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The Effect of 1,4-Dihydropyridines on the Initiation and Development of Gametophore Buds in the Moss *Funaria*[1]

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ABSTRACT

The plant hormone cytokinin stimulates target caulonemata of *Funaria* to form buds that develop into the leafy gametophyte. Previous reports have shown that increases in intracellular Ca^{2+} occur during hormone-activated budding concomitant with an alteration in the polarity of the organelles in the bud site. In order to ascertain the involvement of voltage-dependent Ca^{2+} channels in this phenomenon, we have employed dihydropyridines (DHP), compounds noted for their ability to alter Ca^{2+} flux through potential-sensitive channels. Addition of the DHP agonists (+)202-791 and CGP 28392 (100 micromolar) induces bud initials on every target cell including the tip cell. Application of the DHP antagonist (−)202-791, in the presence of cytokinin (1 micromolar benzyladenine), inhibits budding 96%. Similarly, nifedipine blocks cytokinin-induced budding 87% and its effect on budding can be inactivated with a pulse of ultraviolet light. These results are consistent with the idea that cytokinin induces the budding response by increasing Ca^{2+} entry through voltage-operated channels. We suggest that cytokinin activation of Ca^{2+} channels is the first action of the hormone and that subsequent cytokinin-induced mechanisms are operating to maintain budding, since DHP-induced initials rarely develop into complete buds.

The plant hormone cytokinin stimulates target caulonemata of *Funaria* to form buds leading to the leafy gametophyte. Previous reports (1, 19) have shown that an increase in intracellular membrane-associated Ca^{2+} occurs during hormone-activated budding concomitant with an alteration in the polarity of the organelles in the bud site. In addition artificially increasing $[Ca^{2+}]_i$[3] with the ionophore A23187 induces bud initial formation in the absence of exogenous cytokinin, while growing cells in Ca^{2+}-free medium abolishes budding (20). These data suggest that cytokinin elicits its cellular response through the modulation of $[Ca^{2+}]_i$.

Agents that selectively inhibit or enhance Ca^{2+} entry have been instrumental in identifying the sources and routes of Ca^{2+} mobilization. Of particular interest are the DHP[3] drugs that alter Ca^{2+} flux through potential-sensitive channels in animal cells. DHP are noteworthy as channel ligands since within this group of structurally related compounds are derivatives that function as antagonists as well as agonists to Ca^{2+} entry. Due to their structural similarities, DHP are believed to interact at the same site of the Ca^{2+} channel with agonists preferentially maintaining the open state of the channel and antagonists maintaining the closed state. Ligand binding studies and electrophysiological data support these hypotheses (2–4, 6, 10, 21, 22).

Two DHP derivatives in particular are noted for their exceptional characteristics in altering Ca^{2+} entry through voltage-operated channels. One of these is nifedipine, a photolabile DHP that loses its Ca^{2+} antagonistic capability when irradiated with UV light. The other is 202-791, the stereoisomers of which have opposing effects on Ca^{2+} entry: the (+)enantiomer behaves as a Ca^{2+} agonist while the (−)isomer inhibits Ca^{2+} flux (4). We report here that Ca^{2+}-channel agonists, in the absence of hormone, stimulate bud initiation in target cells, while antagonists block cytokinin-induced budding. The data are consistent with cytokinin inducing its initial response via voltage-dependent Ca^{2+} channels.

MATERIALS AND METHODS

Spores of *Funaria hygrometrica* were aseptically sown onto liquid Laetsch's (7) medium and within 24 h germinating spores were transferred to cellophane discs overlaid onto solidified medium (1% agar). After 10 to 14 d protonemata were transferred to medium containing CGP 28392, nifedipine, or the stereoisomers of 202-791 at 1 to 100 μM. After a 1 h incubation, BA (10^{-6} M) was added to cultures containing Ca^{2+} channel antagonists to induce gametophore bud formation. The nifedipine cultures were divided into two groups, one of which received a UV light ($\lambda < 400$ nm) pulse of 60 s and then both groups were placed in continuous light ($\lambda > 400$ nm). The 202-791 and CGP 28392 cultures were exposed to a daylength of 16 h. Control cultures without DHP were treated in a manner identical to their experimental counterparts. After 48 h all cultures were scored for bud induction using a Wild dissecting microscope at a magnification of ×500. Dry weights of cultures were determined after drying overnight at 80°C and the number of buds per mg dry weight was calculated.

