

Cytokinin stimulates dihydropyridine-sensitive calcium uptake in moss protoplasts

(signal transduction/plant ion channel/phytohormone/bud formation/*Physcomitrella patens*)

KAREN S. SCHUMAKER* AND MICHAEL J. GIZINSKI

Department of Plant Sciences, University of Arizona, Tucson, AZ 85721

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ABSTRACT Ca^{2+} influx through dihydropyridine (DHP)-sensitive Ca^{2+} channels is thought to be an early event in cytokinin-induced bud formation in moss protonema because DHP antagonists inhibit bud formation in the presence of cytokinin and DHP agonists stimulate bud formation in the absence of cytokinin [Conrad, P. A. & Hepler, P. K. (1988) *Plant Physiol.* 86, 684–687]. In the present study, we established the presence of a DHP-sensitive Ca^{2+} transport system by measuring $^{45}\text{Ca}^{2+}$ influx into moss protoplasts. Ca^{2+} influx was stimulated by external KCl (up to 5 mM), indicating that transport is voltage-dependent. K^{+} -induced Ca^{2+} influx was DHP-sensitive with >50% inhibition at 500 nM nifedipine. Ca^{2+} influx was stimulated by increasing concentrations of the DHP Ca^{2+} channel agonist Bay K8644 with half-maximal effects at 25 nM; this stimulation was seen only in the absence of K^{+} , suggesting that the agonist works preferentially on polarized membranes. Ca^{2+} influx was also inhibited by phenylalkylamines (verapamil) and benzothiazepines (diltiazem). The phytohormone 6-benzylaminopurine consistently stimulated Ca^{2+} influx with a K_m value of 1 nM, whereas adenine, indoleacetic acid, and gibberellic acid had no effect on Ca^{2+} transport. The cytokinins kinetin and *trans*-zeatin caused a greater stimulation of Ca^{2+} influx and induced more bud formation than did 6-benzylaminopurine. These results indicate that Ca^{2+} is taken up into moss protoplasts through voltage-dependent DHP-sensitive Ca^{2+} channels on the plasma membrane and that one of the cytokinin effects in the induction of bud formation is regulation of this plasma membrane Ca^{2+} channel.

Plants convert chemical and physical signals (e.g., phytohormones, light, or gravity) into specific growth responses. The pathway from these physiological or environmental cues to a new developmental program involves biochemical and molecular changes in the cell. Controlled changes in cellular Ca^{2+} concentrations are thought to be an important component of this signal transduction pathway. Cytoplasmic Ca^{2+} concentrations are modulated by coordinating passive fluxes and active transport across the plasma membrane and organellar membranes (1–4). Increases in cytoplasmic Ca^{2+} levels activate Ca^{2+} -dependent proteins, stimulate protein phosphorylation, and lead to initiation of processes as diverse as establishment of polarity and phase transition in mitosis (5).

Ca^{2+} acts as a second messenger in vegetative bud formation during the development of moss protonemata. Induction of buds follows a well-established developmental sequence (6–8). Initially the gametophyte (a single-cell spore) germinates to form a thread-like protonema with two histologically different cell types—the chloronema and the caulonema. Caulonema cells accumulate the phytohormone bryokinin,

an adenine-type cytokinin (8), and subsequently, a small initial cell is formed (6). Asymmetric division of the initial cell leads to bud formation and a change from the two-dimensional filamentous protonemata to the three-dimensional “leafy” gametophore.

Cytokinin applied to caulonema cells causes profuse premature bud formation (6), and localized increases in Ca^{2+} precede this cytokinin-induced cell division (9–13). Whole-plant studies indicate that cytokinin-modulated Ca^{2+} entry takes place via dihydropyridine (DHP)-sensitive channels on the plasma membrane (13). DHPs are organic compounds that modulate Ca^{2+} movement through voltage-dependent Ca^{2+} channels in the plasma membrane of animal cells (14); DHP agonists preferentially maintain the open state and DHP antagonists maintain the closed state of the channel (15, 16). In intact moss protonemata, application of DHP Ca^{2+} agonists in the absence of cytokinin stimulates bud formation, whereas DHP Ca^{2+} antagonists block cytokinin-induced bud formation (13).

