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Mechanism of inhibition of cytoplasmic streaming by a myosin inhibitor, 2,3-butanedione monoxime

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Summary. On the basis of the inhibition of myosin by 2,3-butanedione monoxime (BDM), the protein's involvement in various cell activities is discussed. However, it has not been established whether BDM inhibits plant myosin. In the present study, the effect of BDM on isolated plant myosin was analyzed in vitro. The sliding between myosin from lily (*Lilium longiflorum*) pollen tubes and actin filaments from skeletal muscle was inhibited to 25% at a concentration of 60 mM, indicating that BDM can be used as a myosin inhibitor for plant materials. Cytoplasmic streaming was completely inhibited by BDM at 30 mM in lily pollen tubes and at 70 mM in short root hair cells, and at 100 mM in long root hair cells of *Hydrocharis dubia*. However, BDM at high concentrations induced the disorganization of actin filament bundles in lily pollen tubes and short root hair cells. In addition, cortical microtubules were also fragmented in short root hair cells treated with BDM, suggesting a possible side effect of BDM.

Keywords: Actin filament; 2,3-Butanedione monoxime; *Hydrocharis dubia*; In vitro motility assay; Myosin; Pollen tube; Root hair cell.

Abbreviations: AF actin filament; BDM 2,3-butanedione monoxime; MT microtubule.

Introduction

Actin filaments (AFs) are some of the most conspicuous structures in the cytoplasmic matrix of plant cells (Staiger and Schliwa 1987). It is suggested that the actin cytoskeleton is concerned with various physiological functions. To elucidate the involvement of the actin cytoskeleton in physiological events in the cells, application of specific inhibitors permeative to the plasma membrane is one of the most convenient strategies. Since cytochalasins are specific inhibitors of AFs, involvement of AFs in cell function can be examined with these chemicals, e.g., cytoplasmic streaming is inhibited (Williamson 1972, Bradley 1973) and the transvacuolar strand is disorganized (Shimmen et al. 1995, Tominaga et al. 1997) by treatment of cells with cytochalasins.

It is expected that the actin cytoskeleton can manifest its physiological function by interaction with its associated proteins. The most common associated protein is myosin, a motor protein which slides along AFs by using the hydrolysis energy of ATP. However, a specific inhibitor of myosin had not been reported. Chen and Kamiya (1975) reported that N-ethylmaleimide inhibits myosin but not AFs. However, this chemical should exerts effects on all enzymes in which –SH groups are involved in the activities. Recently, extensive studies revealed that 2,3-butanedione monoxime (BDM) reversibly suppressed vertebrate muscle contraction by directly affecting the actin-myosin system (Yagi et al. 1992, McKillop et al. 1994). In vitro studies revealed a direct inhibition of myosin-ATPase by BDM (Higuchi and Takemori 1989, Herrmann et al. 1992, Cramer and Mitchison 1995, Zhao et al. 1995).

On the basis of its inhibition by BDM, the involvement of myosin in various activities of plant cells has been suggested, for example, radial expansion in Arabidopsis roots (Baskin and Bivens 1995), tension of AFs in soybean cell (Grabski et al. 1998), constriction of the neck region of plasmodesmata in root tissues of *Allium cepa*, *Zea mays*, and *Hordeum vulgare* (Radford and White 1998) and development of the Volvox embryo (Nishii and Ogihara 1999). However, inhibitory effects of BDM on plant myosin have not

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been elucidated yet. McCurdy (1999) reported that 10 mM BDM did not inhibit cytoplasmic streaming in *Chara corallina*. Thus, it is important to examine whether BDM really inhibits plant myosin in vitro. Yokota and Shimmen (1994) first succeeded in isolating a functional plant myosin maintaining motility from pollen tubes of lily. This myosin is composed of a 170 kDa heavy chain and calmodulin as a light chain (Yokota and Shimmen 1994, Yokota et al. 1999b). Hereafter this myosin is referred to as 170 kDa myosin. On the basis of the sliding activity in vitro and localization in higher-plant cells, this myosin has been indicated to be a motor protein involved in cytoplasmic streaming (Yokota and Shimmen 1994; Yokota et al. 1995a, b). The effect of BDM on the sliding between AFs and myosin was analyzed by a so-called in vitro motility assay, where fluorescently labeled AFs move on the glass surface coated with myosin (Kron and Spudich 1986). The present study shows for the first time that BDM inhibits sliding between 170 kDa myosin and AFs.