Stock solutions of the stereoisomers of 202-791 (4 mM) were prepared by dissolving the drugs in 4 parts 95% ethanol and bringing up to volume with distilled H_2O. Stock solutions of CGP 28392 (3 mM) and nifedipine (25 mM) were prepared by dissolving the crystals in DMSO (Sigma). For experiments, the stock solutions were serially diluted with medium. The ethanol and DMSO concentrations never exceeded 1% in our experiments, a concentration that had no detectable effect on the cells.

RESULTS

Table I and Figures 1 to 4 illustrate the general effects of 1,4-DHP ligands on stimulating initial formation or blocking cytokinin-induced bud formation in the moss *Funaria*. The Ca^{2+} channel antagonists (−)202-791 and nifedipine at 100 μM inhibit cytokinin-induced bud formation by 94% and 87%, respectively.

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[3] Abbreviations: $[Ca^{2+}]_i$ intracellular Ca^{2+} concentration; BA, benzyladenine; DHP, dihydropyridines.

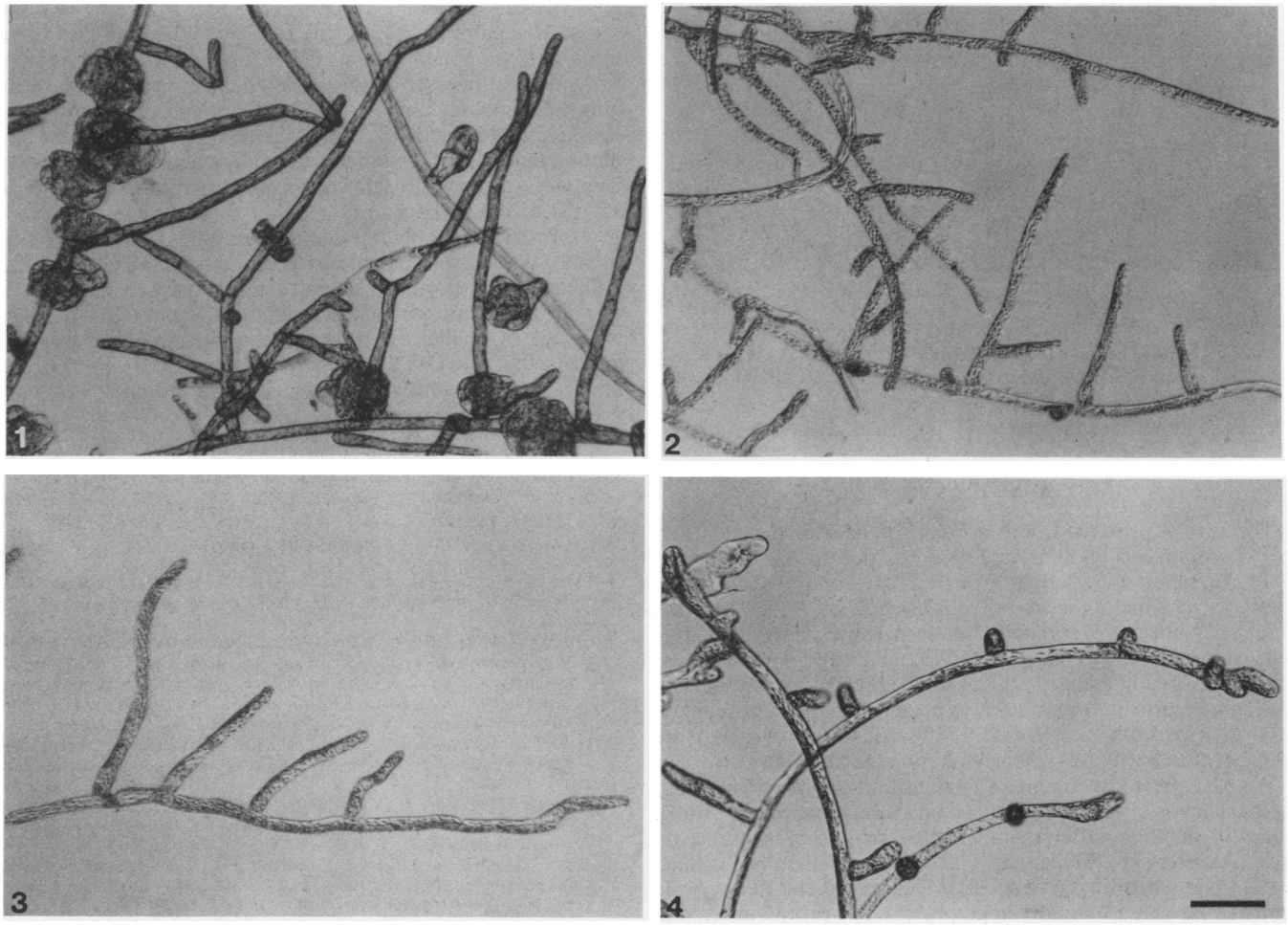
Table I. *Action of 1,4-DHP Derivatives on Bud Formation in the Moss Funaria*

