Observation of polarity induction by cytochemical localization of phenylalkylamine-binding sites in regenerating protoplasts of the moss *Physcomitrella patens*

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Dedicated to Professor Martin Bopp

**Summary.** Different external (e.g., light) and internal (e.g., auxin and calcium gradients) factors control differentiation of the moss protonema. The present investigations demonstrate that exogenously applied auxin, the pharmacological blockade of auxin efflux by naphthylphthalamic acid, and treatment with (−)-bepridil, a calcium channel antagonist, inhibit protoplast division without affecting protoplast viability in the moss *Physcomitrella patens*. A fluorescently labelled phenylalkylamine (DM-Bodipy PAA), another calcium channel antagonist, was used as a probe for in vivo labelling of phenylalkylamine (PAA)-binding sites. The specificity of this binding was demonstrated by competition with (−)-bepridil. Confocal laser scanning microscopy visualized PAA-binding sites on the plasma membrane and along the nuclear membrane as uniformly distributed clusters. During asymmetric division of *P. patens* protoplasts, however, fluorescence labelling particularly increases at the membrane invagination and later along the plate separating the new cells. Intracellular localization of PAA-binding sites, probably at the membranes of vesicles and vacuoles, significantly increases in the smaller daughter cell, destined to later form a polar outgrowth, the first chloronema cell. Thus, a system was established to visualize early events in *P. patens* protoplast polarization at the subcellular level.

**Keywords:** Auxin; Calcium channel; Cell polarity; Phenylalkylamine binding site; *Physcomitrella patens*; Polarity induction.

**Abbreviations:** DHP dihydropyridine; IAA indole-3-acetic acid; NPA naphthylphthalamic acid; PAA phenylalkylamine.

**Introduction**

Isolated protoplasts of mosses regenerate in adequate culture into cell filaments by polar outgrowth, this morphogenetic process being affected by different physical (e.g., direction of light) and chemical (e.g., auxins) factors (Cove 2000, Reski 1998a, Spalding 2000). As in higher-plant embryogenesis (Souter and Lindsey 2000), cell differentiation of the developing moss protonema, namely, the formation and maintenance of caulonema cells, is affected by endogenous as well as exogenous auxins (Bhatla 1992, 1994). Protoplasts of mosses rapidly accumulate indole-3-acetic acid (IAA) from the surrounding medium depending on the pH of the medium. Inhibitors of IAA efflux carrier (such as triiodobenzoic acid and naphthylphthalamic acid [NPA]) stimulate a net uptake of extracellular IAA into the protoplasts of the moss *Funaria hygrometrica* (Geier et al. 1990).

Here, the phytohormone signal may be mediated by changes in cytosolic Ca²⁺ concentrations as it was postulated from cytokinin-induced calcium uptake in isolated moss protoplasts (Schumaker and Gizinski 1993). Such ions are transported via the membranes by ion channels that switch between transporting and nontransporting (open and closed) states by a stochastic process referred to as gating. Functions of calcium channels are commonly studied by a pharmacological approach, using specific antagonists like drugs of the phenylalkylamine (PAA) and dihydropyridine (DHP) families (e.g., verapamil and bepridil),

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which act as L-type Ca\(^{2+}\) channel antagonists in animal
and plant cells (Pineiros and Tester 1997). Coupled
with a fluorescent dye (ST- or DM-Bodipy), calcium
channel antagonists, PAA and DHP, have been used to
localize L-type calcium channels in animal cells
(Knaus et al. 1992). Fucus distichus zygotes (Shaw and
Quatrano 1996a), sunflower protoplasts (Vallée et al.
1997, 1999), and the embryonic and nonembryonic
tissues of sunflower (XuHan et al. 1999). DHP-
sensitive calcium uptake in response to cytokinin has
also been reported in moss protoplasts (Schumaker
and Gizinski 1993). Furthermore, the efficiency of pro-
toplast division in the moss Physcomitrella patens not
only depends on auxin but also on calcium concentra-
tion (Ashton et al. 1990).

In order to establish a system to analyze protoplast
polarization in Physcomitrella patens at the subcellular
level, we quantified the effects of auxin-efflux as
well as of calcium-channel antagonists on protoplast
viability and division capacity and attempted to local-
ize PAA-binding sites, presumed to be markers for
Ca\(^{2+}\) channels, during the onset of polarity induction
and division of regenerating protoplasts. The work
utilizes DM-Bodipy PAA as fluorochrome and
(-)-bepridil as a competitive channel blocker. The
resulting images were analyzed by confocal laser scan-
ning microscopy (CLSM). Results are presented
showing variations in the distribution of PAA-binding
sites in undivided protoplasts, during the initial and
later stages of division, and also during the protrusion
of polar outgrowth leading to first protonema cell
formation.

