The Plant Cell, Vol. 11, 2349–2363, December 1999, www.plantcell.org

Latrunculin B Has Different Effects on Pollen Germination and Tube Growth

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The actin cytoskeleton is absolutely required for pollen germination and tube growth, but little is known about the regulation of actin polymer concentrations or dynamics in pollen. Here, we report that latrunculin B (LATB), a potent inhibitor of actin polymerization, had effects on pollen that were distinct from those of cytochalasin D. The equilibrium dissociation constant measured for LATB binding to maize pollen actin was determined to be 74 nM. This high affinity for pollen actin suggested that treatment of pollen with LATB would have marked effects on actin function. Indeed, LATB inhibited maize pollen germination half-maximally at 50 nM, yet it blocked pollen tube growth at one-tenth of that concentration. Low concentrations of LATB also caused partial disruption of the actin cytoskeleton in germinated maize pollen, as visualized by light microscopy and fluorescent-phalloidin staining. The amounts of filamentous actin (F-actin) in pollen were quantified by measuring phalloidin binding sites, a sensitive assay that had not been used previously for plant cells. The amount of F-actin in maize pollen increased slightly upon germination, whereas the total actin protein level did not change. LATB treatment caused a dose-dependent depolymerization of F-actin in populations of maize pollen grains and tubes. Moreover, the same concentrations of LATB caused similar depolymerization in pollen grains before germination and in pollen tubes. These data indicate that the increased sensitivity of pollen tube growth to LATB was not due to general destabilization of the actin cytoskeleton or to decreases in F-actin amounts after germination. We postulate that germination is less sensitive to LATB than tube extension because the presence of a small population of LATB-sensitive actin filaments is critical for maintenance of tip growth but not for germination of pollen, or because germination is less sensitive to partial depolymerization of the actin cytoskeleton.

INTRODUCTION

Pollen from higher plants is a unique multicellular structure that carries the male germ unit to the ovule (reviewed in Bedinger, 1992; Taylor and Hepler, 1997). The pollen grain consists of a large vegetative cell within which is either a generative cell or two sperm cells derived from a mitotic division of the generative cell. One striking feature of pollen is the production of a polarized cytoplasmic extension, the pollen tube. The pollen tube grows by the addition of membrane and cell wall material to the tip and thereby allows the movement of sperm cells through the pistil. The growth of pollen tubes can be extremely fast; for example, maize pollen tubes grow at rates of ~1 cm/hr (167 μm/min) (Bedinger, 1992).

Pollen is a rich source of actin, which accounts for 2 to 20% of the total soluble protein in this tissue (Andersland et al., 1992; Liu and Yen, 1992; Ren et al., 1997; Vidali and Hepler, 1997). It also contains several actin binding proteins such as profilin, actin depolymerizing factor, spectrin, 115- and 135-kD actin-bundling proteins, and myosin (reviewed in Asada and Collings, 1997; Staiger et al., 1997; de Ruijter and Emons, 1999). The abundance of proteins that modulate actin function suggests that dynamic remodeling of the cytoskeleton is important for pollen function. Despite the presumed importance of actin dynamics, little is known about the proportion of the actin pool that is in polymeric form—that is, filamentous actin (F-actin)—or about changes in F-actin concentrations that may coincide with cellular morphogenesis. The only attempts to quantify F-actin in plant cells have used inhibition of DNase I activity. Such experiments have shown that approximately two-thirds of the actin in *Heracleum sosnowskyi* phloem (Kulikova, 1986; Turkina et al., 1987) and as much as half of the actin in wheat callus culture cells (Turkina et al., 1995) is F-actin. These ratios of F-actin to globular or monomeric actin (G-actin) are similar to those observed in nonplant systems. To our knowledge, measurements of F-actin levels in ungerminated or growing pollen have not been reported.

Previous investigations of actin-dependent processes in plants have relied on the use of cytochalasins, which affect

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actin polymerization by binding to the barbed (fast-growing) end of the actin filament and thereby prevent actin subunits from associating or dissociating (reviewed in Cooper, 1987). The mechanism of cytochalasin action is complex because, in addition to inhibiting polymerization by preventing the addition of actin subunits to the barbed end (Brown and Spudich, 1979), these compounds can also nucleate actin polymerization by forming actin dimers (Goddette and Frieden, 1986). These complications of cytochalasin action in vitro are often reflected in their effects when applied to cells. The primary action of cytochalasins is to prevent new polymerization rather than to depolymerize existing filaments. For example, F-actin levels in resting platelets are not altered by treatment with cytochalasin D (CD), yet CD prevents the increase in F-actin that occurs when untreated platelets are activated (Casella et al., 1981; Fox and Phillips, 1981). Application of cytochalasins to pollen inhibits germination and reduces the growth rate of pollen tubes. At high concentrations, cytochalasins also inhibit cytoplasmic streaming (Franke et al., 1972; Herth et al., 1972; Mascarenhas and LaFountain, 1972). The mechanism for cytochalasin inhibition of pollen tube growth is believed to involve inhibition of vesicle transport to the tip of the pollen tube (Picton and Steer, 1981).

Latrunculins, a class of macrolide toxins isolated from the red sea sponge *Latrunculia magnifica*, have marked effects on actin organization (Spector et al., 1983). These toxins reduce the amount of F-actin that can be detected by immunocytochemistry and generally disrupt actin-based processes at lower concentration than CD (Spector et al., 1983, 1989). Latrunculin A affects actin polymerization in a manner consistent with the formation of a 1:1 latrunculin–G-actin complex (Coué et al., 1987). The latrunculins have been used to inhibit actin function in a wide variety of systems, including animals (Spector et al., 1989), fungi (Ayscough et al., 1997; Gupta and Heath, 1997), and brown algae (Love et al., 1997; Hable and Kropf, 1998). Currently, few reports have been published on the effects of latrunculins on angiosperms (Bibikova et al., 1999; Zonia et al., 1999) and none on the interaction of latrunculin B (LATB) with actin from any organism in vitro.

In this study, LATB treatments revealed that requirements for F-actin differ during key stages of pollen development and morphogenesis. Growth of the maize pollen tube was much more sensitive to LATB than was germination, suggesting that tip growth is extremely sensitive to alteration of F-actin levels or to changes in the ratio of F-actin to G-actin. We measured F-actin levels in pollen and found that the F-actin levels increased slightly upon pollen germination, whereas the amounts of total actin protein remained constant. We also show directly that LATB depolymerized F-actin in pollen and that grains and growing pollen tubes were equally sensitive to LATB treatment. The addition of LATB to solutions of F-actin resulted in rapid depolymerization in vitro, and the decrease in F-actin at steady state was used to determine that LATB has a high affinity for pollen actin. The potent effect of LATB on pollen tube growth is contrasted with the effects of CD and shows that the use of multiple inhibitors can help to reveal novel features of biological processes.

RESULTS

LATB Inhibits Pollen Germination and Tube Growth

LATB caused marked and dose-dependent reductions in pollen germination frequency and pollen tube growth rate. Figure 1 shows the effect of a 1-hr exposure to LATB on maize pollen germination. Pollen that had not been exposed to the inhibitor germinated with a frequency of 40 to 60% and had long, actively growing pollen tubes (Figure 1A). In the presence of 10 to 30 nM LATB, the frequency of germination decreased, and the pollen tubes that emerged were substantially shorter and not as straight as untreated pollen tubes (Figures 1B and 1C). At concentrations >30 nM, germination was blocked almost entirely (Figure 1D); only small protrusions at the germination aperture of some grains were

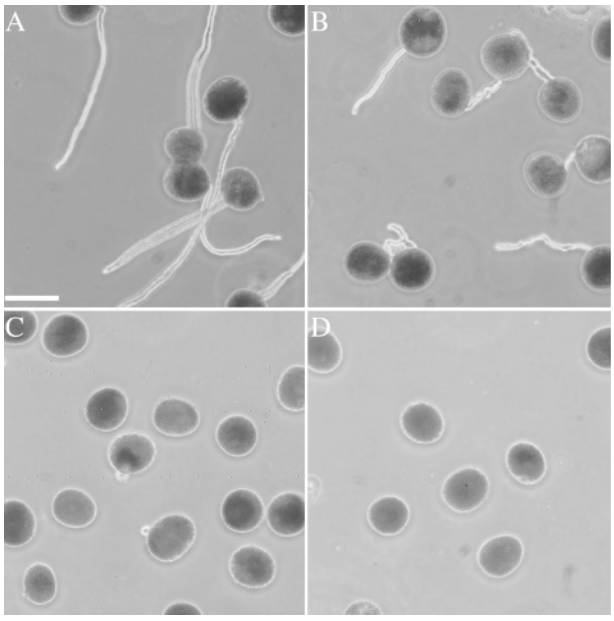

Figure 1. LATB Inhibits Maize Pollen Germination.