Starting from a pioneering study by Kamiya and Kuroda (1956), the involvement of the actin-myosin system in cytoplasmic streaming has been almost established (Shimmen and Yokota 1994). At present, cytoplasmic streaming is believed to occur by sliding of myosin attached to organelles on AFs (Shimmen and Yokota 1994). The velocity of the cytoplasmic streaming directly reflects the sliding velocity of myosin along AFs in situ (Yokota and Shimmen 1994, Yokota et al. 1999a, Igarashi et al. 1999). Therefore, cytoplasmic streaming is a suitable activity to examine whether BDM inhibits myosin-based activity in situ. In the present study, we further investigated the effect of BDM on the cytoplasmic streaming of lily pollen tubes and root hair cells of *Hydrocharis dubia*.

Material and methods

Plant materials

Pollen of *Lilium longiflorum* was allowed to germinate in a culture medium (7% sucrose, 1.27 mM $Ca(NO_3)_2$, 0.162 mM boric acid, 0.99 mM KNO_3, and 3 mM KH_2PO_4, pH 5.2) at 25 °C for 1 to 1.5 h (Yokota and Shimmen 1994). *Hydrocharis dubia* (Blume) Baker was cultured in plastic containers as described previously (Tominaga et al. 1997). Young roots were isolated and kept in 5 mM $CaSO_4$ solution supplemented with 100 mM mannitol before use.

BDM treatment

Stock solution of 1 M BDM (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was prepared by dissolving it into deionized water warmed at 50 °C and stored in the dark at 4 °C. BDM was used for the experiment within two days after preparation of the stock solution. Germinating pollen was treated with various concentrations of BDM in the culture medium for 1 h. Root hair cells of *H. dubia* were treated with BDM in $CaSO_4$ solution supplemented with 100 mM mannitol for 1 h.

Motility of isolated myosin

Isolation of myosin from lily pollen tubes and the in vitro motility assay were carried out according to Yokota and Shimmen (1994). Filamentous (F) actin was prepared from chicken breast muscle according to the method of Kohama (1981). The sliding velocity of pollen tube myosin along AFs isolated from skeletal muscle was almost equal to that along AFs isolated from plant materials (Igarashi et al. 1999). Since large quantities of AFs can be easily obtained from skeletal muscle, AFs isolated from skeletal muscle were used in the present study. The effect of BDM on sliding between pollen tube myosin and fluorescently labeled F-actin of skeletal muscle was analyzed with a solution containing 1 mM ATP, 0.3 μg of rhodamine-phalloidin-labeled F-actin, 0.216 mg of glucose oxidase, 36 μg of catalase, and 4.5 mg of glucose per ml, 30 mM KCl, 5 mM EGTA, 6 mM $MgCl_2$, 30 mM piperazine-N,N′-bis(2-ethanesulfonic acid)-KOH (pH 7.0) (Yokota and Shimmen 1994).

Light microscopy

Cytoplasmic streaming was observed with a microscope equipped with Nomarski optics (Optiphot; Nikon Co., Ltd., Japan) and recorded on videotape with a CCD camera (SSC-M350; Sony Co., Ltd., Japan) and a video recorder (HR-D80; Victor Co., Ltd., Kanagawa, Japan). The velocity of the cytoplasmic streaming was measured by chasing the movements of large cytoplasmic particles.

AF staining

AFs in pollen tubes and root hair cells were stained with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, Oreg., U.S.A.). Pollen tubes were incubated in EMP solution [10 mM EGTA, 2 mM $MgCl_2$, 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid), pH 7.0] supplemented with 0.01% saponin, 7% sucrose, and 0.132 μM rhodamine-phalloidin for 20 min. They were mounted on a glass slide in EMP solution supplemented with 0.1% (w/v) *p*-phenylenediamine and observed under a fluorescence microscope (BX60; Olympus Optical Co., Ltd., Tokyo, Japan).

Root hair cells of *H. dubia* were treated with EMP solution supplemented with 0.1% Triton X-100 for 20 min. After washing with EMP solution three times, root hair cells were stained with 0.132 μM rhodamine-phalloidin in EMP solution for 10 min. After washing with EMP solution three times, they were observed as in the case of pollen tubes.