Cytokinin-induced bud formation during moss development allows direct study of stimulus–response coupling at the cellular and molecular levels. Although physiological studies have established a role for Ca^{2+} in this process, it is essential to define the molecular mechanisms by which Ca^{2+} levels are regulated. Our studies are aimed at elucidating the molecular nature of the plasma-membrane Ca^{2+} channel responsible for stimulus-induced increases in cytoplasmic Ca^{2+} levels leading to bud formation. In this report we show that moss protoplasts accumulate Ca^{2+} in a voltage-dependent DHP-sensitive manner, and we provide evidence that cytokinin stimulates Ca^{2+} influx through this plasma-membrane Ca^{2+} channel.

MATERIALS AND METHODS

Cell Culture and Protoplast Preparation. *Physcomitrella patens* (Hedw.) Br. Eur. was cultured and grown aseptically using a modified Knop’s medium solidified with 1.5% (wt/vol) agar (basal medium) (17). Plants were incubated at 25°C under continuous white light ($55\text{--}70\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) 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 capsules were sterilized by soaking in 3% (wt/vol) sodium hypochlorite/0.1% Triton X-100 for 10 min followed by five rinses in sterile distilled water. Capsules were opened with sterile forceps, and the spores were dispersed in sterile distilled water; plates were inoculated with 1 ml of spore suspension (10^4 viable spores per ml).

To isolate protoplasts, protonemata were incubated with 2% (wt/vol) driselase (laminarinase, xylanase and cellulase,

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Abbreviations: BA, 6-benzylaminopurine; BTP, 1,3-bis[tris(hydroxymethyl)methylamine]; DHP, 1,4-dihydropyridine.

*To whom reprint requests should be addressed.

Sigma) in 8% (wt/vol) mannitol (18). Prior to use, the driselase solution was clarified by centrifugation at $2500 \times g$ for 5 min and sterilized by passage through a Millipore filter (pore size, $0.22 \mu\text{m}$). Seven-day-old protonemata (1–2 g) were harvested and added to the enzyme preparation [10 ml of driselase per g (wet weight)]; cells were incubated in driselase for 30 min at 22°C with gentle agitation (90 revolutions per min). Protoplasts were separated from large cell debris by filtration through a stainless steel sieve (pore size, $100 \mu\text{m} \times 100 \mu\text{m}$), harvested by low-speed centrifugation ($200 \times g$ for 3 min), washed twice in sterile 8% mannitol and resuspended in the appropriate solution. Protoplast yields were consistently 10^7 protoplasts per g (wet weight) of protonema. All experiments were performed on the day of protoplast isolation; protoplasts were stored on ice for 1–2 h until use.

Stimulation of bud formation by phytohormones was measured by culturing protonemata from spore suspensions on cellophane discs on basal medium for 9 days. The protonemata were then transferred with the cellophane disc to basal medium containing adenine or the indicated phytohormone at $1 \mu\text{M}$. Filaments were observed at 12-h intervals after transfer using a dissecting microscope and scored for the number of buds produced per colony 5 days after transfer to phytohormone.

Ca²⁺ Transport Assays. Ca²⁺ influx was measured by a filtration method (19) with modifications (2). In a typical experiment, $0.5\text{--}2 \times 10^7$ viable protoplasts (based on fluorescence measurements with fluorescein diacetate) were preincubated in buffer (0.5–1.5 ml) containing 8% mannitol and 25 mM Hepes buffered to pH 7.4 with 1,3-bis[tris(hydroxymethyl)methylamine] (BTP), with or without Ca²⁺ channel blocker for 60 min at 4°C . Ca²⁺ influx was initiated by addition of 0.1 mM CaCl₂ and ⁴⁵Ca²⁺ at $0.7 \mu\text{Ci/ml}$ (1 Ci = 37 GBq). Ionophores, inhibitors, or phytohormones dissolved in ethanol or isopropanol were added to reaction mixtures to give a final solvent concentration of 0.5–1.0% (ethanol and isopropanol concentrations up to 1.0% had no effect on Ca²⁺ influx). After appropriate intervals, duplicate aliquots of 150–300 μl were filtered under reduced pressure through Millipore filters (pore size, $0.45 \mu\text{m}$) (2). The filtration procedure involved wetting a Millipore filter with 1 ml of wash solution (8% mannitol/25 mM Hepes-BTP, pH 7.4/1 mM CaCl₂) at 4°C , filtering an aliquot of the reaction mixture, and quickly rinsing with 4 ml of ice-cold wash solution. The filters were allowed to air dry, and the radioactivity was determined by liquid scintillation counting. Experiments using DHP derivatives, verapamil, kinetin, and zeatin were performed in subdued lighting due to the light sensitivity of these compounds.