Material and methods

Chemicals

DM-Bodipy PAA (5-[3-(4,4-difluoro-5,7-dimethyl-3a,4a-diaza-4-borao-3-indacene-3-y1)propionamido]phenethyl-N-methylamino]-2-
iso propyl-2-(3,4,5-trimethoxyphenyl)-valeronitrile) and Bodipy FL
(Molecular Probes, Eugene, Oreg., U.S.A.) were prepared as 100 µM
stock solutions in dimethyl sulfoxide. (-)-Bepridil (Sigma Chemical
Co., St. Louis, Mo., U.S.A.) was prepared as 10 mM stock in 95%
nitrogen, Naphthalphthalamic acid (NPA) (gift from Dr. Michalke,
Biologie III, Freiburg University, Freiburg, Federal Republic
Germany), synthesized according to Thomson et al. (1973), was
prepared as a 10 mM stock in dimethyl sulfoxide, whereas IAA (Sigma)
was made as a stock of 5 mM in 95% ethanol.

Plant material

Physcomitrella patens (Hedw.) B.S.G. was cultivated as described
previously (Strepp et al. 1998).

Protoplast isolation

Isolation of protoplasts was carried out according to Rother et al.
(1994). Protoplast viability was determined by treating them with
fluorescein diacetate (0.1% [w/v]) solution made from a stock of 5
mg of fluorescein diacetate in acetone. Viable protoplasts appear
bright green when examined in blue light with a fluorescence micro-
scope (excitation wavelength, 499 nm).

Protoplast culture

Protoplasts were suspended at a final density of 5 × 10⁶ protoplasts
per ml of regeneration medium (Knop’s medium, pH 5.7, addition-
ally containing 5% glucose and 3% mannitol) (Rother et al. 1994)
and cultured in 400 µl aliquots in multiwell steriplates (17.8/16 mm,
24-well plates; Greiner Labortechnik, Frickenhausen, Federal
Republic of Germany, for suspension cultures). Cultures were main-
tained at 25 °C under 55 microeinstein light from fluorescent tubes,
16 h diurnally. The division rate was regularly monitored and finally
estimated after 10 days of culture as the percentage of protoplasts
undergoing divisions out of a population of viable protoplasts.

All data presented here are averages of four observations and
all experiments were repeated twice. In experiments using IAA,
(-)-bepridil, NPA, or a combination of any of these chemicals, these
substances were added to the protoplast suspensions at the desig-
nated concentrations from the dilutions of stock solutions made in
regeneration medium.

DM-Bodipy PAA labelling of protoplasts

An incubation period of 25–30 min was found to result in efficient
labelling of protoplasts when subjected to 1 µM DM-Bodipy PAA.
The fluorochrome was added as a 5 µM stock (made in regeneration
medium) to 20 µl of protoplast suspension transferred to a
microscope slide from the suspension culture, giving a final concen-
tration of 1 µM. Incubation was carried out in the dark at 20 °C. A
cover slip was placed on the incubation mixture thereafter, and the
slide was used for observations under a fluorescence microscope
(Axiophot; Zeiss, Oberkochen, Federal Republic of Germany)
and/or a CLSM (Leica, Wetzlar, Federal Republic of Germany). Pro-
toplasts initially selected under visible light were exposed to blue
light for a minimum period of observation and photography to min-
imize photobleaching.

The specificity of DM-Bodipy PAA labelling was studied by
competition with (-)-bepridil, a nonfluorescent competitor of PAA.
(-)-Bepridil strongly inhibits DM-Bodipy PAA fluorescence when
added before or after the probe (Fig. 2A–C). Protoplasts were first
incubated for 30 min in DM-Bodipy PAA (1 µM) and (-)-bepridil
was then added to the medium at a final concentration of 100 µM.
Fluorescence was recorded after further 10 min of incubation.

In order to rule out nonspecific dye accumulation, protoplasts
were incubated in the unconjugated form of dye, Bodipy FL (1 µM)
for 30 min.

