Myosin Is Involved in Postmitotic Cell Spreading

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Abstract. We have investigated a role for myosin in postmitotic Potoroo tridactylis kidney (PtK2) cell spreading by inhibitor studies, time-lapse video microscopy, and immunofluorescence. We have also determined the spatial organization and polarity of actin filaments in postmitotic spreading cells. We show that butanedione monoxime (BDM), a known inhibitor of muscle myosin II, inhibits nonmuscle myosin II and myosin V adenosine triphosphatases. BDM reversibly inhibits PtK2 postmitotic cell spreading. *Listeria* motility is not affected by this drug. Electron microscopy studies show that some actin filaments in spreading edges are part of actin bundles that are also found in long, thin, structures that are connected to spreading edges and substrate (retraction fibers), and that 90% of this actin is oriented with barbed ends in the direction of spreading. The remaining actin in spreading edges has a more random orientation and spatial arrangement. Myosin II is associated with actin polymer in spreading cell edges, but not retraction fibers. Myosin II is excluded from lamellipodia that protrude from the cell edge at the end of spreading. We suggest that spreading involves myosin, possibly myosin II.

Forward movement of cell edges is necessary for locomotion of a cell across a substrate and for many types of cell shape change. Actin filaments play a key role in both cell locomotion and morphogenesis. The behavior of actin filaments has been studied in motile cell types for a number of years, particularly in a specialized type of cell edge called the lamellipodium (the thin band of actin-rich, protrusive cytoplasm found at the edge of many motile cells). It is thought that lamellipodia protrusion is driven at least in part by forces deriving from actin polymerization (Abercrombie, 1980; Cooper, 1991; Singer and Kupfer, 1986; Theriot and Mitchison, 1991). Protrusive force in lamellipodia may be related to the force that propels the bacterium *Listeria monocytogenes* forward in infected cells (Sanger et al., 1992; Theriot and Mitchison, 1992). Recently, however, it has been shown that forward movement of other types of cell edges is not driven by actin polymerization, but by other actin-based forces (Bement et al., 1993; Martin and Lewis, 1992; Young et al., 1993; for review see Cramer et al., 1994).

We have studied actin filament dynamics in a type of cell motility called postmitotic cell spreading. During postmitotic cell spreading, cell edges move outward over long, thin, actin-rich fibers (retraction fibers, described in Mitchison [1992]), which are connected to the spreading edge and the substrate (Cramer and Mitchison, 1993). Our observations of the behavior of actin filaments in this system could be best explained by the activity of an actin-dependent motor. In this study we use inhibition and localization experiments to investigate this possibility. We suggest that at least one myosin is likely to play a role in postmitotic cell spreading. This type of myosin activity may generally contribute to forces that drive cell locomotion.

Materials and Methods

Handling Butanedione Monoxime

We store butanedione monoxime (BDM)[1] powder (Sigma Chemical Co., St. Louis, MO) at room temperature in the dark and typically make a fresh 0.5-M stock in water on the day of the experiment, which we keep at room temperature for half a day (it needs vigorous vortexing), but we have occasionally used a −20°C 5-M DMSO stock, both stored in the dark. We standardly dilute BDM to 10 mM from the stock concentration immediately before an experiment. We have found that BDM is inactivated by imidazole in aqueous solution. It is stable at 37°C in tissue culture media for at least 60 min.

Purification of Nonmuscle Myosin II and In Vitro BDM Studies

Human platelet nonmuscle myosin II was purified as described (Daniel and Sellers, 1992) except we used a sephacryl S-400 HR or S-500 HR (Sigma Chemical Co.) gel filtration column. ATPase activities of individual, active, purified motor proteins were measured enzymatically in a spectrophotometer (Catterall and Pederson, 1971) in a final assay volume of 80–100 μl. 0.1–1 mg/ml platelet myosin II in 0.5 M NaCl or KCl, 10 mM MOPS, 20 mM Hepes, pH 7.5, 5 mM MgCl$_2$, 0.1 mM EGTA, 1 mM DTT, 3 mM NaN$_3$, was added to reaction mix (1 mM ATP, 0.5 mM phosphoenol pyruvate, 0.25 mM NADH, 0.1% lactate dehydrogenase, and 0.2% pyru-

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1. *Abbreviations used in this paper*: BDM, butanedione monoxime; PtK2, Potoroo tridactylis kidney.