RESULTS

K⁺ Stimulation of Ca²⁺ Influx. Moss protoplasts accumulated Ca²⁺ in a time-dependent manner (Fig. 1); Ca²⁺ influx increased significantly upon addition of A23187 (data not shown), an ionophore that mediates an electroneutral exchange of protons for Ca²⁺. Measurement of Ca²⁺ binding to isolated moss plasma membranes showed no increase in the presence of A23187 (data not shown), indicating that the protoplast assays measure Ca²⁺ influx into the protoplast and not binding to the exterior surface of the protoplast. When protoplasts were permeabilized with low levels (0.012%) of Triton X-100 (data not shown), Ca²⁺ associated with the membranes was <15% of the total influx seen in the absence of Triton.

K⁺ gradients ($K_{\text{out}}^+ > K_{\text{in}}^+$) have been used to alter the voltage across membranes (depolarize the membrane potential) in plant, fungal, and animal cells (20–23). To determine whether Ca²⁺ influx across the moss plasma membrane was voltage-dependent, the effect of increasing concentrations of

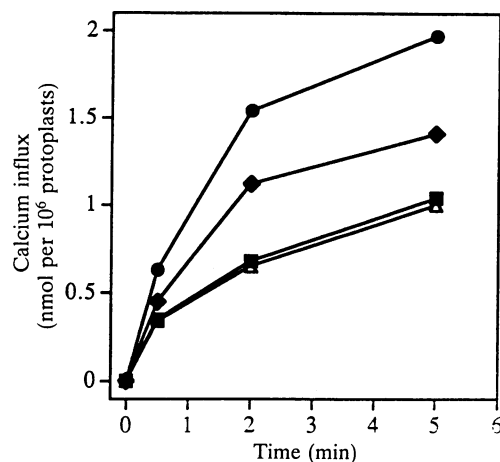


FIG. 1. Effect of K⁺ on Ca²⁺ influx into moss protoplasts. Protoplasts were incubated in the absence (■), or the presence of 1 (◆) or 5 (●) mM KCl or 5 mM BTP Cl (△) at 4°C for the times indicated. ⁴⁵Ca²⁺ remaining on the filter without protoplasts accounted for <1% of total radioactivity. Results are from one representative experiment of two.

KCl (K⁺) in the incubation medium on Ca²⁺ influx was measured. Moss protoplasts incubated in buffer without K⁺ (polarizing conditions) showed time-dependent ⁴⁵Ca²⁺ accumulation (Fig. 1). Protoplasts incubated in buffer with increasing K⁺ levels up to 5 mM (depolarizing conditions) showed an increase in Ca²⁺ accumulation. Ca²⁺ influx in the presence of 5 mM K⁺ was 2-fold higher than influx in the absence of K⁺. In contrast, BTP Cl at concentrations where maximal KCl stimulation was observed had no effect on Ca²⁺ influx.

Inhibition of Ca²⁺ Influx by DHPs, Phenylalkylamines, and Benzothiazepines. A characteristic feature of voltage-dependent Ca²⁺ channels (L-type channels) on the plasma membrane in animal cells is a sensitivity to DHPs. To determine whether influx of Ca²⁺ into moss protoplasts takes place through DHP-sensitive channels, Ca²⁺ influx induced by 5 mM K⁺ was measured in the presence of nifedipine. Ca²⁺ influx was inhibited by >80% by 25 μM nifedipine (Fig. 2) and was virtually eliminated by 1 mM nifedipine (Fig. 3A). The nifedipine-sensitive Ca²⁺ influx component (the difference in influx in the absence and presence of inhibitor)

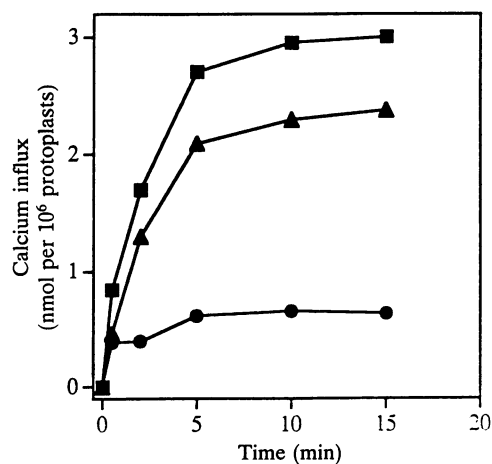


FIG. 2. Effect of the organic Ca²⁺ channel blocker nifedipine on Ca²⁺ influx into moss protoplasts. Protoplasts were incubated with or without Ca²⁺ channel blocker for 1 hr at 4°C . Nifedipine-sensitive influx (▲) was calculated from the difference in influx in the absence (■) and presence (●) of nifedipine. Results are from one representative experiment of three.