Double fluorescence staining of AFs and MTs

Both AFs and MTs in root hair cells were visualized as previously described (Tominaga et al. 1997) with slight modifications. Ethylene glycol-bis-succinimide succinate (Dojindo Laboratories Co., Kumamoto, Japan) was used for preservation of MTs (Abdella et al. 1979). It was dissolved in dimethylsulfoxide at 200 mM as a stock solution. Root hair cells were treated in EMP solution supplemented with 1 mM ethylene glycol-bis-succinimide succinate and 0.1% Triton X-100 for 40 min. After washing with EMP solution three times, root hair cells were fixed with 2% paraformaldehyde in EMP solution for 1 h. In the case of root hair cells of *H. dubia*, antibodies could be introduced into the cell without enzymatic digestion of the cell wall (Tominaga et al. 1997). Root hair cells were

washed with EMP solution three times and incubated with a monoclonal antibody against α-tubulin (Amersham Pharmacia Biotech, Little Chalfont, U.K.) in phosphate-buffered saline (PBS) for 1 h. After washing with PBS three times, root hair cells were incubated in PBS supplemented with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (American Qualex, San Clemente, Calif., U.S.A.) and 0.132 μM rhodamine-phalloidin for 40 min. They were washed three times with PBS, mounted on a glass slide in PBS supplemented with 0.1% (w/v) *p*-phenylenediamine, and observed with a fluorescence microscope (BX60, Olympus).

Results

In vitro motility assay

The direct effects of BDM on the sliding activity of isolated plant myosin, 170 kDa myosin, were analyzed by the in vitro motility assay (Fig. 1). This is the first study to analyze the effect of BDM on the sliding activity of a plant myosin. AFs purified from chicken skeletal muscle actively moved on the glass surface coated with 170 kDa myosin. The velocity ranged from 6.0 to 9.8 μm/s with an average of 7.7 μm/s (Yokota and Shimmen 1994). This sliding activity was suppressed by BDM in a concentration-dependent manner (Fig. 1). However, motility was still preserved even at 60 mM.

Cytoplasmic streaming and elongation of lily pollen tubes

The effects of BDM on the velocity of cytoplasmic streaming in pollen tubes were analyzed. The velocity of cytoplasmic streaming was measured after the incubation of pollen tubes with BDM at various concentrations for 1 h (Fig. 2a). BDM suppressed cytoplasmic streaming in a concentration-dependent manner and completely inhibited it at 30 mM (Fig. 2a). After the cytoplasmic streaming was completely stopped in the presence of 30 mM BDM, the saltatory movement of organelles and vesicles was still observed (data not shown). The inhibitory effect of BDM on the cytoplasmic streaming of pollen tubes was reversible as shown in Fig. 2b. After complete inhibition of the cytoplasmic streaming by treatment with 50 mM BDM in pollen tubes, BDM was washed out. About 1 h after removal of the drug, the velocity of the cytoplasmic streaming recovered to the original level.

In pollen tubes, actin-myosin-dependent migration of secretory vesicles is believed to be indispensable for cell growth (Franke et al. 1972). Next, we examined the effect of BDM on the growth of pollen tubes. After pollens were germinated for 1 h in the absence of BDM, BDM at various concentrations was added. Then, the length of pollen tubes was measured after 1 h. In the absence of BDM, pollen tubes elongated to

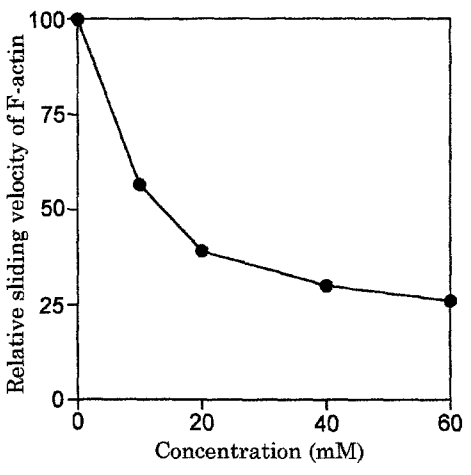

Fig. 1. Effect of BDM on sliding velocity of actin filaments on glass surface coated with 170 kDa myosin. The velocity in the absence of BDM was 7.7 μm/s