(A) Pollen germinated without LATB.
(B) Pollen germinated in the presence of 10 nM LATB. The tubes were substantially shorter and not as straight as untreated pollen tubes.
(C) Pollen treated with 30 nM LATB. A few pollen grains had small protrusions.
(D) In the presence of 1 μM LATB, germination was almost entirely blocked, and very few grains had small protrusions.
Bar in **(A)** = 100 μm for **(A)** to **(D)**.

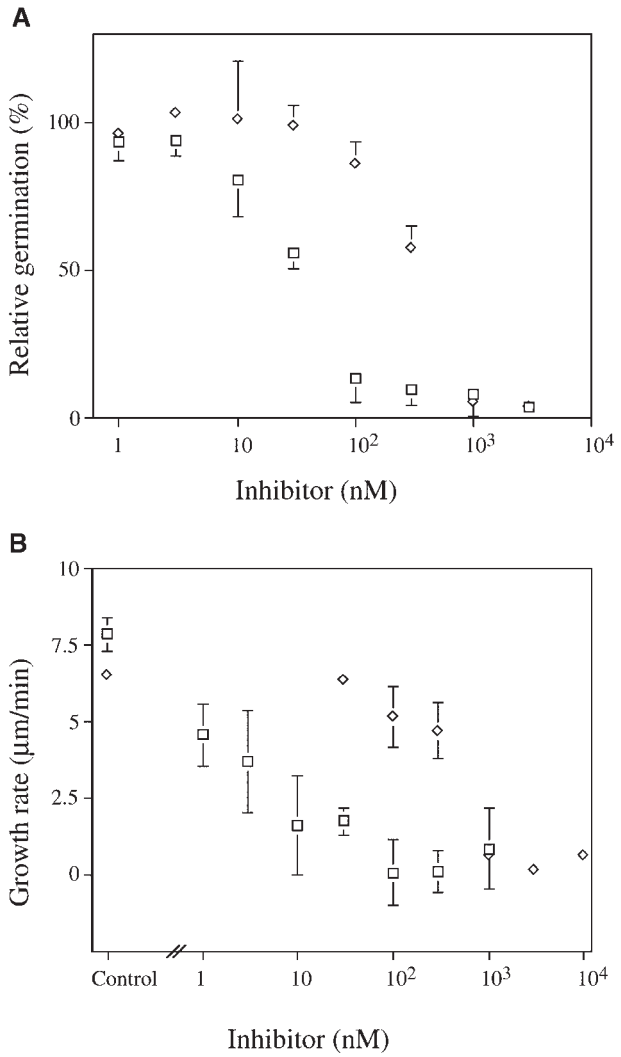

Figure 2. Germination Frequency and Pollen Tube Growth Rates Are Reduced by LATB.

(A) Inhibition of germination in the presence of various concentrations of LATB (squares; $n = 4$) or CD (diamonds; $n = 4$). Values for germination frequency were normalized relative to those for DMSO-treated controls, of which 40 to 60% germinated. The half-maximal inhibition of germination occurred at ~50 nM LATB (400 nM for CD). The error bars represent SD.

(B) Inhibition of pollen tube growth in the presence of LATB (squares; $n = 4$) or CD (diamonds; $n = 2$). Growth rate was calculated as the difference in pollen tube length divided by the duration of exposure to the drug. Half-maximal inhibition of growth occurred at ~5 nM LATB and 500 nM CD. The error bars represent SD.

observed. Quantitative analysis of the effects of a 1-hr LATB treatment is shown in Figure 2A. The germination frequency was substantially reduced in the presence of submicromolar concentrations of LATB, the half-maximal inhibition occurring at ~50 nM. Thirty-minute treatments gave identical results (data not shown), verifying that the pollen did not acclimate to LATB. The effect of CD on germination was substantially less pronounced, the half-maximal inhibition occurring at ~400 nM (Figure 2A).

Examination of the effect of LATB on pollen tube growth rate showed that the addition of LATB substantially slowed the growth of the pollen tubes; after 30 min of treatment, the average length of the pollen tubes was markedly less than untreated pollen grown for the same period (data not shown). In addition to being shorter, the LATB-treated pollen tubes appeared distorted in comparison with untreated pollen tubes. In Figure 2B the effect of LATB treatment over a range of concentrations is illustrated. The average results from four experiments are shown. Pollen tube growth was extremely sensitive to LATB, the half-maximal inhibition occurring at ~5 nM. Although growth was markedly inhibited, cytoplasmic streaming was still observed in many tubes exposed to 10 to 30 nM LATB (data not shown). Nonetheless, the streaming was not normal with respect to the size and number of particles moving but resembled the patterns observed after recovery from LATB treatment (see below; Tables 1 and 2). Streaming was not observed at higher concentrations of inhibitor. CD was less potent than LATB for inhibiting pollen tube growth, requiring ~500 nM for half-maximal inhibition (Figure 2B)—approximately the same CD concentration required for half-maximal inhibition of pollen germination.

To verify that the differential sensitivity of pollen germination and pollen tube growth to LATB treatment was not unique to maize pollen, we measured germination frequency and growth rates for Tradescantia pollen. The response of this pollen to LATB was similar to that observed for maize pollen. Germination was inhibited half-maximally at 40 nM LATB. Tradescantia pollen grew at a faster rate (13.5 μm/min) than did maize pollen (7.9 μm/min), but, like maize, tube elongation was half-maximally inhibited at 7 nM. As these results indicate, the differential response of pollen germination and pollen tube extension to LATB treatment is a general phenomenon.

The disruption of germination and pollen tube growth by LATB was partially reversible. Ungerminated maize pollen was treated with LATB for 1 hr and then washed with several changes of medium and allowed to recover for 3 hr. Only a small proportion of the pollen grains could initiate growth to form a long pollen tube under these conditions (Table 1). Because the experiments described earlier had revealed cytoplasmic streaming in many pollen tubes treated with similar concentrations of LATB, we analyzed the recovery of growing pollen tubes after treatment with LATB to clarify the effect of washing out the inhibitor. In untreated growing pollen tubes, cytoplasmic particles moved toward the tip down one side

Table 1. Recovery of Germination in Pollen Grains Treated with LATB

Treatment[a] (nM)	% Recovery[b] (mean ±SD [*n*])
30	32.7 ± 18.5 (3)
100	6.1 ± 2.0 (3)
300	1.2 ± 1.1 (3)

[a] Pollen was sown on medium containing the indicated concentration of LATB and allowed to germinate for 1 hr. The inhibitor was washed out with several exchanges of medium, and the grains were allowed to recover for 3 hr.

[b] The percentage of pollen grains that had recovered and had long pollen tubes were counted. Values were expressed relative to untreated controls.

of the tube, reversed direction several micrometers behind the tip, and moved away from the tip on the opposite side of the tube. When growing pollen was treated with concentrations of LATB that stopped growth, and the drug was subsequently washed out, streaming typically resumed. At the lowest concentration of LATB tested (10 nM), growth resumed in a substantial portion of the population (Table 2). At higher concentrations of LATB (30 to 100 nM), despite the observation of vigorous streaming, growth did not resume. The streaming pattern in nongrowing pollen tubes that had recovered from LATB treatment was frequently aberrant, following two abnormal patterns. The most frequently observed pattern looked normal except that the streaming extended to the extreme tip of the pollen tube. The second pattern had a transverse rotation of organelles around the cortical cytoplasm of the pollen tube. Occasionally, the two patterns were observed in the same tube, in which case the transverse cortical rotation generally occurred near the tip, but the longitudinal movement of material was observed subapically. Maize pollen was unable to recover from treatment with 300 nM LATB.