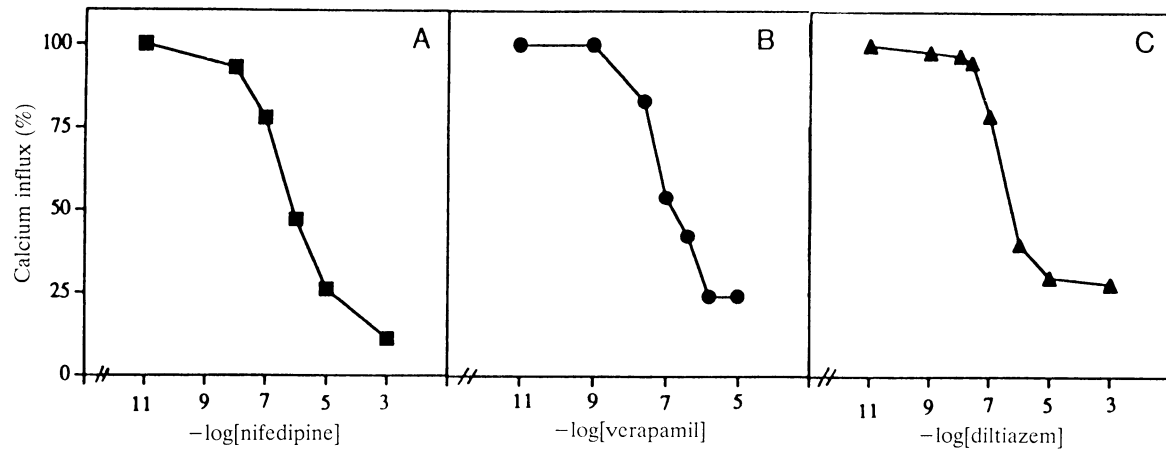


FIG. 3. Inhibition of Ca^{2+} influx into moss protoplasts by increasing concentrations of the organic Ca^{2+} channel inhibitors nifedipine (A), verapamil (B), and diltiazem (C) in high K^+ medium (5 mM KCl). Protoplasts were incubated for 1 h in the presence of increasing amounts of antagonist (0.1–1 mM). Time of Ca^{2+} influx was 5 min after addition of Ca^{2+} . Concentrations giving half-maximal inhibition were 158, 320, and 500 nM for verapamil, diltiazem, and nifedipine, respectively. Results represent means of three to four experiments.

remained constant when external K^+ concentrations were varied between 5 and 100 mM (data not shown).

DHP-sensitive Ca^{2+} channels in animal cells are also inhibited by the phenylalkylamines and benzothiazepines, suggesting that these channels contain at least three distinct binding sites for the Ca^{2+} channel blockers (24). Influx into moss protoplasts (Fig. 3) was measured as a function of inhibitor concentration (nifedipine, verapamil, and diltiazem, Fig. 3 A–C, respectively) under conditions of net Ca^{2+} influx at 4°C. The concentrations of the different inhibitors producing half-maximal inhibition (I_{50}) varied between 0.1 and 0.5 μM . The rank order of potency is verapamil > diltiazem > nifedipine.

Stimulation of Ca^{2+} Influx by the DHP Ca^{2+} Channel Agonist [(±)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate] (Bay K8644). Relatively small structural modifications of DHP antagonists lead to the formation of a distinct class of drugs such as Bay K8644 that activate Ca^{2+} entry in animal cells (21, 25). To determine the effect of the agonist on Ca^{2+} transport into moss protoplasts, net Ca^{2+} influx was measured in K^+ -free

medium with increasing concentrations of Bay K8644 (Fig. 4). Ca^{2+} influx was stimulated with increasing Bay K8644 concentration; half-maximal stimulation occurred at 25 nM Bay K8644. No stimulation of Ca^{2+} influx was seen in the presence of K^+ (data not shown), suggesting that Bay K8644 increases the probability of opening Ca^{2+} channels under polarized conditions.