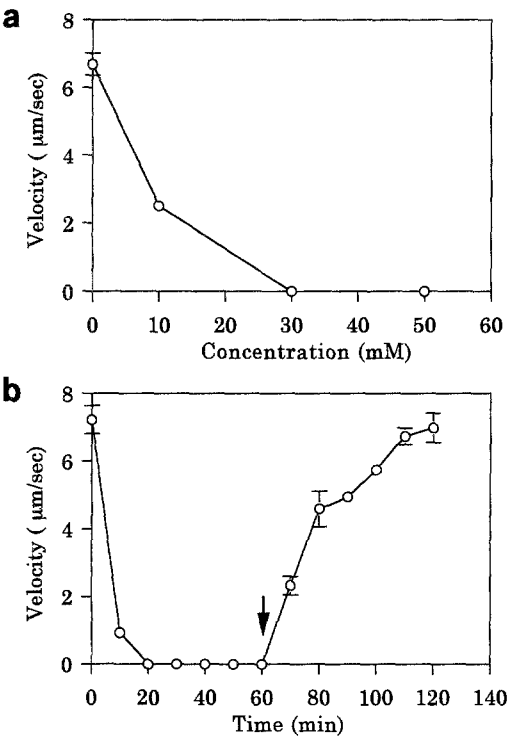

Fig. 2a, b. Effect of BDM on cytoplasmic streaming of pollen tubes. **a** Concentration dependency. Pollen tubes were treated with BDM at various concentrations for 1 h and the velocity of cytoplasmic streaming was measured. **b** Reversibility. At time zero, 50 mM BDM was added, and it was removed at 60 min (arrow). Cytoplasmic streaming recovered completely. Results show average values of 15 measurement with standard error

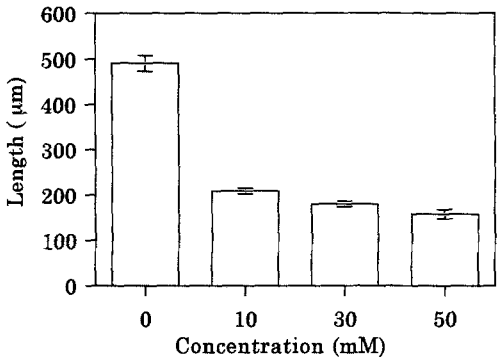

Fig. 3. Effect of BDM on elongation of pollen tubes. After pollen grains were germinated for 1 h in the absence of BDM, BDM at various concentrations was added. The length of pollen tubes was measured after 1 h of treatment. Results show average values of 15 measurement with standard error

about 500 μm. In the presence of BDM, elongation of pollen tubes was significantly inhibited (Fig. 3).

Cytoplasmic streaming in root hair cells

The effect of BDM on the cytoplasmic streaming was also analyzed in root hair cells of *H. dubia*. Young and short root hair cells are developed at the tip region of roots, old and long root hair cells at the basal region. In short root hair cells, so-called reverse fountain streaming is observed, i.e., cytoplasm streams acropetally at the subcortical region and basipetally in a transvacuolar strand longitudinally penetrating the central vacuole (Shimmen et al. 1995; Tominaga et al. 1997, 1998). In longer root hair cells, however, a transvacuolar strand is not observed and both acropetal and basipetal streamings occur at the subcortical region. Roots were treated with BDM at various concentrations for 1 h, and the velocity of cytoplasmic streaming was measured. The sensitivity to BDM was different between short and long root hair cells. Cytoplasmic streaming was completely stopped by 70 mM and 100 mM BDM in short and long root hair cells, respectively (Fig. 4a). The inhibition by BDM was reversible also in root hair cells (Fig. 4b). When long root hair cells were treated with 100 mM BDM, cytoplasmic streaming was completely stopped after 20 min, and it reversibly recovered by removing BDM.

Organization of AFs

The effect of BDM on the organization of AFs in pollen tubes was examined by fluorescence microscopy.