LATB Disrupts Microfilament Organization

The actin cytoskeleton of germinated pollen was investigated by light microscopy and fluorescent-phalloidin staining. Microfilament distribution in untreated maize pollen is shown in Figure 3. Before germination, cortical actin filaments radiated toward the germination aperture and surrounded numerous organelles (Figure 3A). An intensely stained patch of F-actin, positioned just beneath the single germination aperture, was also observed. After pollen germination, the actin became aligned parallel to the growth axis, even in very short pollen tubes (Figure 3B). The actin in older pollen tubes consisted of long filaments that were arranged longitudinally within the cytoplasm and extended toward the tip of the pollen tube. A "collar" of actin, or a dense array of fine filaments, was frequently observed ~5 μm behind the apex of the pollen tube, and relatively few thick filaments extended beyond the collar into the apex (Figures 3C, 3D, and 4A to 4D).

The effect of LATB treatment on actin microfilaments in pollen tubes is shown in Figure 4. The effects of low concentrations of LATB were visible within 5 min, and marked changes in the organization of actin in the tip region were quite obvious. The initial effect of LATB was to cause the microfilament bundles to extend into the extreme apex of the pollen tube (Figures 4E and 4F); moreover, the collar of fine actin filaments behind the tip was eliminated (Figures 4E

Table 2. Recovery of Cytoplasmic Streaming and Pollen Tube Growth after Treatment with LATB

		Nongrowing but Streaming[b]			
Treatment[a] (Recovery Time)	Growing % ±SD	Longitudinal % ±SD	Cortical % ±SD	Mix % ±SD	No Growth or Streaming % ±SD
10 nM (1.5 hr)	81 ± 10	9 ± 5	4 ± 3	2 ± 3	4 ± 2
30 nM (1.5 hr)	48 ± 21	11 ± 4	14 ± 7	13 ± 7	18 ± 8
100 nM (1.5 hr)	2 ± 4	11 ± 7	12 ± 10	5 ± 6	71 ± 22
300 nM (1.5 hr)	0	0	0	0	100
10 nM (3 hr)	83 ± 6	5 ± 3	2 ± 3	5 ± 2	6 ± 3
30 nM (3 hr)	45 ± 30	20 ± 14	10 ± 11	5 ± 3	22 ± 15
100 nM (3 hr)	7 ± 9	32 ± 14	4 ± 3	5 ± 6	54 ± 17
300 nM (3 hr)[c]	0	0	0	0	100

[a] Pollen was grown for 30 min before application of LATB and then treated with inhibitor for 30 min. After several washes with germination medium, the streaming pattern and growth of pollen tubes at 1.5 and 3 hr were measured. $n = 4$ for each treatment.

[b] Many cells recovered streaming but did not initiate new growth. Three streaming patterns were observed: longitudinal and similar in nature to the pattern observed in growing pollen tubes but sometimes extending to the extreme apex; cortical and transverse to the growth axis; and a mixture of both patterns at different locations along the tube axis.

[c] $n = 3$.

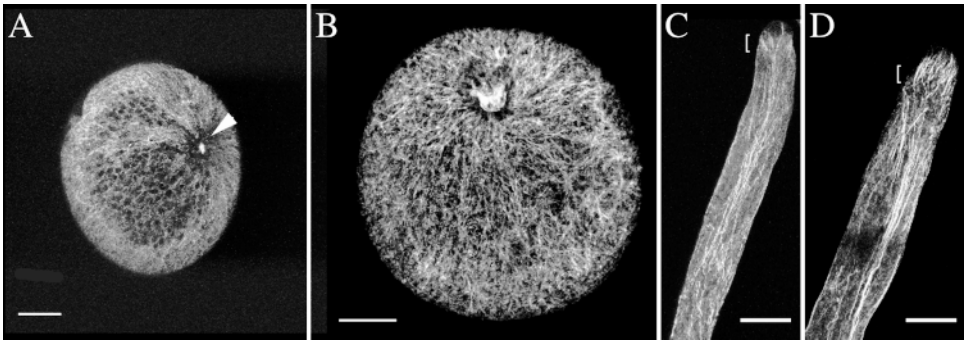

Figure 3. Fluorescent-Phalloidin Staining of F-Actin in Maize Pollen.

(A) Before germination, actin filaments radiated toward the germination aperture (arrowhead), and an intensely stained patch of actin was present under the germination aperture.
(B) After ~5 min, a small pollen tube was visible and stained brightly with fluorescent phalloidin. Many microfilaments in the tube were aligned parallel to the growth axis.
(C) Thirty minutes after germination, another pollen tube was ~250 μm long and filled with thick axial microfilaments. A region of bright and fine filamentous staining, or "collar," was frequently observed ~5 μm behind the pollen tube tip (bracket).
(D) After 60 min, a pollen tube was ~500 μm long, and the microfilaments remained oriented along the growth axis. The collar of fine filaments behind the tip was also observed after 60 min of pollen tube growth (bracket).
Bars in **(A)** to **(D)** = 15 μm.

and 4F). Some tubes showed more pronounced disorganization in the tip region, with several microfilament bundles becoming aligned transverse to the growth axis (Figure 4G). After 15 min of LATB treatment, the proportion of filaments observed in the transverse orientation had increased substantially (Figures 4H and 4I). The most brightly stained transverse filaments were often located in the region occupied by the collar of actin filaments in untreated cells (Figure 4I). After 30 min of LATB treatment, some cells continued growing slowly. In these tubes, the actin filament organization was similar to that in the untreated control tubes (Figure 4J). Most tubes, however, ceased growing and had primarily transversely oriented microfilaments (Figure 4K). Pollen tubes treated with 30 nM LATB showed a pronounced disruption of the actin cytoskeleton. Some cells had transversely arranged microfilaments (Figure 4L), but most had large aggregates of phalloidin-stained material in the cortical cytoplasm, with only a few short bundles of filaments (Figure 4M). Treatment with 1 μM LATB resulted in the loss of nearly all filamentous staining, and only aggregates of actin remained in the cytoplasm (Figures 4N and 4O).

Actin filament organization after 3 hr of recovery from treatment with LATB is shown in Figure 5. A representative tube treated with 30 nM LATB for 30 min is shown in Figure 5A. The actin organization was similar to that in the tubes shown in Figures 4L and 4M, which received the same treatment. The actin staining in pollen tubes that had resumed growth after the inhibitor had been washed out was similar to that in the controls, but the diameter of the recovered pollen tubes was frequently much smaller (Figure 5B). Many of the pollen tubes that did not resume growing had aberrant actin arrangements that were consistent with the streaming patterns observed after recovery. Some pollen tubes had bundles of actin filaments that extended to the extreme tip (Figure 5C), whereas others had cortical transverse "hoops" of actin at the tip (Figure 5D). These patterns were never observed in untreated pollen tubes.

LATB Reduces the Amount of F-Actin in Pollen

The effect of LATB treatment on F-actin levels was investigated by quantifying fluorescent-phalloidin binding sites and compared with the amount of total actin protein in similar populations of pollen. Actin in extracts of pollen before and after germination was measured by using an immunoassay with a polyclonal antiserum raised against maize pollen actin. Figure 6A shows that the antiserum reacts strongly with purified maize actin (lanes 1) and with a 42-kD protein from extracts of pollen prepared as described in Methods (lanes 2 and 3). There were also minor bands at 94 and 37 kD. Most likely, the smaller of the two polypeptides was a degradation product of actin. The preimmune serum had no reactive bands (data not shown). The efficiency of the pollen extraction procedure was estimated to be ~75 to 80%, as determined by densitometry of the protein blots prepared with equal volumes of the soluble supernatant and cell pellet solubilized in sample buffer (cf. lanes 2 and 3 in Figure 6A).