vate kinase; all from Sigma Chemical Co.) with 0.3–2.5 mg/ml skeletal muscle actin polymer stabilized with phalloidin (2.5-fold the concentration of myosin II). Active (5.1 ATP/second/dimer after dialysis) chick brain myosin V (gift from Richard Cheney, Yale University Medical School, New Haven, CT), purified as described (Cheney et al., 1993) was further dialyzed in 10 mM Hepes, pH 7.4, 100 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, and 0.1 mM EGTA and immediately assayed at 1–10 μg/ml in 75 mM KCl, 20 mM Hepes, pH 7.4, 2.5 mM MgCl₂, 1 mM DTT, 1 mM CaCl₂ and reaction mix with 0.5 mg/ml phalloidin–actin. A *Drosophila* protein fraction (0.1 mg/ml) with ATPase activity (2.6 μmol/minute/milligram) enriched in cytoplasmic myosin and myosin VI and also containing other unidentified proteins (proteins eluted with nucleotide from an actin affinity column as described in Miller et al. [1991], gift from Chris Field, University of California, San Francisco, CA) was assayed in 50 mM Hepes, pH 7.5, 50 mM KCl, 2 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM MgCl, and reaction mix with 0.3 mg/ml phalloidin–actin. Active kinesin ATPase activity (9.8 ATP/second/dimer) was measured at 7 μg/ml (human partial length recombinant kinesin heavy chain comprising the first 560 amino acids; Romberg, L., personal communication) in 80 mM Pipes, 20 mM Hepes, pH 7, 5 mM MgCl₂, 1 mM EGTA, 25 mM KCl with reaction mix and taxol-stabilized 5-mg/ml microtubules (gift from Lisa Belmont, University of California, San Francisco, CA). Control ATPase activities were monitored for 1–5 min at room temperature and compared with that of motor proteins that had been preincubated at room temperature with a final concentration of 1–20 mM BDM for 1–5 min.

Purified skeletal muscle actin monomer was polymerized in F-buffer (50 mM Tris, pH 8, 50 mM KCl, 4 mM MgCl₂, 1 mM ATP) overnight at 4°C in the presence of 0–20 mM BDM. The polymer was pelleted and the protein concentration measured and compared with total protein.

Cell Culture

Potoroo tridactylis kidney (PtK2) cells, an epithelial cell line, were grown, plated onto glass coverslips, and transferred into a chamber for time-lapse videomicroscopy as described (Cramer and Mitchison, 1993) except that live cells were observed at 34–36°C. PtK2 cells were infected with *L. monocytogenes* strain 10403S as described (Dabiri et al., 1990) and grown and observed as for uninfected cells except that streptomycin and penicillin were omitted from the media. Both fixed and live postmitotic cells are very easily identified. They have a characteristic shape unique to postmitotic cells that is clearly distinct from all other stages of the cell cycle (described in detail in Cramer and Mitchison [1993]).

Fluorescent Staining of Actin Filaments and Myosin

To be able to observe actin filaments and myosin in spreading cells in double label experiments, we developed a method that not only preserved the delicate structure of these cells, but was also optimal for observing both proteins with phalloidin and the antimyosin antibody, respectively. PtK2 cells on glass coverslips were extracted in cytoskeleton buffer (Small, 1981; Symons and Mitchison, 1991) with 0.32 M sucrose, 0.1% Triton X-100, and 1 μg/ml phalloidin (Sigma Chemical Co.) for 45 s. They were then fixed in 4% formaldehyde in cytoskeleton buffer with 0.32 M sucrose for 20 min, rinsed in TBS (0.15 M NaCl, 0.02 M Tris-Cl, pH 7.4), and permeabilized with TBS-0.5% Triton X-100 for 10 min. This method minimizes loss of free myosin by only briefly extracting (seconds) and improves the myosin staining with the antibody in formaldehyde fixed cells (methanol is usually used for this antibody, but methanol partially destroys postmitotic spreading cells, and phalloidin does not bind denatured actin filaments). The inclusion of sucrose and phalloidin in the extraction buffer and fixing in formaldehyde preserves actin structure, and phalloidin also prevents loss of any actin filaments that may have been able to bind myosin. Indirect immunofluorescence was performed using affinity-purified rabbit anti–human platelet nonmuscle myosin II (Biomedical Technologies Inc., Stoughton, MA). Cells were then stained with FITC-conjugated affinipure goat anti–rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 0.5–1 μg/ml rhodamine–phalloidin (Molecular Probes, Inc., Eugene, OR) and mounted on antifade (FITC-guard; Testog Inc., Chicago, IL).

Cells to be pretreated with BDM were incubated in 10 mM inhibitor (concentration known to inhibit cardiac and smooth muscle contraction without having other major effects; Blanchard et al., 1990; Spurgeon et al., 1992; Osterman et al., 1993) in media at 37°C for ~5 min. To observe myosin II, cells were then fixed in −20°C methanol for 1–2.5 min and stained as above, using rhodamine-conjugated affinipure goat anti–rabbit IgG

(H+L) (Jackson ImmunoResearch Laboratories, Inc.) as a secondary antibody. To observe actin filaments, cells were fixed and stained as described (Cramer and Mitchison, 1993).