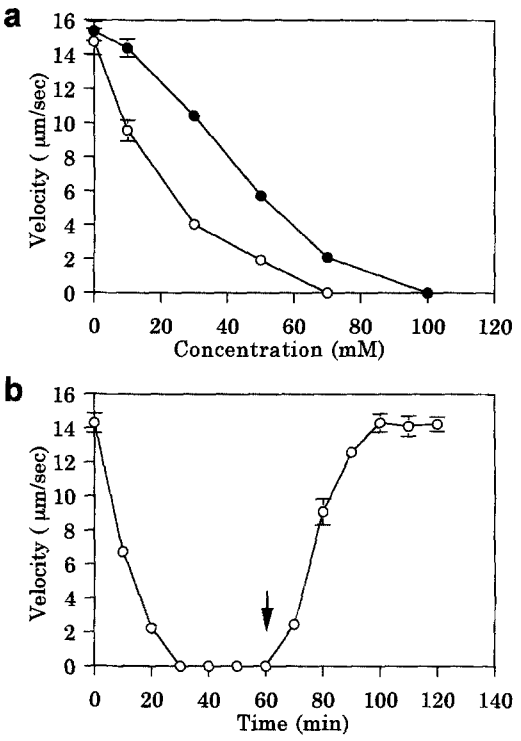

Fig. 4a, b. Effects of BDM on cytoplasmic streaming of root hair cells of *H. dubia*. **a** Concentration dependency. Roots were treated with BDM at various concentrations for 1 h and the velocity of cytoplasmic streaming was measured. Results of both young and short (○), and old and long root hair cells (●) are shown. **b** Reversibility in long root hair cells. At time zero, 100 mM BDM was added and the cytoplasmic streaming was completely stopped at 20 min. When BDM was removed at 60 min (arrow), the cytoplasmic streaming recovered completely. Results show average values of 15 measurement with standard error

In untreated pollen tubes, AFs were oriented parallel to the longitudinal direction (Fig. 5a). Treatment with BDM at 10 mM for 1 h did not induce any changes in the organization of AFs (Fig. 5b). When pollen tubes were treated with BDM at higher concentrations which caused complete inhibition of cytoplasmic streaming (30 mM and 50 mM), the organization of AFs was severely disrupted (Fig. 5c, d).

The effect of BDM on AFs was also analyzed in root hair cells. As reported previously (Shimmen et al. 1995, Tominaga et al. 1997), AFs formed discrete bundles in control cells (Fig. 6a). In short root hair cells, the organization of AFs was disturbed by BDM in a concentration-dependent manner (Fig. 6a–c). In long root hair cells, however, no effects on the organization of AFs were observed even after treatment with 100 mM BDM (Fig. 6d), which completely inhibited cytoplasmic streaming (Fig. 4a).

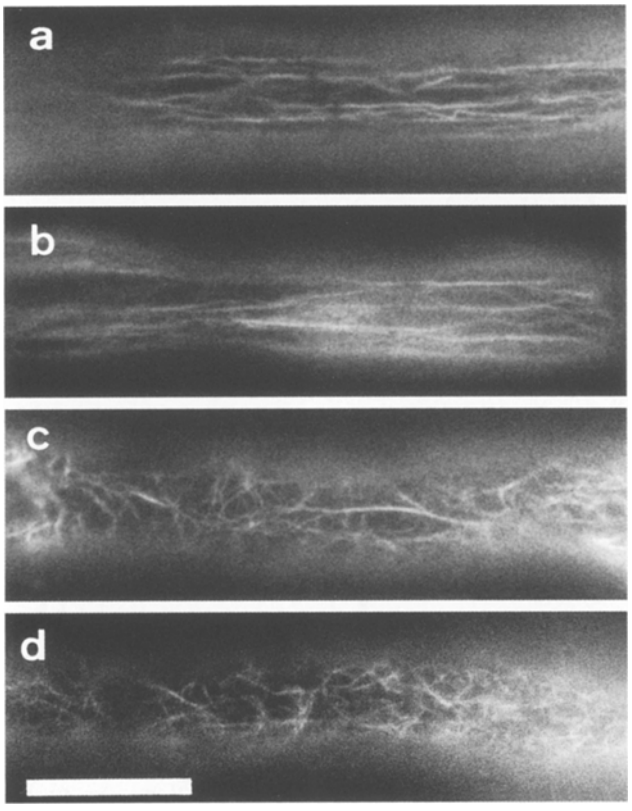

Fig. 5a–d. Fluorescence staining of AFs in pollen tubes. **a** Before treatment. **b–d** Pollen tubes treated with 10 mM (**b**), 30 mM (**c**), and 50 mM (**d**) BDM for 1 h. Bar: 20 μm