To quantify the amount of total actin protein, we coated the soluble extracts, as well as known amounts of purified maize pollen actin, onto the wells of a microtiter plate. Purified

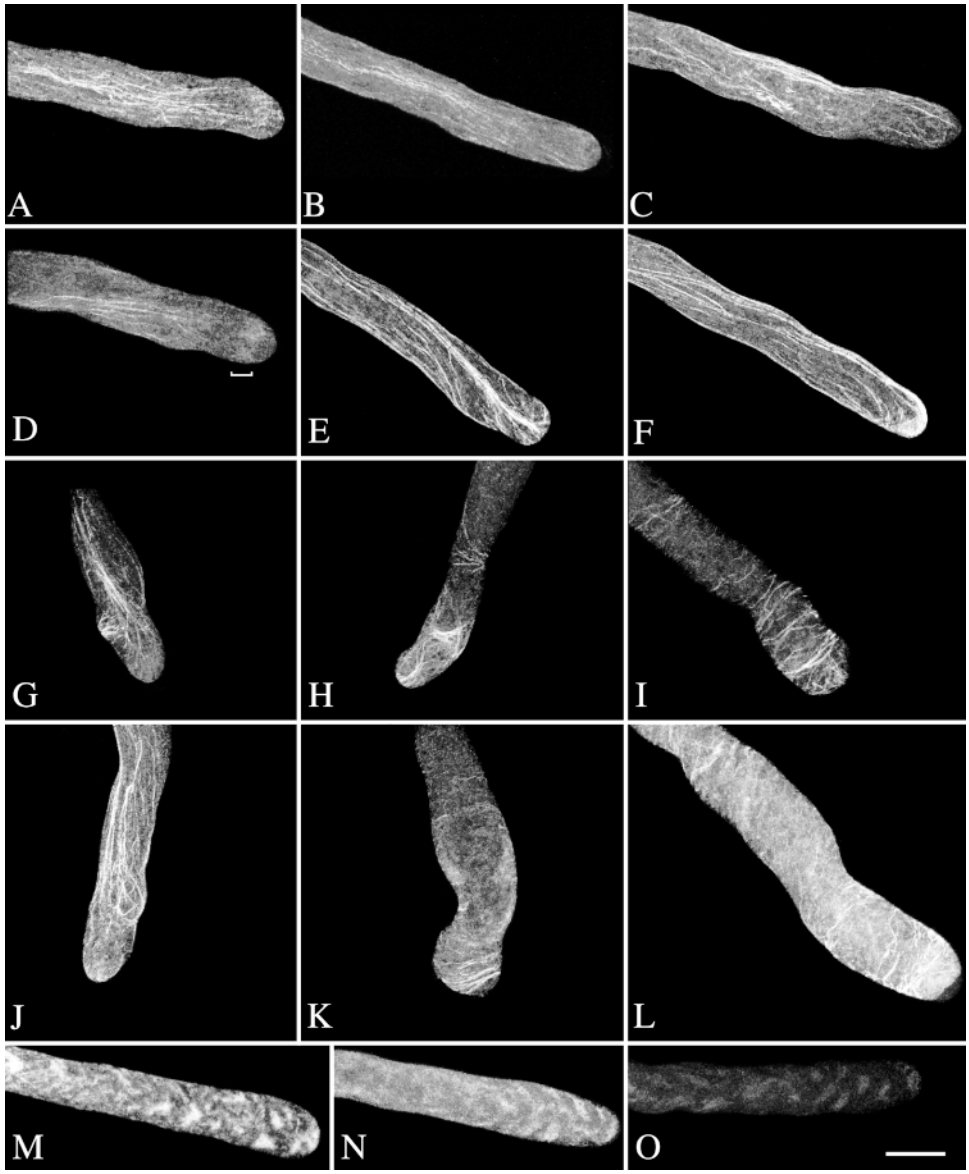

Figure 4. LATB Perturbs Actin Distribution in Pollen Tubes.

(A) and **(B)** Untreated pollen tubes that were grown for 30 min.
(C) and **(D)** Representative pollen tubes grown for 60 min. The bracket in **(D)** marks the region of brighter staining just behind the tip of the pollen tube.
(E) to **(G)** Pollen tubes treated with 10 nM LATB for 5 min. Many microfilaments remained, but their organization was disrupted. Long microfilaments remained in the central cytoplasm, but large bundles extended into the extreme tip. In some cells, the microfilaments were more severely disrupted and a few transversely oriented bundles were observed **(G)**. The collar of actin behind the tip was not observed in LATB-treated pollen tubes.
(H) and **(I)** Pollen tubes treated with 10 nM LATB for 15 min. The organization of microfilaments was more severely perturbed, and many filament bundles were observed oriented transverse to the growth axis. The brightest filaments were frequently located in the region where the collar of actin was observed in untreated tubes.
(J) and **(K)** Pollen tubes treated with 10 nM LATB for 30 min. A few pollen tubes continued growing slowly in LATB and probably correspond to those in which the organization of actin microfilaments appeared quite normal **(J)**. Most tubes, however, ceased growing and had severely disrupted microfilament organization **(K)**.
(L) and **(M)** Pollen tubes treated with 30 nM LATB for 30 min. Some tubes had clearly defined microfilament bundles that were often arranged transverse to the growth axis **(L)**. The majority of tubes had large aggregates of phalloidin-stained material in the cytoplasm with a few short microfilament bundles remaining in the cortex **(M)**.
(N) and **(O)** Pollen tubes treated with 1 μM LATB for 30 min. Tubes treated with high concentrations of LATB had some phalloidin-stained aggregates in the cytoplasm, but the intensity of the staining was generally much less than in untreated tubes or tubes treated with lower concentrations of the inhibitor.
All micrographs were obtained with similar settings on the confocal microscope. Bar in **(O)** = 15 μm for **(A)** to **(O)**.

pollen actin was used to generate a standard curve for determining the amount of actin in the extracts. Actin was detected by using anti-actin antiserum and an alkaline phosphatase–conjugated secondary antibody with *p*-nitrophenyl phosphate as the substrate. The immunoassay revealed that the total actin protein concentrations in pollen did not change over time, nor did they change when pollen was treated with 30 nM LATB (Figure 6B). Actin represented ~5% of total extractable protein, which corresponds to a cytosolic concentration of ~127 μM after correcting for the loss of actin in the cellular debris and using the assumptions for estimating cytoplasmic volume stated in Methods.

To measure F-actin in pollen, we fixed ~5-mg samples of grains or tubes with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), permeabilized the samples, and incubated them with saturating concentrations of Alexa 488–phalloidin. Bound phalloidin was extracted with methanol and analyzed by fluorometry. Figure 6C shows that the concentration of F-actin increased slightly after germination relative to ungerminated pollen. The quantity of extracted Alexa 488–phalloidin was determined by comparison with standard curves of known Alexa 488–phalloidin concentrations, and the concentration of F-actin in the samples was calculated as described in Methods. F-actin in ungerminated pollen was calculated to be 11.2 ± 2.6 μM ($n = 10$) and increased to 12.7 ± 3.4 μM ($n = 5$) and 13.1 ± 3.5 μM ($n = 10$) at 30 and 60 min, respectively. F-actin concentrations at 30 or 60 min after germination were not significantly different from those in ungerminated pollen (*t* test; $P > 0.05$).

Pollen that was treated with LATB showed substantial reductions in the quantity of bound fluorescent phalloidin, consistent with a reduction in F-actin content (Figure 6D). To verify that the difference in sensitivity to LATB observed between germination and growth was not due to differential stability of the actin cytoskeleton, we measured the F-actin in ungerminated pollen and pollen tubes that had been growing for 30 min. The amount of F-actin that depolymerized at LATB concentrations that inhibited tip growth (10 nM) was small. However, as Figure 6D clearly shows, the sensitivity of ungerminated grains or growing pollen tubes to LATB was not different.

The concentration of F-actin and the proportion of F-actin to total actin were low. Therefore, we measured the quantity of profilin, the major G-actin binding protein in pollen, by using an immunoassay with an antiserum raised against recombinant maize profilin 3 (Karakesisoglou et al., 1996). The results of this analysis are shown in Table 3. The amount of profilin in maize pollen remained constant during germination or treatment with LATB, as had been observed for actin protein. In contrast to actin, however, 90 to 95% of the profilin was found to be in the soluble protein extracts. Profilin represented ~2.5% of total soluble protein, which corresponds to a cellular concentration of ~125 μM. This concentration of profilin in maize pollen is consistent with the low concentration of F-actin observed in maize pollen (see Discussion).

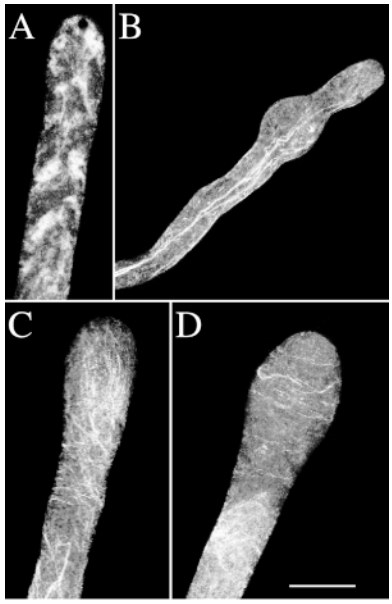

Figure 5. Pollen Tubes after Recovery from Treatment with 30 nM LATB.