Treatment	Bud Induction	
	% control	
BA (1 μM)	100	
CGP 28392 (100 μM)	100	
(+)202-791 (100 μM)	100	
BA/(−)202-791 (100 μM)	5.9	
BA/(−)202-791 (10 μM)	59.1	
BA/(−)202-791 (1 μM)	82.3	
		UV Light
BA/nifedipine (100 μM)	12.9	85.9
BA/nifedipine (10 μM)	55	98
BA/nifedipine (1 μM)	82.7	96

Reducing the concentration of antagonist in the culture medium resulted in a decrease in the ability of these drugs to block hormone-induced budding. In addition, when nifedipine was photoreversed to its inactive form with a pulse of UV light, cytokinin-induced bud formation remained at near normal values (control levels). A reduction in the number of buds appears to be the only obvious effect of active antagonists since treated cells are morphologically identical to controls (compare Figs. 2 and 3).

Culturing protonema in medium containing the DHP agonists (+)202-791 or CGP 28392 had the opposite effect on *Funaria* protonema. These agents stimulated initial formation on virtually every cell, including the tip cells (Table I, Fig. 4). Frequently, more than one initial formed per cell. In a few cases the agonist-induced initial cells divided in three planes and eventually formed buds, but in most cases the cells continued to grow as branches. Application of 1 μM BA stimulated complete bud development in these cells.

DHP agonists also affected growth in other ways. Within the first 24 h, the tip region of apical cells of both main filaments and side branches became swollen. When cells are grown in agonist-containing medium for extended periods of time, tip growth proceeds but at a reduced rate. By the time division occurs in the apical cell, there is a 200% increase in cell diameter and a 50% reduction in cell length. This pattern of growth continues as long as the cells are exposed to the Ca^{2+} channel agonists and consequently results in filaments consisting of exceedingly short, wide cells compared to controls (Fig. 3 *versus* Fig. 5) Subculturing the protonema in medium lacking agonists reversed the effects and gave rise to normal cells (Fig. 6).



FIGS. 1–4. The effect of DHP on bud formation.

FIG. 1. Normal appearance of protonemal filaments and buds formed after the application of 1 μM BA.

FIG. 2. Cells cultured in cytokinin-containing medium plus the Ca^{2+} channel antagonist nifedipine are unable to produce buds.

FIG. 3. Typical appearance of caulonema filaments and side branches growing in control medium.

FIG. 4. Cells cultured in medium containing the Ca^{2+} channel agonist (+)202-791; initials are produced on every cell, including the tip cells. Bar length equals 100 μm.

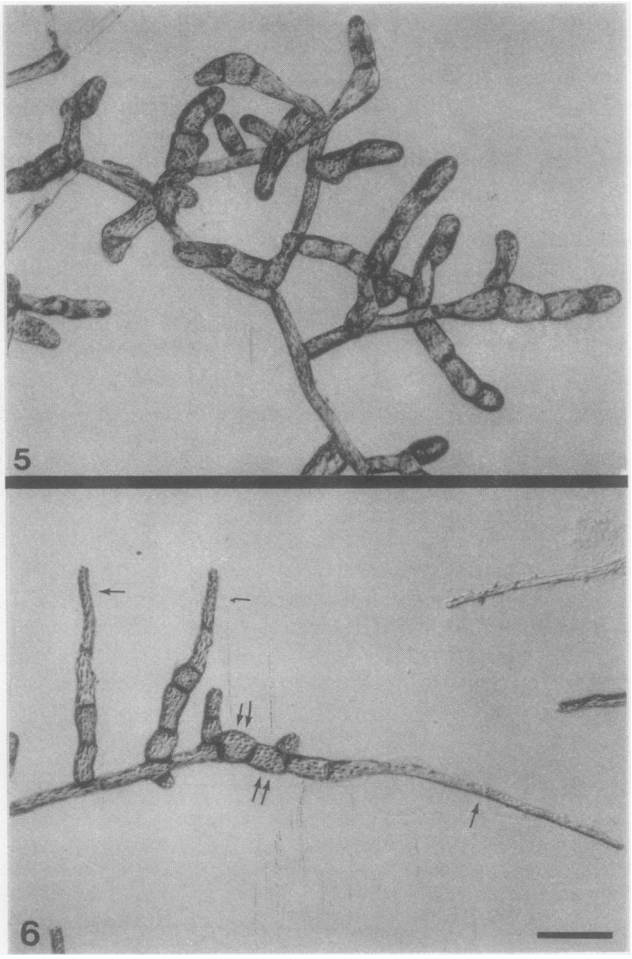


FIG. 5. Continuous culture in (+)202-791 results in a decrease in cell length and an increase in width, creating filaments of short, wide cells.