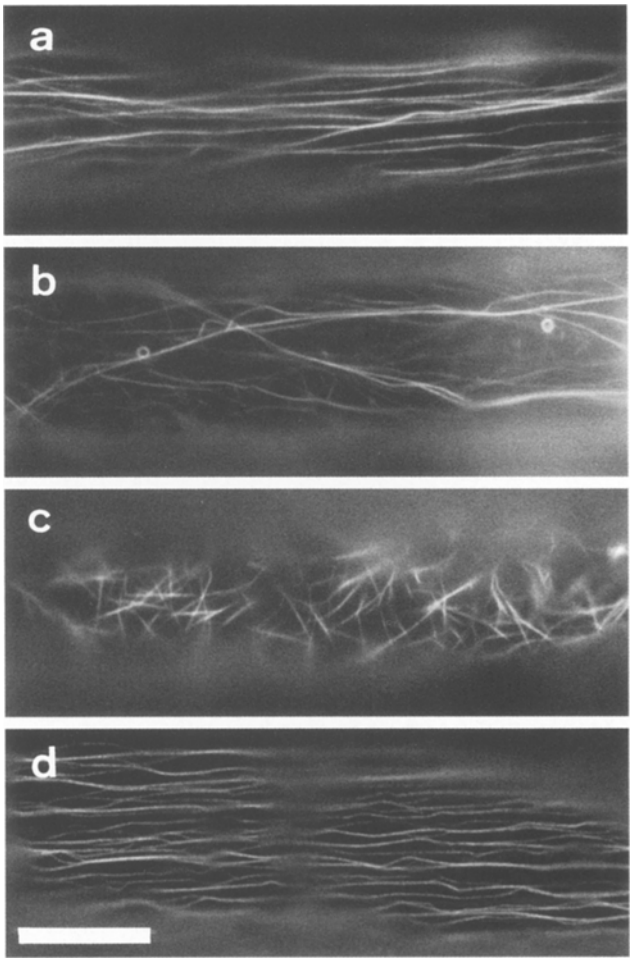

Fig. 6a–d. Fluorescence staining of AFs in root hair cells of *H. dubia*. **a–c** Short root hair cells treated with 30 mM (**a**), 50 mM (**b**), and 70 mM (**c**) BDM for 1 h. **d** Long root hair cells treated with 100 mM BDM for 1 h. Bar: 20 μm

MTs organization in root hair cells

Young root hair cells were treated with 100 mM BDM for 1 h and both AFs and MTs were stained. By the treatment, both AFs and MTs were severely fragmented (Fig. 7a, b). Interestingly both fragmented MTs and AFs did not colocalize in the presence of BDM, although both cytoskeletal components are well colocalized in untreated cells (Tominaga et al. 1997). In long root hair cells, however, 100 mM BDM had no effect on the organization of both AFs and MTs, and their colocalization was observed at the subcortical region (Fig. 7c, d).

Discussion

AFs are one of the ubiquitous cytoskeletons in plant cells and are suggested to have roles in various activities of plant cells (Staiger and Schliwa 1987). At least, two physiological roles have been elucidated for AFs: as a skeleton to support intracellular structure and as a track for a motor protein, myosin. The role of the actin-myosin system had been most extensively studied in cytoplasmic streaming; myosin bound to organelles slides along bundles of AFs (Shimmen and Yokota 1994). On the other hand, it is becoming evident that plant cells contain more than one myosin (Miller et al. 1995, Yokota et al. 1999a). It is suggested that myosins are concerned with not only cytoplasmic streaming but also other activities of plant cells. To examine whether myosin is concerned with a targeted physiological phenomenon of plant cells, use of an inhibitor of myosin is one of the strategies. From studies on muscle contraction, BDM has appeared as an inhibitor of myosin. This drug is also used for plant cells to examine the possible involvement of myosin (Baskin et al. 1995, Nishii and Ogihara 1999, Sato et al. 1999). However, it has not been studied whether BDM inhibits the activity of plant myosin. The present study demonstrated by an in vitro motil-