Confocal laser scanning microscopy

For imaging, a CLSM (model TCS 4D, Leica), equipped with an
argon laser scanner (488 nm), a dichroic splitter at 510 nm and a
520–550 nm emission wavelength filter was used. Image acquisition
was achieved with a ×100 (numerical aperture, 1.4) oil objective
(NPL Fluotar) in scan mode with a pinhole (diameter, 70 µm). The
emission for PAA-binding sites is at 511 nm, whereas the autoflu-
orescence emission of chlorophyll is observed at 685 nm.

Fluorescence could also be detected to some degree by using
standard epifluorescence microscopy.
Results

Division of Physcomitrella protoplasts in culture

Isolated protoplasts of the moss *P. patens* exhibit a significant degree of nonsynchronous division both with regard to time and pattern of division. However, about 20% of the protoplasts start dividing within seven days in adequate culture conditions. Most protoplasts exhibit first unequal division, leading to polar tube formation from the smaller daughter cell. Some divide into two equal cells, one of which divides further by an oblique septum and subsequently forms a polar tube (data not shown).

In the presence of extracellular auxin (IAA), the protoplast division rate decreases significantly above 1 μM IAA (Fig. 1A) and remains similarly low between 5 and 50 μM extracellular IAA. NPA, a specific blocker of auxin efflux carrier protein, completely inhibits protoplast division, without affecting protoplast viability (Fig. 1B). Co-addition of IAA (50 μM) does not affect the response due to NPA. (−)-Bepridil, an antagonist of calcium channels, restricts protoplast division in a way similar to IAA but at a much lower concentration range, i.e., 1 to 20 μM (Fig. 1C). Viability of protoplasts largely remains unaffected by lowest concentrations of IAA (1 μM), NPA (0.1 μM), and bepridil (1 μM), but it is slightly reduced at highest concentrations of IAA (50 μM) and (−)-bepridil (20 μM). Interestingly, the suppression of protoplast division by 20 μM NPA is not accompanied with any effect on protoplast viability, and protoplasts remain healthy and viable as judged by fluorescein acetate treatment (data not shown).

Specificity of DM-Bodipy PAA labelling

Protoplasts, observed in the excitation wavelength (488 nm) after incubation in the presence of 1 μM Bodipy FL (the fluorophore used to bind PAA) for 30 min, do not exhibit any unspecific fluorescence on the protoplast membrane, thereby confirming the specific binding of the PAA derivative of DM-Bodipy to the PAA affinity sites. Such protoplasts simply show red autofluorescence in the chloroplasts. Binding specificity of the fluorescent probe for PAA-specific sites can be tested by incubating the protoplast suspension in the absence or presence of calcium ion channel antagonists. Verapamil and (−)-bepridil strongly inhibit PAA fluorescence by competition, when added before or after the addition of the probe (DM-Bodipy PAA). In the present investigation, protoplasts initially incubated for 30 min in the presence of DM-Bodipy PAA (1 μM) and then treated with 100 μM of (−)-bepridil for 10 min, exhibited no obvious fluorescence bound to any subcellular structure (Fig. 2A–C). Already bound fluorescence could be completely competed out by the calcium channel inhibitor. The drop in fluorescence due to (−)-bepridil treatment was rapid (1–2 min after addition of the competitor) and stabilized within 5 min.
Fig. 2 A–I. CLSM images of *P. patens* protoplasts and cells in culture. A–C As a control, protoplasts were initially incubated for 30 min in 1 μM DM-Bodipy PAA, followed by 10 min of incubation in 100 μM bepridil, showing no obvious green fluorescence of subcellular structures due to competition between unlabelled bepridil and labelled PAA. A and B Isolated protoplast. Images constructed from a combination of optical scans observed at a gap of 1 μm scanned only for chlorophyll autofluorescence (red) (A) or for both chlorophyll autofluorescence (red) and PAA-binding sites (green) (B). C Median confocal optical section of the protoplast in A and B scanned for PAA-binding sites only. D–I Protoplasts and cells were incubated in 1 μM DM-Bodipy PAA for 30 min prior to observation for fluorescence at PAA-binding sites. D Median confocal optical section of an undivided protoplast scanned for PAA-binding sites only. E Median confocal optical section of a just-dividing protoplast scanned for PAA-binding sites only. F Median confocal optical section of an asymmetrically divided protoplast scanned for PAA-binding sites only. G Combined confocal optical images of a protoplast showing bipolar division scanned for both chlorophyll autofluorescence and PAA-binding sites. H Distribution of PAA-binding sites in an apical cell formed from the germ tube as seen in combined confocal optical images scanned for both chlorophyll autofluorescence and PAA-binding sites. I Median confocal optical section of an apical cell formed from the germ tube scanned for PAA-binding sites only. Bars: 20 μm
Distribution of DM-Bodipy PAA-binding sites in undivided and in dividing protoplasts