Cells were imaged on a photomicroscope (Carl Zeiss, Inc., Thornwood, NY) with color reversal (p1600; Eastman Kodak Co., Rochester, NY) or hypersensitized Technical Pan film (Alpha Photo Products Inc., Oakland, CA). A red filter was used to minimize any bleedthrough of rhodamine fluorescence into the fluorescein channel in double label experiments. In immunofluorescence BDM studies (Fig. 2), all photography and printing conditions were the same.

Video Microscopy, In Vivo BDM Studies, and Data Collection

Time-lapse video recording of phase contrast images of live cells and data collection were done as described (Cramer and Mitchison, 1993). A 40× Neofluar 0.75 NA objective with a 1× eyepiece was used. Cells infected with *L. monocytogenes* were observed 3–4 h after infection. BDM was added directly to cells under observation to final concentrations of 1–20 mM. BDM was washed out with three to four changes in PBS and then two to three changes in media.

S1 Decoration of Actin Filaments in Postmitotic Spreading Cells

PtK2 cells were extracted (Tilney et al., 1992) in 0.01–0.125% Triton X-100 and 1 μg/ml phalloidin for 1–5 min, incubated with S1 myosin (gift from Kathy Franks, Department of Biochemistry and Biophysics, University of California, San Francisco, CA) (Tilney et al., 1992) for 30 min, fixed, embedded, and sectioned (60–80 nm thick) for EM (Hayat, 1989; McDonald, 1984).
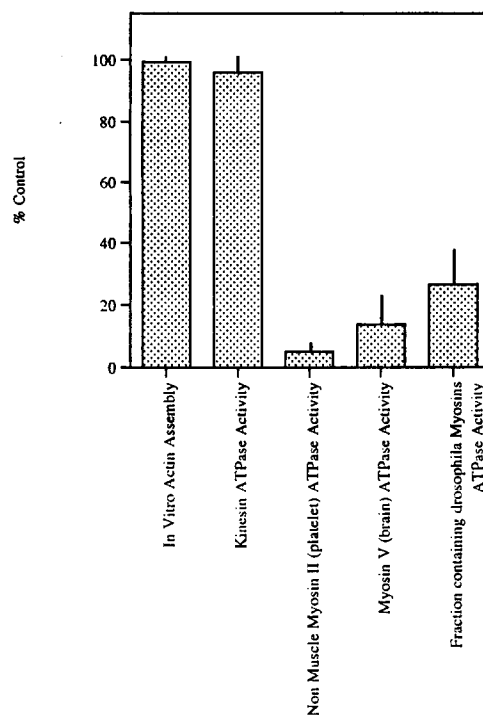