(A) Pollen tube treated with 30 nM LATB for 30 min and then fixed. Brightly stained aggregates of actin were located throughout the cytoplasm, and some short bundles of microfilaments were in the cortex.
(B) Representative pollen tube that resumed growth after removal of LATB. Many of the tubes that resumed growth were narrower than the original pollen tubes and grew in a distorted fashion.
(C) and **(D)** Pollen tubes that did not resume growth after washing out LATB. Some of these tubes had bundles of microfilaments that extended to the extreme tip **(C)** or that were arranged in transverse cortical "hoops" **(D)**.
Bar in **(D)** = 15 μm for **(A)** to **(D)**.

LATB Binds Pollen Actin in Vitro

The biochemical basis for the potent effects of LATB was investigated with purified maize pollen actin (Ren et al., 1997). Depolymerization of F-actin in vitro was monitored by 90° light scattering. Adding LATB to solutions of pollen F-actin produced a rapid decrease in the total amount of polymer (Figure 7), the extent of depolymerization increasing with the concentrations of LATB. Furthermore, LATB was substantially more effective than an equivalent concentration of another G-actin binding factor, recombinant maize pollen profilin 1 (ZmPRO1). These data suggested that LATB is a better G-actin–sequestering factor than ZmPRO1, and this was verified by measuring the affinity of LATB for maize pollen actin in vitro.

Increasing concentrations of actin were polymerized in the absence or presence of 0.8 or 1 μM LATB. The K_d values for LATB binding to actin were determined by measuring the

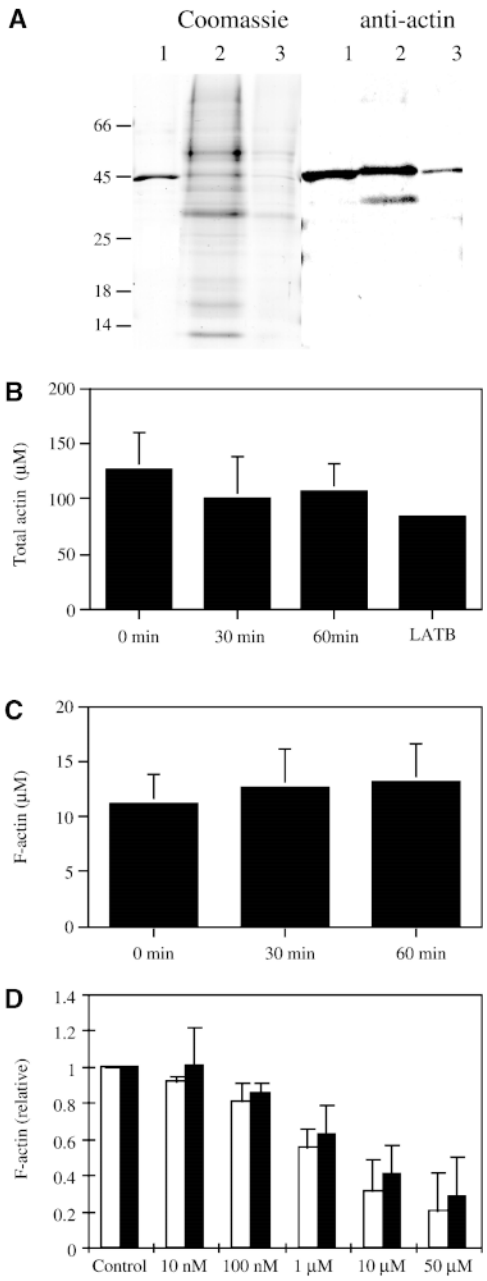

Figure 6. LATB Reduces F-Actin Levels in Pollen.

(A) Characterization of the polyclonal antiserum raised against maize pollen actin. Lanes 1 contain 1 μg of purified maize pollen actin; lanes 2, 10 μg of soluble proteins from maize pollen; lanes 3, an equal volume of cell pellet solubilized in protein sample buffer. Numbers at left indicate the position of molecular weight standards. Coomassie, Coomassie Brilliant Blue R.

(B) Total actin in pollen extracts was measured with an immunoassay. The amount of actin does not change over time or in the presence of LATB. Actin represents ~5% of total soluble protein. For each bar $n = 6$, except for the LATB-treated samples, for which $n = 4$. Error bars represent SD.

shift in the critical concentration (C_c) for actin polymerization at steady state equilibrium. The C_c is the actin concentration at which filaments begin to form, and it can be estimated from plots of actin concentration versus light scattering. Figure 8 shows the data from single representative experiments for pollen actin (Figure 8A) and rabbit skeletal muscle α-actin (Figure 8B). The intercept of the regression line on the ordinate is the C_c, and the shift of the intercept in the presence of LATB can be used to calculate the K_d of the interaction, as described in Methods. Using the data for the representative experiment shown in Figure 8, we calculated K_d values of 55 nM for LATB binding to pollen actin (Figure 8A) and 23 nM for LATB binding to muscle actin (Figure 8B). The average K_d (±SD) for LATB binding to pollen actin was 74 ± 60 nM ($n = 12$) and for muscle actin was 35 ± 31 nM ($n = 3$). These values are 25-fold less than the apparent K_d of 1.1 μM for ZmPRO1 binding to maize pollen actin measured previously (Gibbon et al., 1998).

DISCUSSION

The data presented here demonstrate that LATB bound with high affinity to pollen G-actin and had differential effects on pollen germination and pollen tube growth. The shift in C_c values for actin assembly was used to calculate a K_d of 74 nM for LATB binding to purified maize pollen G-actin in vitro. The concentration of LATB required for half-maximal inhibition of pollen germination was 40 to 50 nM, whereas pollen tube extension was much more sensitive, requiring only 5 to 7 nM LATB for half-maximal inhibition. To understand the effects of LATB on actin in pollen, we used a fluorometric assay to quantify F-actin and an immunoassay to quantify total actin protein. The amounts of total actin protein did not change after germination or when pollen was treated with LATB. In contrast, treatment with LATB caused a dose-dependent decrease of F-actin in pollen. Furthermore, ungerminated pollen grains and growing pollen tubes were

(C) F-actin was quantified by fixing pollen and incubating it with a saturating concentration of Alexa 488–phalloidin. Each bar represents the mean of five to 10 measurements from five experiments, and the error bars represent SD.

(D) The effect of LATB on the F-actin concentration was measured for ungerminated pollen (white bars) and for pollen germinated 30 min before application of the inhibitor (black bars). The pollen was exposed to LATB 30 min before fixation. The results were normalized relative to untreated controls that correspond to the time at which LATB was added to allow for direct comparison. The initial F-actin concentration in the ungerminated grains was 12.1 ± 1.3, compared with 13.9 ± 2.3 μM in the grains that had germinated for 30 min. The means are four measurements for each bar from three independent experiments. Error bars represent SD.

Table 3. Actin and Profilin Protein Levels in Maize Pollen

Treatment	Actin Protein (ng Actin/μg Total)	Cytosolic Concentration (μM)[a,b]	Profilin Protein (ng Profilin/μg Total)	Cytosolic Concentration (μM)[b]
Ungerminated	51.7 ± 13.2 (4)[c]	127 ± 32 (4)	24.1 ± 6.1 (4)	126 ± 32 (4)
Germinated (30 min)	50.3 ± 18.5 (4)	101 ± 37 (4)	28.4 ± 5.4 (4)	114 ± 22 (4)
Germinated (60 min)	51.2 ± 12.0 (4)	107 ± 25 (4)	26.1 ± 6.1 (4)	107 ± 25 (4)
LATB treated (60 min)	44.0 ± 0.4 (3)	84 ± 1 (3)	27.9 ± 9.0 (3)	110 ± 35 (3)

[a] Data taken from Figure 6B.

[b] Cytosolic concentration calculated as outlined in Methods.

[c] The quantity of actin and profilin protein in soluble extracts of maize pollen was measured by an immunoassay using actin or profilin purified from maize pollen as a standard. All values reported as mean ±SD (*n*).

equally sensitive to the inhibitor. These data are consistent with high-affinity binding of LATB to maize pollen G-actin, thereby causing partial depolymerization of F-actin and inhibition of tip growth. These findings suggest that pollen germination and tip growth have different requirements for F-actin, with tip growth being highly sensitive to perturbation of the F-actin pool.