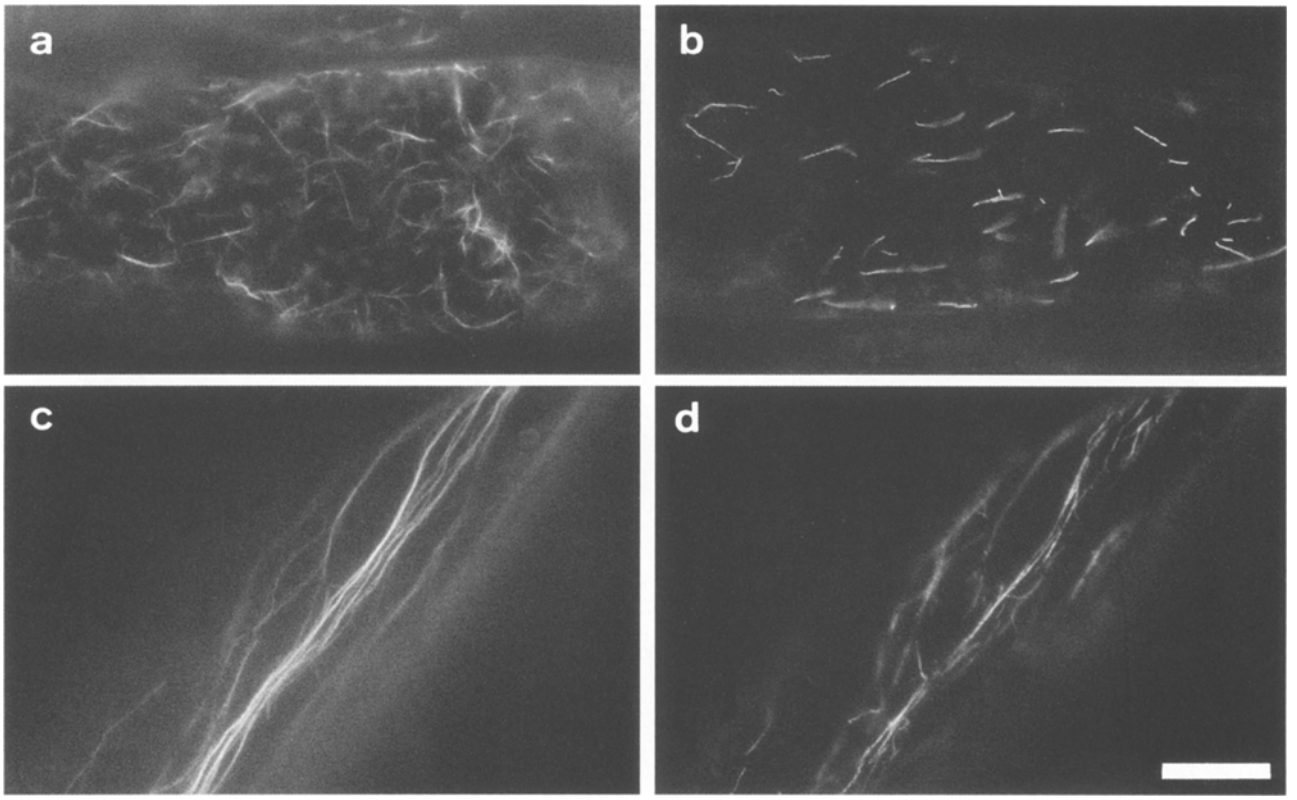

Fig. 7a–d. Double fluorescence staining of AFs and MTs in root hair cells of *H. dubia*. AFs (**a**) and MTs (**b**) of short root hair cells treated with 100 mM BDM for 1 h. AFs (**c**) and MTs (**d**) of long root hair cells treated with 100 mM BDM for 1 h. Bar: 20 μm

ity assay that BDM really inhibits the activity of plant myosin (Fig. 1).

The sliding activity of myosin along AFs was still observed in the presence of 60 mM BDM (Fig. 1). This suppressive effect of BDM is similar to the effect on myosin of skeletal muscle (Horiuti et al. 1998, Higuchi and Takemori 1989, Herrmann et al. 1992, Zhao et al. 1995), although the effective concentrations of BDM on the 170 kDa myosin activity are higher than those on skeletal muscle fibers. Although 25% of the activity of 170 kDa myosin of pollen tubes remained in motility assay in vitro even at 60 mM BDM (Fig. 1), the cytoplasmic streaming of pollen tubes was completely inhibited at 30 mM BDM (Fig. 2 a). Two possibilities are suggested for this discrepancy.

The in vitro motility assay was carried out in a rather simple solution. However, the cytoplasm is not a simple solution and should have high viscosity (Kamiya and Kuroda 1973). In addition, it is expected that the cytoplasm has non-Newtonian characteristics. If this would be the case for pollen tubes, the cytoplasm should not move upon application of a force smaller than the yielding point of the cytoplasm. Thus, it is suggested that the sliding force generated by AFs and myosin in the presence of BDM at higher concentrations was smaller than the yielding point of the cytoplasm. However, the occurrence of saltatory movement in the presence of BDM at high concentrations obscures this possibility.