Stimulation of Ca^{2+} Influx and Bud Formation by Cytokinins. Cytokinins, which provide the primary stimulus for bud formation in mosses, were tested for their ability to stimulate or inhibit Ca^{2+} influx into moss protoplasts. The cytokinin 6-benzylaminopurine (BA) stimulated Ca^{2+} influx in a concentration-dependent manner with half-maximal stimulation at 1 nM (data not shown). Other cytokinins also stimulate Ca^{2+} influx with the rank order of kinetin > *trans*-zeatin > BA \gggg *cis*-zeatin (Table 1). The hormonally inactive but chemically related adenine and the phytohormones indoleacetic acid and gibberellic acid had no effect on influx. To correlate the ability of cytokinins to stimulate Ca^{2+} influx

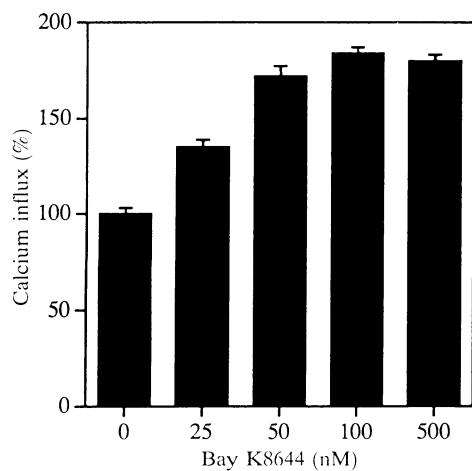


FIG. 4. Effect of the organic Ca^{2+} channel agonist Bay K8644 on Ca^{2+} influx into moss protoplasts in medium without K^+ . Protoplasts were incubated for 1 h in the presence of increasing amounts of agonist (25–500 nM) at 4°C. Time of Ca^{2+} influx was 5 min after addition of Ca^{2+} and is expressed as percent uptake in untreated cells (control). Control influx for Ca^{2+} after 5 min was 0.7 ± 0.07 nmol per 0.5×10^6 protoplasts (mean \pm SEM). Results are from three experiments.

Table 1. Effect of phytohormones on Ca^{2+} influx into moss protoplasts and on bud formation in intact plants

Hormone	$^{45}\text{Ca}^{2+}$ influx, %		Buds per colony, no.
	0.001 μM	0.1 μM	
None (control)	100	100	2 ± 0.38
Adenine	97 ± 3.58	101 ± 1.55	0
Cytokinin			
BA	123 ± 3.03	160 ± 1.14	12 ± 3.08
Kinetin	205 ± 6.80	262 ± 2.76	62 ± 4.39
<i>trans</i> -Zeatin	155 ± 5.23	192 ± 4.58	34 ± 3.10
<i>cis</i> -Zeatin	113 ± 4.77	138 ± 6.63	6 ± 5.09
Gibberellic acid	106	100 ± 3.91	0
Indoleacetic acid	94	102 ± 4.20	0

For Ca^{2+} transport assays, reaction mixtures contained 5 mM KCl. Net Ca^{2+} influx was measured at 5 min and is expressed as percent influx of untreated cells (control). Mean control influx for Ca^{2+} ranged from 1.7 to 2.1 nmol per 10^6 protoplasts (SEM \pm 0.04 to 0.07). Adenine and phytohormones (at the concentrations indicated) were added to each reaction mixture immediately prior to the start of the assay. Results represent the mean \pm SEM with $n = 4$ (higher concentrations of adenine and phytohormones) or $n = 3$ or 4 (lower adenine and cytokinin concentrations). For the lower gibberellic acid and indoleacetic acid concentrations, data represent the mean of two experiments. For measurement of spontaneous and cytokinin (1 μM)-induced bud formation, 9-day-old protonemata were transferred to nutrient medium without addition or with adenine or phytohormone. Results represent the mean \pm SEM, $n = 3$ colonies from three experiments.

with their ability to induce bud formation, the moss was treated with phytohormone, and the number of buds produced per colony was compared with untreated cells. As expected, added cytokinin caused budding in caulonema cells before natural bud formation occurred. Kinetin and *trans*-zeatin induced the greatest number of buds per colony relative to noncytokinin-treated plants. Adenine, indoleacetic acid, and gibberellic acid did not stimulate bud formation. BA had effects on bud formation intermediate between kinetin and untreated control and *cis*-zeatin caused a slight stimulation of bud formation.