Cytochalasins have been used to show that pollen germination and tube growth depend on the actin cytoskeleton (Franke et al., 1972; Herth et al., 1972; Mascarenhas and LaFountain, 1972; Speranza and Calzoni, 1989; Geitmann et al., 1996), but the concentrations used in these studies are ~10- to 50-fold higher than the concentrations of LATB that are sufficient to inhibit growth and germination. Despite the fact that cytochalasins are applied at high concentrations, it is not apparent that such treatments result in the depolymerization of actin filaments. Several studies have shown that microfilaments remain, and can even be reorganized, in the presence of cytochalasins (Tang et al., 1989; Tiwari and Polito, 1990; Collings et al., 1995). These effects could be caused by the capping of existing filaments or the nucleation of new filaments by CD. Together, these studies suggest that CD inhibits actin dynamics rather than depolymerizing actin filaments.

The most surprising finding was that pollen tube growth was 10-fold more sensitive to LATB than was germination. Treatment with only 5 nM LATB caused 50% inhibition of growth; this is similar to the concentration of LATB that causes 50% inhibition of photopolarization in Pelvetia embryos (Hable and Kropf, 1998) and progression through microsporogenesis in tobacco (Zonia et al., 1999). In contrast, Arabidopsis root hair elongation requires >50 nM LATB for 50% inhibition (Bibikova et al., 1999), possibly indicating differential sensitivity to LATB among organisms. Different effects were observed for CD, to which germination and pollen tube extension of maize pollen were equally sensitive. The effect of CD is consistent with a previous report showing that tobacco pollen germination has a half-maximal sensitivity at 200 nM CD (Geitmann et al., 1996), a value similar to the 400 nM CD that we determined for maize pollen. These data indicated that pollen tube extension was more sensitive to changes in F-actin or to the ratio of F-actin to G-actin. To better understand the effect of LATB on pollen, we measured the concentrations of total actin protein and F-actin in pollen treated with the inhibitor.

One possible explanation for the difference in sensitivity to LATB is that the amount of total actin protein in pollen tubes is less than in ungerminated pollen grains. However, the amount of total actin protein, as measured by an immunoassay, did not change after germination or during treatment with LATB. The actin protein concentration of 127 μM

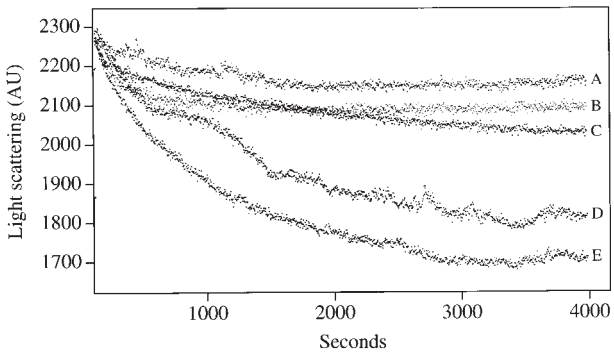

Figure 7. LATB Causes Pollen F-Actin Depolymerization in Vitro.

In a representative experiment, pollen F-actin (3 μM) was mixed with (A) buffer, (B) 6 μM ZmPRO1, (C) 1.5 μM LATB, (D) 3 μM LATB, or (E) 6 μM LATB. Recombinant ZmPRO1 was substantially less effective than equivalent amounts of LATB at depolymerizing F-actin but had a much faster initial rate of depolymerization (B versus E). LATB had pronounced effects on polymer concentrations even at a ratio of 1:2 LATB:actin (C), and the effect on depolymerization was dose dependent. AU, arbitrary units.

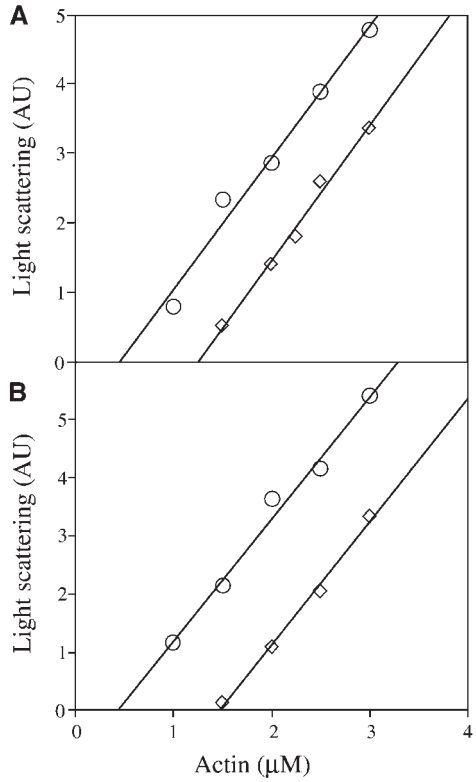

Figure 8. LATB Binds with High Affinity to G-Actin.

(A) A representative experiment with maize pollen actin to determine the K_d for LATB binding to pollen G-actin. The scattered light increased linearly for increasing concentrations of actin alone (circles). In the presence of 0.8 μM LATB, the scattering observed at each actin concentration was reduced (diamonds). The C_c for pollen actin alone was 0.46 μM, whereas in the presence of LATB the apparent C_c was 1.25 μM. From these values, a K_d of 122 nM was determined. The average K_d (±SD) from 12 similar experiments was 74 ± 60 nM.

(B) A representative experiment with muscle actin. The C_c for actin alone (circles) was 0.43 μM, and the apparent C_c in the presence of LATB (diamonds) was 1.47 μM. The K_d calculated from these values was 25 nM, and the average K_d (±SD) from three similar experiments was 35 ± 31 nM.

AU, arbitrary unit.

in ungerminated maize pollen is four- to fivefold higher than has been reported for ungerminated lily pollen (Vidali and Hepler, 1997). The proportion of actin in extracts from maize pollen was consistent with prior determinations that used inhibition of DNase I as a measure (Andersland et al., 1992).

Because the amount of total actin protein did not change, we considered an alternative model in which F-actin levels are reduced upon the transition from germination to tip growth. F-actin levels, measured by quantifying phalloidin binding sites, revealed a slight but not statistically significant increase when pollen germinated. The concentration of F-actin in pollen was estimated to be ~11 to 13 μM, or ~10% of the total actin pool. In nonplant systems, F-actin typically accounts for 20 to 50% of the total actin pool (Blikstad et al., 1978; Haugwitz et al., 1994). The concentration of the major G-actin binding protein profilin in maize pollen and its affinity for G-actin are consistent with the size of the G-actin pool, as calculated from our determinations of total actin and F-actin concentrations in pollen. The concentration of profilin is roughly equimolar to the total actin in maize pollen, 125 μM, and the apparent K_d of native pollen profilin binding to G-actin is 0.9 μM (D.R. Kovar, B.K. Drybak, and C.J. Staiger, manuscript submitted). From these values, the concentration of the profilin–actin complex in ungerminated pollen is calculated to be 115 μM, the G-actin concentration 0.5 μM (the C_c), and the F-actin concentration 9.5 μM. This predicted value is very close to the measured concentration of F-actin.

Treatment of pollen with LATB resulted in a dose-dependent decrease in F-actin. At the lowest concentration tested (10 nM), there was only a modest decrease in the F-actin concentration. Given that the K_d of LATB for pollen actin is 74 nM, one can estimate the quantity of G-actin bound when pollen is incubated with the inhibitor. The equilibrium situation can be described by Equation 1:

$$K_d = \frac{[\text{LATB}] \times [\text{actin}]}{[\text{LATB:actin}]} \quad (1)$$

Assuming that the volume of medium containing LATB is large relative to the cytoplasmic volume of the pollen, the molar quantity of LATB bound to actin in the cell is small relative to the total molar quantity of LATB in the medium, and [LATB] does not change significantly. If LATB is freely permeable across the membrane, then $[\text{LATB}] = [\text{LATB}]_{\text{internal}}$ and the change in $[\text{actin}] = [\text{actin}]_{\text{total}} - [\text{LATB:actin}]$, where [LATB:actin] is the concentration of LATB bound to actin in the pollen grain. Substitution of these terms into Equation 1 and solving for [LATB:actin] yields Equation 2:

$$[\text{LATB:actin}] = \frac{[\text{LATB}]_{\text{internal}} \times [\text{actin}]_{\text{total}}}{[\text{LATB}]_{\text{internal}} + K_d} \quad (2)$$

If we assume that actin is in equilibrium with actin binding proteins, such as profilin or actin depolymerizing factor, and that the effect of LATB is additive, then at the new equilibrium, the G-actin that is bound must come from the F-actin pool. Therefore, the actin available to interact with the inhibitor (i.e., $[\text{actin}]_{\text{total}}$) in ungerminated pollen would be ~12 μM, and only 0.8 μM actin would be depolymerized in the presence of 5 nM LATB. This is an estimate of the maximum amount of depolymerization because at the lowest concentrations of inhibitor our assumption that [LATB] does not change may not be true, which would result in less destruction of actin. This analysis shows that at low concentrations a small shift (<10%) in the F-actin concentration would be expected, and this was consistent with our observations. Alternatively, the small amount of depolymerization we observed could result from the binding of LATB to the large

pool of profilin–actin. Importantly, however, when we tested the sensitivity of F-actin in ungerminated pollen grains and growing pollen tubes to LATB-induced depolymerization, no differences were observed. These data clearly show that slight depolymerization of F-actin occurs under conditions that stop growth and that F-actin is equally sensitive to depolymerization by LATB before and after germination.