Since cytoplasmic streaming is induced by the sliding of myosin along AFs, it is suggested that BDM affected not only myosin but also AFs. BDM at high concentrations severely damaged the organization of AFs. It is suggested that saltatory movement was induced by the sliding of partially inhibited myosin on disorganized AFs in the presence of BDM. A disorganization of AFs by BDM has been also reported in *Schizosaccharomyces pombe* (Steinberg and McIntosh 1998, May et al. 1998) and PtK2 cells (Cramer and Mitchison 1995). When PtK2 cells were treated with BDM for a long period, stress fibers became shorter and the intensity of staining of AFs by rhodamine-phalloidin decreased. It was suggested that the destabilization of stress fibers was caused by a loss of myosin activities (Cramer and Mitchison 1995). Grabski et al. (1998) suggested the involvement of

myosin in the generation of tension of AF bundles in soybean cells, on the basis of BDM experiments. Thus, it is suggested that the disorganization of AFs by BDM in pollen tubes and young root hair cells was caused by the inhibition of putative myosin involved in the organization of AFs.

We have reported that AFs and microtubules (MTs) are colocalized and that MTs regulate the organization of AFs in the subcortical region of root hair cell of *H. dubia* (Tominaga et al. 1997, 1998). Hence, there is a possibility that the organization of MTs is also changed by BDM. From activated sea urchin coelomocytes, it has been reported that BDM acts as an inhibitor of organelle movement along MTs (Krendel et al. 1998). BDM at high concentrations severely fragmented MTs in our material. At present, it is difficult to imagine the molecular mechanism for the disorganization of MTs by BDM. Furthermore, assuming that BDM affected only the actin-myosin system, it is hard to explain the difference in sensitivity to BDM between short and long root hair cells. Although BDM has been used as an inhibitor of myosins in animal cells, several studies reported that BDM affected the intracellular Ca^{2+} concentration (Zhu and Ikeda 1993, Gyorke et al. 1993, Xiao and McArdle 1995, Byron et al. 1996, Ferreira et al. 1997, Allen et al. 1998) or potassium channels (Schlichter et al. 1992, Lopatin and Nichols 1993, Smith et al. 1994) and modulated protein kinase activity (Ye and McArdle 1996, Verrecchia and Herve 1997) or altered the activity of certain kinases (Huang and McArdle 1992, Ye and McArdle 1996). Thus, the possibility is also suggested that cytoskeletons were affected by BDM via a disturbance of the intracellular homeostasis, especially in young root hair cells. We have reported that cytoplasm acidification induced the disorganization of AFs and MTs in root hair cells of *H. dubia* (Tominaga et al. 1998). An increase in free Ca^{2+} in the cytoplasm may be also considered to induce the disorganization of MTs in the presence of BDM in pollen tubes and in short root hair cells. Yoshida (1994) reported that low temperature severely lowered cytoplasmic pH, which is supposed to be maintained by the proton pump of the membrane, in cultured mung bean cells at the early stage of growth, but did not in cells of a late stage of growth. The plasma membrane is permeabilized by plasmolysis at low temperature in the absence of extracellular Ca^{2+} in young Nitella cells, but not in old cells (Shimmen and MacRobbie 1987). It seems that the membrane of young cells is more susceptible to physical and chemical perturbation. Hence, it is suggested that BDM at high concentrations disturbs the intracellular homeostasis in young root hair cells but not in old long root hair cells.

BDM severely inhibited growth of pollen tube. Migration of secretory vesicles responsible for tip growth is mediated by the actin-myosin system in pollen tubes (Franke et al. 1972). In fission yeast, the secretion of invertase transported by myosin along AFs was inhibited by BDM (Steinberg and McIntosh 1998). The inhibition of the elongation of pollen tubes might be due to the inhibitory effect of BDM on actin-myosin-dependent vesicle transport (Franke et al. 1972), although the side effects of BDM must be also considered as described above.

The present study first indicated that BDM suppresses the activity of plant myosin in vitro and the cytoplasmic streaming supported by the actin-myosin system. Therefore, BDM can be used to study myosin-related activities in plant cells. Cytochalasin B is generally used as inhibitor of AFs. However, this chemical also inhibits the activity of the hexose transporter of the plasma membrane (Karim et al. 1987, Lin and Spudich 1974, Rampal et al. 1980). Therefore, cytochalasin B is used with caution as an inhibitor of AF in studies using cells (Wayne and Tazawa 1988). The situation for BDM is similar to that for cytochalasin B. Since BDM does not inhibit kinesin (Cramer and Mitchison 1995), it may be a "specific" inhibitor of myosin in studies of motor proteins in vitro. However, side effects must be considered, when used in vivo.

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