DISCUSSION

Regulation of Ca²⁺ Uptake into Moss Protoplasts by Voltage. Ca²⁺ transport into moss protoplasts appears to be under voltage control since K⁺ concentrations up to 5 mM stimulate Ca²⁺ influx (Fig. 1). This stimulation is due to K⁺, as BTP, a large impermeant cation, did not stimulate influx. Ca²⁺ influx in the absence of K⁺ may represent accumulation through DHP-sensitive channels in cells where the membrane is already depolarized. At K⁺ concentrations >5 mM, no further increase in influx was seen (data not shown). Based on internal K⁺ measurements in algal (26) and fungal (27) cells, internal K⁺ levels in cells of *Physcomitrella patens* are estimated to be between 100 and 180 mM so an external K⁺ concentration of 5 mM would generate a membrane depolarization of ≈40 mV. Measuring membrane potential as a function of external K⁺ concentration, Slayman showed (20) that 5 mM K⁺ in the external medium resulted in a 40-mV depolarization in cells of *Neurospora crassa*. K⁺ depolarization of the membrane potential leading to increased Ca²⁺ influx has been shown for chick cardiac cells (21), aneurally cultured human muscle (22), and internodal cells of *Chara corallina* (23); however, maximum depolarization was seen at higher K⁺ concentrations. In contrast to Ca²⁺ influx in the moss, Ca²⁺ uptake in *Chara* decreased at concentrations >20 mM K⁺. Reid and Smith (23) speculate that Ca²⁺ influx in *Chara* may not represent voltage-dependent activity, in part because of the drop in influx at high K⁺ concentrations.

Regulation by Calcium Channel Antagonists and Agonists. Ca²⁺ channels in animal cells are distinguished largely on the basis of pharmacological criteria, with L channels being the site of action of DHPs (28). We investigated the effect of nifedipine on Ca²⁺ influx into moss protoplasts using conditions that depolarize or polarize the membranes. Time-dependent Ca²⁺ influx in the absence of K⁺ is not affected by nifedipine (data not shown). The increased Ca²⁺ influx observed in the presence of 5 mM K⁺ is virtually eliminated by nifedipine (Figs. 2 and 3A). These results are similar to those seen in chick cardiac cells (21) and aneurally cultured human muscles (22) where the actions and binding of DHPs are voltage-dependent, with inhibitors acting with higher affinity on depolarized compared to polarized preparations. In moss protoplasts, the half-maximal effect of nifedipine is observed at 500 nM, indicating that Ca²⁺ influx inhibition by nifedipine is pharmacologically linked to inhibition of bud formation; in intact cells, bud formation was reduced >50% by 10 μM nifedipine (13). While there is growing evidence for an effect of DHPs in a number of physiological processes in plants (29–31), there has not been consistent evidence for DHP-sensitive Ca²⁺ transport and DHPs have shown little effect at the membrane level (32–35). The results presented here demonstrate DHP-sensitive Ca²⁺ influx into single cells in a system previously shown to respond to these compounds at the whole plant level.

There is strong evidence for three Ca²⁺-antagonist receptor sites on the animal Ca²⁺ channel, one for DHPs, one for phenylalkylamines, and one for benzothiazepines, and all of these sites are allosterically coupled (24). Inhibitors of the

phenylalkylamine (verapamil) and benzothiazepine (diltiazem) series inhibit Ca²⁺ influx in moss protoplasts with K_{0.5} = 158 and 320 nM, respectively (Fig. 3). In contrast to the complete sensitivity of moss Ca²⁺ influx to DHPs (Fig. 3A), verapamil and diltiazem were unable to completely block Ca²⁺ accumulation (Fig. 3B and C), possibly reflecting a population of channels insensitive to these inhibitors or channels in which the antagonist receptor sites have been altered. Verapamil has been reported to inhibit cytokinin-stimulated bud formation in the moss (11) with 50% inhibition at 15 μM verapamil. Verapamil-sensitive Ca²⁺ influx in moss protoplasts is consistent with phenylalkylamine-sensitive Ca²⁺ transport in carrot protoplasts (32, 36); however, Ca²⁺ transport in carrot protoplasts was not inhibited by any DHP tested. Current studies looking at DHP binding to moss plasma membranes should allow us to determine whether the difference in DHP sensitivity is due to a greater abundance of DHP-sensitive channels in the moss membranes than in higher plant membranes or whether the moss membrane DHP receptors have a higher affinity for DHPs, facilitating their detection.