The differences in sensitivity to LATB, but not to CD, suggest that germination and pollen tube extension proceed by distinct mechanisms. An analogous situation is observed in root hairs; hair initiation occurs by a mechanism that is independent of calcium, whereas root hair growth occurs by a calcium-dependent tip growth process (Wymer et al., 1997). Moreover, the initiation of root hairs can be genetically uncoupled from tip growth (Schiefelbein and Somerville, 1990). The sensitivity of pollen tube extension to LATB suggests that a specific F-actin concentration, or a specific ratio of F-actin to G-actin, is critical for maintaining tip growth, whereas germination does not show such a strict dependence on F-actin. From the data presented here, however, we cannot discriminate between the requirement for a specific amount of F-actin or whether the ratio of F-actin to G-actin is important.

The function of actin in pollen tube extension is widely believed to be solely the delivery of secretory vesicles to the tip (reviewed in Taylor and Hepler, 1997; Kost et al., 1998). However, this study and recent work by Miller et al. (1999) indicate that this model may be too simple. The observation that pollen tubes recovering from LATB treatment could resume vigorous streaming but not tip growth suggests that a LATB-sensitive population of F-actin is required for extension of the tip. Similarly, treatment of root hairs with low concentrations of CD stopped extension of the tip, caused the disappearance of fine actin bundles behind the tip, and allowed large bundles of actin to extend into the tip (Miller et al., 1999). Together, these studies indicate the existence of a highly dynamic population of actin filaments that is sensitive to depolymerization or stabilization by actin inhibitors such as LATB or CD. The possibility of a separate population of actin that is more sensitive to inhibitors must be confirmed by visualizing these filaments directly. However, dynamic polymerization of new actin filaments is apparently not sufficient for extension of the pollen tube because abundant microfilaments appear after LATB is removed from the medium. Therefore, we think it likely that actin is required for functions that are independent of vesicle transport to the tip.

In conclusion, we found differential sensitivity of pollen germination and tube growth to treatment with LATB, the pollen tube growth being extremely sensitive to perturbation by the inhibitor. The effect of LATB was caused by depolymerization of F-actin, as shown by fluorescence microscopy and quantitative fluorescent-phalloidin binding. The strict dependence of pollen tube growth on F-actin was not observed in prior studies that used cytochalasins. These results demonstrate the value of careful evaluation of the physiological and biochemical activities of inhibitors to elucidate the role of the actin cytoskeleton in plant cells. Furthermore, direct measurements of actin dynamics are warranted to facilitate understanding of the potential differences in cytoskeletal behavior at key developmental stages.

METHODS

Reagents

Latrunculin B (LATB; Calbiochem, San Diego, CA) and cytochalasin D (CD; Sigma, St. Louis, MO) were dissolved at final concentrations of 2 and 5 mg/mL, respectively, in anhydrous DMSO (Aldrich, Milwaukee, WI) or absolute ethanol (McCormick Distilling Co., Weston, MO). All other reagents were obtained from Sigma unless otherwise noted. Gel-filtered rabbit skeletal muscle actin was obtained from Cytoskeleton Inc. (Denver, CO). Maize pollen actin was purified according to Ren et al. (1997), with minor modifications. Recombinant human profilin was added during grinding, and the concentration of ATP in the extraction buffer was increased to 0.8 mM, as described by Staiger et al. (1999). Recombinant maize profilin 1 (ZmPRO1) was purified by affinity chromatography on poly-L-proline–Sepharose, as described previously (Karakesisoglou et al., 1996; Gibbon et al., 1997).

Inhibition of Pollen Germination and Tube Growth

Pollen was harvested from greenhouse-grown maize plants (*Zea mays*; inbred lines A188 or OH43) or from newly opened flowers of *Tradescantia virginiana.* Freshly shed pollen (2 to 5 mg) was sown onto 35-mm diameter plastic dishes containing 1 mL of maize germination medium (GM; modified from Lin and Dickinson, 1984; 550 mM sucrose, 1.27 mM $Ca[NO_3]_2$, 1 mM KNO_3, 200 μM H_3BO_3) or Tradescantia GM (O'Driscoll et al., 1993; 10% sucrose, 10 mM $CaCl_2$, 1.6 mM H_3BO_3, 5 mM KH_2PO_4/K_2HPO_4, pH 5.8) and allowed to germinate at room temperature. Inhibition of germination was investigated by including LATB or CD in the GM. The maximum concentration of DMSO or ethanol was 60 ppm, and equivalent concentrations of these solvents were used for all controls. After 1 hr of growth, any protrusion from the germination aperture was scored as positive for germination. At least 200 pollen grains were counted in each dish, and duplicate dishes were used in each experiment. No differences in germination frequency were observed between 1-hr or 30-min treatments with LATB. In experiments investigating the inhibition of tube growth, photographs of a field of pollen germinated for 20 min were taken. LATB or CD was added and mixed with gentle agitation, and the pollen was incubated for an additional 30 min. A photograph of another field of cells was taken, and the length of the treatment period was recorded. The average length of 20 to 50 pollen tubes was measured before and after addition of the inhibitor from digitized negatives by using the National Institutes of Health Image software (version 1.61; Bethesda, MD). The growth rate was calculated as the difference in average lengths divided by the time of exposure to inhibitor.

The recovery of pollen tubes from LATB treatment was monitored after extensive washing. Pollen was germinated for 20 min before LATB treatment, and the tubes were exposed to the inhibitor for 30 min. After treatment with LATB, the inhibitor was washed out with five changes (1 mL each) of fresh GM over 30 min. The recovery of streaming or pollen tube growth, measured 1.5 and 3 hr after the washes were begun, was classified into five groups: growing,

streaming along the growth axis, streaming in the cortex transverse to the growth axis, a mixture of these two streaming patterns, and no recovery of growth or streaming. For each treatment, 30 to 100 cells were counted in duplicate dishes.

Phalloidin Staining

Ungerminated and newly germinated pollen grains were permeabilized in microtubule stabilizing buffer (MTSB; 50 mM Pipes, 5 mM EGTA, 5 mM $MgSO_4$, pH 6.9 [Goodbody and Lloyd, 1994]) with 0.05% (v/v) Nonidet P-40, 500 mM mannitol, and 50 to 70 nM rhodamine–phalloidin or Alexa 488–phalloidin (Molecular Probes, Eugene, OR). Pollen was mounted and images were captured within 1 hr of exposure to MTSB. The staining pattern of permeabilized cells was identical to that of cells that were fixed for 30 min with 300 μM 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS). Germinated pollen could not be treated with MTSB because the pollen tube ruptured immediately upon contact with MTSB. Pollen was germinated for 30 min and fixed by addition of 1 volume of GM plus 1 M sucrose, 0.1% Nonidet P-40, 600 μM MBS, and 0.5% glutaraldehyde (Grade I; Sigma). LATB was added at various concentrations and the mixture was incubated 5 to 30 min before fixation. The pollen was fixed for 30 min, rinsed twice in GM plus 0.05% Nonidet P-40, and gradually exchanged into TBS-Tween (50 mM Tris, 200 mM NaCl, 0.05% Tween-20, pH 7.4) plus 300 mM sucrose and 5 mM DTT to block any remaining free reactive sites from the MBS. Cells were stained with 1 μM Alexa 488–phalloidin for 1 hr, washed once with TBS-Tween plus 300 mM sucrose, and mounted in the same medium. Fluorescence micrographs were obtained on a laser scanning confocal microscope (Model MRC 1024; BioRad, Hercules, CA) with a ×60 1.4 N.A. PlanApo lens (Nikon, Melville, NY). The fluorophore was excited with the 488- or 568-nm line of a Kr/Ar laser as appropriate, and three to four Kalman-filtered scans were averaged for each optical section.