A protoplast suspension containing 1 µM DM-Bodipy PAA was examined under white light or at excitation wavelength (488 nm). When illuminated by the excitation light, the protoplasts emitted bright green-yellow fluorescence, while the background remained uniformly dark. Fluorescence due to the dye binding appeared to be irregularly distributed along the cell membrane as discrete spots. Both fluorescence microscopy and confocal imaging further indicated the presence of PAA-binding sites along the nuclear membrane. Signals were never localized inside the vacuoles (Fig. 2D).

Protoplasts began to divide within seven days of culture. This was accompanied with remarkable changes in the distribution of PAA-binding sites. The peripheral distribution of the binding sites exhibited an overall increase along the plasma membrane as seen from CLSM images (Fig. 2E). The intensity of localization of PAA-binding sites was, however, highest near the margins of the cell plate and new cell walls. PAA-binding sites were maximally located on either side of the new cell plate (Fig. 2E). With asymmetric division, the smaller cell exhibited a much higher overall presence of the PAA-binding sites all along the plasma membrane and also inside the cell, probably representing vesicular membranes. Also, a very strong signal was seen along the edges of the new cell plate (Fig. 2F). Like a unipolar germ tube, bipolar germ tube formation showed an abundance of PAA-binding sites at the septum between the parent and the daughter cell (Fig. 2G).

As the daughter cell elongated to form a germ tube, the distribution of PAA-binding sites changed substantially, now showing an increased intracellular distribution. In the subsequent cell formed from the germ tube, a relatively higher intracellular fluorescence along the vesicular membranes close to the tip was seen (Fig. 2H). The transverse septum between the basal region of the apical cell did not reveal any binding sites. This is in contrast to the strong fluorescence in this region soon after asymmetric division between the parent cell and the daughter cell. The nuclear membrane which exhibited strong fluorescence due to PAA-binding sites in undivided and just-dividing cultured protoplasts, did not show any fluorescence in the fully formed germ tube (Fig. 2I).

Discussion

Moss protonemata exhibit polar transport of auxin from tip to more basal cells and this continuous transport is a prerequisite for the stability of protonema differentiation (Rose and Bopp 1983). Freshly isolated protoplasts of the moss Funaria hygrometrica rapidly accumulate IAA from the culture medium within 15 min of incubation. Co-incubation of the protoplasts in the presence of 10 or 100 µM NPA (auxin efflux blocker) enhances IAA accumulation by the protoplasts by 30%; possibly due to the blocked efflux of the phytohormone (Geier et al. 1990).

The NPA-binding protein, which may belong to the tyrosine kinase family (PTK), is thought to regulate auxin efflux via phosphorylation and dephosphorylation. Thus, the NPA-binding protein can integrate signals from different receptors for light, gravity, ethylene, an other environmental stimuli and can transmit this information to the auxin efflux carrier via phosphorylation (Venis and Napier 1997).

In the present work, we show that auxin efflux from isolated P. patens protoplasts is a prerequisite for the establishment of cell polarity and subsequent cell division since treatment with NPA as well as addition of auxin to the culture medium inhibited protoplast division. Thus, the auxin balance between the cell and the medium, or auxin transport across the plasma membrane itself, may be a prerequisite for the induction of cell polarity and subsequent cell division. This is in accordance with a proposed auxin receptor located at the plasma membrane, leading to a phosphorylation of anion channels and a subsequent depolarization of the plasma membrane, which in turn stimulates the opening of calcium channels (Marten et al. 1991, Zimmermann et al. 1994).

Such L-type calcium channels mediate voltage-controlled Ca²⁺ entry into the excitable cell and their activity can be modulated by different classes of drugs, e.g., DHP and PAA, well known as potent Ca²⁺ channel antagonists, which bind to the pore forming ß₂-subunit of these channels. These antagonists, when fluorescently labelled as, e.g., DM-Bodipy PAA, are used as markers for PAA- and DHP-binding sites in animal and plant cells (Knaus et al. 1992). As PAA binds to the plasma membrane (Vallée et al. 1997), inhibits Ca²⁺ influx (Pineros and Tester 1997), and binds to isolated Ca²⁺ channel peptides (Terry et al. 1992), and DHP and PAA sites colocalize with calcium channels (Knaus et al. 1992), it is assumed that
DHP- and PAA-binding sites in plant cells represent Ca\(^{2+}\) channels.