The Ca²⁺-channel agonists CGP 28392 and (+)-202-791 at micromolar concentrations stimulate moss bud formation in the absence of cytokinin (13). In moss protoplasts, stimulation of Ca²⁺ influx was seen between 10 and 100 nM Bay K8644 (Fig. 4); Bay K8644 was unable to stimulate activity (increase the probability of opening the channel) in the presence of KCl (data not shown). Bay K8644 activates Ca²⁺ entry in polarized chick (21) and rat (25) cardiac cells. Agonist-stimulated Ca²⁺ influx into moss protoplasts shows that the Ca²⁺ channel in the moss plasma membrane has similar agonist sensitivity at the whole-plant and membrane levels.

Regulation by Phytohormones. Membrane control of Ca²⁺ transport leads to changes in cytoplasmic Ca²⁺ concentrations that mediate the action of phytohormones (9–13, 37). To study the role of Ca²⁺ as a second messenger in bud formation, we looked at the action of cytokinins on Ca²⁺ transport in moss protoplasts. The synthetic cytokinin BA consistently caused a stimulation of Ca²⁺ influx without prior incubation of the protoplasts with the phytohormone (Table 1), suggesting a primary effect. Cytokinin stimulation was seen when the effect of BA on Ca²⁺ influx into protoplasts from cotyledons of *Amaranthus tricolor* was studied; however, maximum stimulation required much higher levels of BA than was seen for moss protoplasts (1 mM vs. 0.1 μM), and experiments were not done to determine whether transport was taking place through DHP-sensitive Ca²⁺ channels (38).

The stimulation of Ca²⁺ influx into moss protoplasts by BA appears to be specific for cytokinin, as adenine, which is hormonally inactive but chemically related, did not stimulate Ca²⁺ influx (Table 1). This is in contrast to stimulation of electrogenic pumping in soybean cultures where adenine elicited a membrane hyperpolarization, indicating that cytokinin effect was related to adenine or its metabolism and not hormonal action (39). The *cis* isomer of zeatin has been shown to be hormonally inactive as a cytokinin in several plant systems (40). In *Physcomitrella patens*, *cis*-zeatin consistently produced a stimulation of bud formation and Ca²⁺ influx (relative to controls); however, the stimulation was lower than any active cytokinin tested. These results may reflect impurities in the compound (the Ca²⁺ channel is sensitive to nanomolar concentrations of the active cytokinins), and the presence of enzymes that mediate the interconversion of *cis* to the favored *trans* isomer (40). A comparison between active and inactive cytokinins showed differential ability to stimulate Ca²⁺ influx and corresponding ability to promote bud formation in whole plants (Table 1). Cytokinin stimulation of Ca²⁺ influx into moss protoplasts indicates that cytokinins regulate the channel. This is in

agreement with whole-plant studies where cytokinin treatment increased the inward current 2-fold along the length of moss cells (41). Within minutes, current decreased in the nuclear zone and at the proximal end, while increasing at the distal end of target cells at the site of future bud formation. These results led to the conclusion that cytokinin may activate plasma-membrane Ca^{2+} channels, which are subsequently redistributed to the distal ends of the target cells by a microfilament-dependent process (41).

Studies with intact plants have demonstrated that chloronema cells are unable to form buds even though cytokinin-induced increases in Ca^{2+} levels can be detected in these cells (12). Our studies with protoplasts from 7-day-old protonemata (primarily chloronema cells) provide evidence that the channel responsible for the increases in Ca^{2+} is present in the plasma membrane of chloronema cells. These results indicate that cell-specific expression of the Ca^{2+} channel in chloronema and caulonema cells is not responsible for the differential ability of these cell types to form buds but that competence for bud formation must be a property of the caulonema cells and lie further down the signal transduction pathway.

The present study shows that voltage-dependent Ca^{2+} influx into moss protoplasts displays similar sensitivities to DHPs and phenylalkylamines as does bud formation in whole plants. The ability of cytokinins to stimulate Ca^{2+} influx into moss protoplasts points to a role for the phytohormone in the regulation of the channel either directly or via a regulatory protein in the membrane. These results coupled with the simple yet highly ordered cellular development of the moss indicate that the moss will be an excellent experimental system in which to study the pathway by which plant cells transduce hormonal stimuli into developmental changes.

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