Generation of Anti-Actin Polyclonal Antiserum

A polyclonal antiserum to purified maize pollen actin was produced in a New Zealand white rabbit according to standard procedures. Briefly, 100 μg of purified maize pollen actin was suspended in Freund's complete adjuvant and injected into the animal. Additional boosts of 50 μg suspended in Freund's incomplete adjuvant were injectedinto the animal at weekly intervals. The preimmune serum and serum collected after injection of the antigen were screened against purified maize pollen actin and total protein extracts of maize pollen. SDS-PAGE and immunoblotting were performed with the mini-Protean II system (BioRad), according to the manufacturer's instructions. The preimmune serum applied at 1:1000 dilution displayed no reactivity to purified pollen actin or total pollen proteins. High-titer antiserum was observed after the third boost, and the animal was bled at weekly intervals three times.

Quantification of Actin and Profilin in Pollen

Total actin and profilin in soluble pollen extracts was measured with an enzyme-linked immunosorbent assay (Kemeny, 1997). Extracts were prepared by sonication in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 9.6). Debris was removed by centrifugation at 10,000*g*, and the supernatants were frozen and stored at −80°C. Densitometry of protein blots containing equal volumes of the extract and cellular debris solubilized in sample buffer indicated that ~80% of the actin and 90 to 95% of the profilin were extracted by this procedure.

The extracts were diluted to 2 μg/mL in coating buffer, and 50 ng was applied to duplicate wells of a microtiter plate. Purified maize pollen actin or profilin was used as a standard and was applied at 0.2 to 10 ng per well. The proteins were allowed to bind overnight at 4°C, washed once with PBS plus Tween-20 (PBST; 0.05 M phosphate, 145 mM NaCl, 0.05% [v/v] Tween-20, pH 7.4), and blocked for 1 hr with 2.5% (w/v) BSA in PBS at 37°C. The blocking solution was removed, and the wells were washed with one change of PBST. The wells were incubated for 2 hr at room temperature with a 1:1000 dilution of the appropriate polyclonal antibody. The wells were washed three times with PBST and incubated with a 1:2000 dilution of alkaline phosphatase–conjugated anti–rabbit IgG (Sigma) for 1 hr at 4°C. The wells were washed three times with PBST, and the substrate *p*-nitrophenyl phosphate was added at 1 mg/mL in diethanolamine buffer (50 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). The reaction was stopped after 20 min with 3 M NaOH, and the optical density at 405 nm was measured with a microplate reader (Molecular Devices, Menlo Park, CA).

No color development above background was observed in wells containing pollen extracts exposed to secondary antibody alone. A standard curve was plotted with the known samples, and the amount of actin in the crude extracts was estimated by extrapolating the measured optical density to the standard curve. The total intracellular actin concentration was calculated from these measurements, as described for F-actin.

F-actin was measured indirectly by measuring phalloidin binding sites in fixed pollen (Howard and Oresajo, 1985; Southwick et al., 1989; Yeh and Haarer, 1996). A measured amount of maize pollen (~5 mg) was added to 1 mL of GM and incubated in the presence of various concentrations of LATB. The pollen was fixed and washed as described above for fluorescence microscopy, except that glutaraldehyde was omitted because it was found that the phalloidin binding capacity of the pollen decreased ~10-fold when aldehydes were included. Pollen was transferred to microcentrifuge tubes, and 50 μL of 4 μM Alexa 488–phalloidin (Molecular Probes) in GM plus 0.05% Nonidet P-40 was added. We estimated that this would result in approximately twofold molar excess of phalloidin to F-actin. The cells were incubated for 1 hr and washed with three changes of 500 μL of GM plus 0.05% Nonidet P-40. The final wash was removed, and the fluorescent phalloidin was extracted overnight at 4°C in 1 mL of methanol. The fluorescence from 200 μL of the extracted material, diluted in a final volume of 2 mL, was read on a spectrofluorometer (model SLM 8000; SLM Instruments, Urbana, IL) by exciting at 494 nm and monitoring emission at 517 nm. The quantity of phalloidin extracted was determined by comparison with titrations of Alexa 488–phalloidin at known concentrations into methanol. The intracellular concentration of F-actin was calculated by using the following assumptions: phalloidin binds mol:mol with F-actin at saturation, 1 g of pollen occupies 1.2 mL, the cytoplasmic volume is 50% of the total pollen volume, and for germinated pollen the cytoplasmic volume remains constant over time.

Actin Binding

The effect of LATB on the concentrations of actin polymer in vitro was monitored by 90° scattering of 450-nm light with a spectrofluo-

rometer (Cooper and Pollard, 1982). CD, LATB, and ZmPRO1 were examined for the ability to cause depolymerization of assembled actin filaments. A solution of 3 μM pollen actin was polymerized overnight in LSF buffer (5 mM Hepes, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM ATP, pH 7.0). A baseline scattering value was obtained, and depolymerizing agents or an equivalent volume of buffer alone was added to the cuvette and stirred for several seconds. Recording was resumed after 2 min, to allow small air bubbles to clear from the light path, and continued for ~1 hr. DMSO at a concentration equivalent to the highest concentration used for LATB had the same effect as addition of buffer alone. The maximum dilution of the reaction cuvette contents was ≤2%.

Equilibrium dissociation constant (K_d) values for LATB binding to maize pollen and muscle actin were calculated by measuring the shift in the critical concentration (C_c) for actin assembly in the presence of LATB. The C_c is the lowest concentration at which a solution of actin forms filaments. Similar measurements were performed previously to determine K_d values for profilin binding to plant actin (Gibbon et al., 1998). The concentration of two lots of LATB was verified by measuring UV absorbance of the compound diluted in methanol at a wavelength of 212 nm and using an extinction coefficient of 17,250 M^{-1} cm^{-1} (Groweiss et al., 1983). The UV absorbance of these lots was found to be ~20% lower than expected, and the concentration of LATB in all biochemical analyses was based on that obtained by direct measurement by UV absorbance. A constant concentration of LATB (0.8 or 1.0 μM) was added to samples of actin (in LSF buffer minus KCl and $MgCl_2$) ranging in concentration from 1 to 3 μM. The light scattering of G-actin alone and G-actin plus LATB was measured, and polymerization was initiated by adding KCl and $MgCl_2$ to final concentrations of 100 and 5 mM, respectively. Steady state light scattering was measured after polymerization had proceeded overnight. The difference between the initial and the final light scattering values was plotted versus actin concentration. The C_c is the intercept of the regression line on the ordinate. The C_c for actin alone and the apparent C_c for actin in the presence of LATB were calculated. Assuming that the C_c for actin assembly did not change, we calculated the K_d according to Equation 3:

$$K_d = \frac{[\text{LATB}]_{\text{free}} \times C_c}{[\text{LATB:actin}]} \quad (3)$$

where [LATB:actin] is equal to the difference in C_c for actin alone and the apparent C_c of actin in the presence of LATB, and $[\text{LATB}]_{\text{free}}$ is the total concentration of LATB minus [LATB:actin].

ACKNOWLEDGMENTS

We thank Haiyun Ren and Sharon Ashworth for help harvesting pollen, Dave Kniola for care of plants in the greenhouse, and our colleagues in the laboratories of David Asai, Joann Otto, and Peter Hollenbeck for helpful comments. The confocal microscope was partially supported by a grant from the National Science Foundation (Grant No. BIR 9512962), and additional microscopy facilities were supported by a grant from the Showalter Fund to C.J.S. This work was supported by Grant No. 97-35304-4876 from the U.S. Department of Agriculture National Research Initiative Grants Program to C.J.S. and a National Science Foundation Plant Genetics Traineeship (Grant No. 9355012 GEF) to B.C.G. This study was performed by B.C.G. in partial fulfillment of the Ph.D. requirements of the Purdue Genetics Program.

Received June 28, 1999; accepted September 25, 1999.

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