The specificity of PAA binding in the moss *P. patens* was demonstrated in the present investigation in two steps: neither Bodipy FL alone nor the fluorophore bound to PAA led to any unspecific fluorescence on subcellular structures of Physcomitrella protoplasts. Labelling of subcellular structures with DM-Bodipy PAA was completely abolished by an excess of a non-fluorescent competitor, (-)-bepridil, another potent calcium channel antagonist (Fig. 2B, C).

A competition of PAA and (-)-bepridil for the same binding sites has recently been found also in protoplasts of sunflower (Vallée et al. 1997, 1999). Additionally, calcium channel blockers have been used to study protonema differentiation in *P. patens*, demonstrating that DHP inhibits cytokinin-inducible bud formation (Conrad and Hepler 1988) and demonstrating a cytokinin-induced, DHP-sensitive calcium uptake into protoplasts (Schumaker and Gizinski 1993).

In the present investigation, protoplast division was efficiently blocked by the auxin efflux antagonist NPA as well as by the calcium channel antagonist (-)-bepridil (Fig. 1), thus connecting auxin and Ca\(^{2+}\) fluxes with Physcomitrella protoplast polarization. (-)-Bepridil concomitantly abolished labelling of subcellular structures with DM-Bodipy PAA (Fig. 2B, C).

In freshly isolated Physcomitrella protoplasts the PAA-binding sites were distributed on or close to the plasma membrane and were organized into clusters (Fig. 2D). This location is in accordance with ion flux all through the plasma membrane in a nonpolar cell or protoplast. After a few days of culture, PAA-binding sites were no longer restricted to the plasma membrane but were also present inside the cell, along the cytoplasmic strands, on the vesicles and tonoplasts and were highly concentrated on the nuclear membrane, suggesting that the binding sites observed inside the cultured protoplasts correspond to internal ion channels or to newly synthesized subunits of ion channels, respectively. These observations are in agreement with earlier observations on protoplasts of sunflower (Vallée et al. 1999). Shaw and Quatrano (1996a, b) also observed the labelling of the perinuclear region of *Fucus distichus* eggs by ST-Bodipy-DHP fluorescence.

A possible involvement of PAA-binding sites in the division process is highlighted by the nonuniform distribution of fluorescence accompanying cell division in Physcomitrella protoplasts. The beginning of cell plate formation was marked with a clustering of the binding sites to the margins of new cell plates (Fig. 2E-G). Calcium channels clustering is known in animal cells (Yazaki et al. 1995) and has been observed in dividing protoplasts of sunflower (Vallée et al. 1999).

Once the protoplast has undergone asymmetric division which leads to the formation of a polar germ tube, the pattern of PAA-binding sites changed to a significant increase in cytosolic distribution (Fig. 2H, I). The role of calcium in cell division is well known (Hepler 1992, Meng 1994) and calcium gradients are linked to polarization processes in a variety of species (Shaw and Quatrano 1996a, b; Love et al. 1997).

These redistributions of PAA-binding sites during polarization of Physcomitrella protoplasts and their subsequent division leading to the germ tube may highlight a redistribution of calcium channels, necessary for polarized growth. As shown by inhibitor studies, efflux of auxin is necessary for the redistribution of these calcium channels in Physcomitrella protoplasts. It is conceivable that also light plays a crucial role in this system, as has been well documented for *Ceratodon purpureus* protoplasts (Cove et al. 1996).

Having developed a marker system for protoplast polarization in *P. patens*, future efforts will be made to elucidate the sequence of events (light, auxin fluxes, calcium channels, ion fluxes) leading to this first morphogenetic step in plant protoplast culture, consequently using the unique properties of this moss to study gene function by targeted gene knockout (Reski 1998b).

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**References**


Bhatla SC (1992) Endogenous IAA after tryptophan application in the wild type and an auxin-insensitive mutant (86.1 = CAL 1) of the moss *Funaria hygrometrica* Hedw. J Plant Physiol 139; 758–760


Geier U, Werner O, Bopp M (1990) Indole-3-acetic acid uptake in isolated protoplasts of the moss Funaria hygrometrica. Physiol Plant 80: 584–592


– (1996b) The role of targeted secretion in the establishment of cell polarity and the orientation of the division plane in Fucus zygotes. Development 122: 2623–2630