FIG. 6. Subculturing cells into medium lacking (+)202-791 reversed the agonist-induced effects and gave rise to normal cells (double arrows, cells grown in agonist-containing medium; single arrow, cells formed after subculturing in control medium). Bar length equals 100 μm.

DISCUSSION

The results presented here are consistent with the idea that cytokinin-induced budding in *Funaria* is due to the hormone's ability to increase Ca^{2+} flux through voltage-operated channels. The Ca^{2+}-channel agonists (+)202-791 and CGP 28392, in the absence of hormone, stimulate initial formation on every target cell, while the antagonists (−)202-791 and nifedipine block cytokinin-induced budding. In addition, inactivating nifedipine with UV light returns bud formation to normal values. Although agonist-induced initial stages rarely differentiate further, they are competent to form buds when cytokinin is added to the medium.

We have previously reported, in an ultrastructural and morphometric analysis of cytokinin-induced bud formation, that the initial stages of budding closely parallel those of side branches, a tip growing system (1). Maintenance of a Ca^{2+} gradient is a distinct feature of tip growing systems as evidenced by chlortetracycline fluorescence (11, 12), autoradiography (5), and proton-induced x-ray microanalysis (13, 14). In *Funaria*, cytokinin also induces a Ca^{2+}-gradient in the presumptive bud site (19). We maintain that cytokinin first induces a Ca^{2+} gradient via voltage-gated channels in the future bud site causing a repositioning of the polar axis of the cell. This alteration in the cell's polarity is manifested in a reorganization of the organelles into distinct zones in the bud site and the activation of tip growth perpendicular to the main axis of the filament (1). These gradients in Ca^{2+} and organelle distribution are dissipated prior to the division cutting off the bud initial, as evidenced by the vibrating probe experiments of Saunders (18) and our ultrastructural data (1), and diffuse growth is established. Since Ca^{2+} channel openers stimulate initial formation and these DHP-induced initials rarely, if ever, produce complete buds, we further stipulate that cytokinin activation of Ca^{2+} channels is the hormone's immediate action and that subsequent cytokinin-induced mechanisms are operating to maintain bud formation. These prolonged actions of cytokinin as yet remain unclear, but preliminary experiments suggest that components of the phosphatidylinositol cycle may play a key role in this process.

It is interesting to note that, like cytokinin, both Ca^{2+}-channel agonists and the Ca^{2+} ionophore A23187 induce budding exclusively in the distal region of the caulonema cell. While both the ionophore and DHP-agonist serve as vehicles for Ca^{2+} translocation across membranes, they are believed to act by different mechanisms: A23187 forms neutral membrane permeant complexes with Ca^{2+} that indiscriminately allow Ca^{2+} movement in the cytoplasm (8) while DHP stimulate Ca^{2+} flux through voltage-sensitive channels already in existence. If DHP alone stimulated initial formation, one may deduce that potential-sensitive channels associated with budding are limited to, or solely activated within, the distal portion of the cell. Since both compounds give virtually the same response, we feel that only the distal region is capable of responding to Ca^{2+} influx and that the cytoplasmic components here may be exclusively primed for bud formation. Conversely, a few studies have shown that ionophore-induced responses are sensitive to the organic Ca^{2+} antagonists verapamil and D 600 (9, 16, 17) lending credence to the speculation that A23187 may activate voltage-dependent Ca^{2+} channels via a mechanism leading to membrane depolarization (9, 15–17). If this is the case then either Ca^{2+} channels involved in bud formation are only operating in the distal portion of the cell, or only this area is capable of responding to changes in Ca^{2+} homeostasis. Regardless, ultrastructural observations show that one of the first morphological reactions to cytokinin is a realigning of the organelles exclusively in this portion of the cell (1). It appears that a Ca^{2+} flux, whether global or specific, is communicated to a specific cellular region where morphological events occur.

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