gradients, K_{out}⁺ > K_{in}⁺) stimulate calcium influx (21). This depolarization-induced calcium influx suggests that we are most likely measuring [³H]azidopine binding to right-side out membrane vesicles and that the binding activity of the channel may also be stimulated by a depolarization of the

plasma membrane.

[³H]Azidopine binding to moss plasma membranes has an absolute requirement for the presence of calcium (Fig. 7). [³H]Azidopine binding in the absence of calcium was very low (0.18 pmol/mg of protein, Fig. 7, Table II); additions of calcium as low as 10 nM significantly stimulated binding. Similar calcium-dependence has been seen with DHP binding to calcium channels in a number of animal cells (33, 50–53). While the site of DHP binding is still uncertain (54–56), a conserved region in the cytoplasmic domain adjacent to segment IVS6 has been shown to bind DHPs in the purified rabbit skeletal muscle calcium channel (54). This conserved DHP binding site contains a putative calcium binding site (an EF-hand domain). Binding of calcium to this region may be necessary to induce correct folding of the DHP binding domain and may explain the calcium stimulation of azidopine binding. The ability of various cations to stimulate or inhibit [³H]azidopine binding correlates with their known agonist or antagonist activities at calcium channels in animal cells (34). Strontium and barium are known animal calcium channel agonists and mimic the effects of calcium in stimulating [³H]azidopine binding to receptors in moss plasma membranes (Table II). Lanthanum and cobalt, classical animal calcium channel antagonists, reduce [³H]azidopine binding to moss receptors (Table II). Inorganic calcium antagonists (manganese and cobalt) can also stimulate [³H]azidopine binding at low concentrations, suggesting that these ions possess agonist as well as antagonist properties at the calcium channels depending on their concentration. Similar effects were seen with binding of the DHP-antagonist [³H]nitrendipine to brain membranes (34). The relative potencies of these ions in stimulating or inhibiting [³H]azidopine binding may be related to their ionic crystal radii. Maximal stimulation occurs at a diameter corresponding to that of strontium with substantially lesser effects for ions with smaller or larger diameters (Table II). These results suggest that it will be important to link [³H]azidopine binding data with studies correlating ionic crystal radius and action at the moss calcium channel.

Previous studies showed that cytokinins stimulate calcium influx into moss protoplasts (21). These effects were seen without prior incubation of the protoplasts with the hormone, suggesting a primary effect. To understand the interaction of cytokinins with the channel, we examined the action of hormones on [³H]azidopine binding to moss plasma membranes. Low levels of cytokinins consistently caused a stimulation of [³H]azidopine binding in a manner qualitatively similar to their ability to stimulate calcium influx into moss protoplasts. These cytokinin effects on binding were also seen without prior incubation of the membranes with the hormone (Table III), providing additional evidence for a primary effect. The stimulation of binding to moss plasma membrane receptors by cytokinins appears to be specific as adenine did not stimulate azidopine binding (Table III). As with studies of cytokinin effects on calcium influx into moss protoplasts, the less active cytokinin, *cis*-zeatin, was less effective in stimulating azidopine binding (Table III). Cytokinin may stimulate [³H]azidopine binding by interacting with the channel and altering its conformation, facilitating inhibitor binding.

Whole cell studies of the effect of DHPs on bud formation in moss suggested that cytokinin-stimulated bud formation is modulated by calcium entry through calcium channels (20). Subsequent studies characterizing calcium influx into moss protoplasts established that calcium influx is stimulated by DHP agonists and inhibited by DHP antagonists (21). The current study establishes the presence of abundant, high affinity sites for DHP binding in the moss plasma membrane. The ability to monitor the [³H]azidopine receptor activity demon-

strated in this study should allow the identification of the protein responsible for this ligand interaction and ultimately lead to an understanding of the expression and regulation of the calcium channel during moss development. In addition, information learned about the moss DHP-sensitive calcium channel may serve as a model to allow characterization and identification of the channel homolog in higher plants.

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