Figure 1. BDM inhibits the ATPase activity of nonmuscle myosin II and myosin V. Human platelet myosin II is inhibited to an average 5.1% (*n* = 26) of control ATPase activity after 1–5 min preincubation with 10 mM BDM, chick brain myosin V is similarly inhibited to an average 13.8% (*n* = 13) of control activity, and a *drosophila* protein fraction enriched in cytoplasmic myosin is inhibited to an average 26.6% (*n* = 10) of control. Kinesin ATPase activity and in vitro assembly of actin is not inhibited by 10 mM BDM.
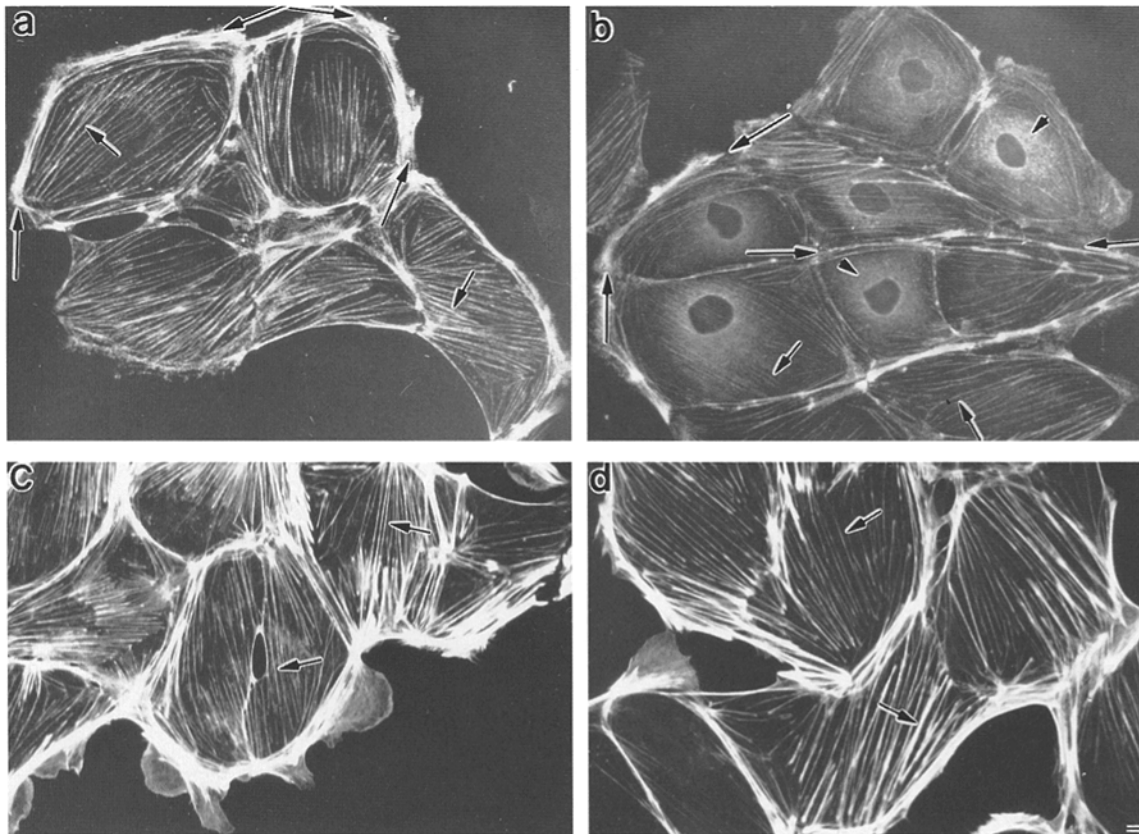
Figure 2. BDM affects a myosin, but not actin filaments, in PtK2 cells. PtK2 cells were incubated with 10 mM BDM for ~5 min and then fixed, permeabilized, and stained for myosin II and actin filaments. (*a*) Control cells illustrate myosin II staining on stress fibers (*short arrows*) and circumferential rings (*long arrows*). (*b*) Treated cells have reduced staining on stress fibers (*short arrows*) and circumferential rings (*long arrows*) and also stain diffusely in the cytoplasm (*arrowheads*). (*c* and *d*) Actin filaments are unaffected after 5 min BDM treatment (*c, arrows*) compared with control cells (*d, arrows*). Bar, 10 μm.

Results

BDM Inhibits the ATPase Activity of Nonmuscle Myosin II and Myosin V

To test the possibility that a myosin may play some role in postmitotic PtK2 cell spreading we wished to generally disrupt the myosins in spreading cells. Progress in understanding the biological roles of other cytoskeletal proteins, for example, actin and tubulin, has been helped by the use of pharmacological agents. In the absence of known high-affinity inhibitors of myosin, we have further characterized one potentially useful drug (BDM) that inhibits contraction of intact and permeabilized muscle and septation in a fungus with low affinity (Horiuti et al., 1988; Higuchi and Takemori, 1989; Blanchard et al., 1990; Spurgeon et al., 1992; Yagi et al., 1992; Osterman et al., 1993; Thompson-Coffe and Zickler, 1993). In vitro, this drug inhibits skeletal myosin II ATPase (Higuchi and Takemori, 1989). From these data it seemed possible that BDM could be a general inhibitor of myosins. To test this, we purified nonmuscle myosin II from platelets essentially as described (Aldelstein et al., 1971; Daniel and Sellers, 1992). Our purified myosin II fraction sometimes contained myosin rod domain and actin (as observed in the original platelet my-

osin II purification; Aldelstein et al., 1971). We estimate these proteins combined represented 0.5–3% of the myosin II protein concentration. Since we wished to study actin-activated myosin II ATPase activity and were adding excess exogenous actin to any platelet actin, we did not purify the myosin II further. We obtained an average actin-activated nonmuscle myosin II ATPase activity of 6.7 μmol/minute/milligram as measured spectrophotometrically, which is similar to other reports of actin-activated myosin ATPase activities. 10 mM BDM inhibits this ATPase activity to 5.1% of control (Fig. 1).

We also determined that 10 mM BDM inhibits the ATPase activity of purified myosin V (to 13.8% of control) and of a *drosophila* protein fraction (to 26.6% of control) that mostly contains cytoplasmic myosin and myosin VI as well as other unidentified actin-binding proteins. BDM does not inhibit kinesin ATPase activity nor the in vitro polymerization of rabbit muscle actin (Fig. 1).

BDM Affects a Myosin, but Not Actin Filaments, in PtK2 Cells

To confirm that BDM would also affect a myosin, but not actin, in PtK2 cells, we treated cells with 10 mM BDM for 5 min and stained them for nonmuscle myosin II and actin
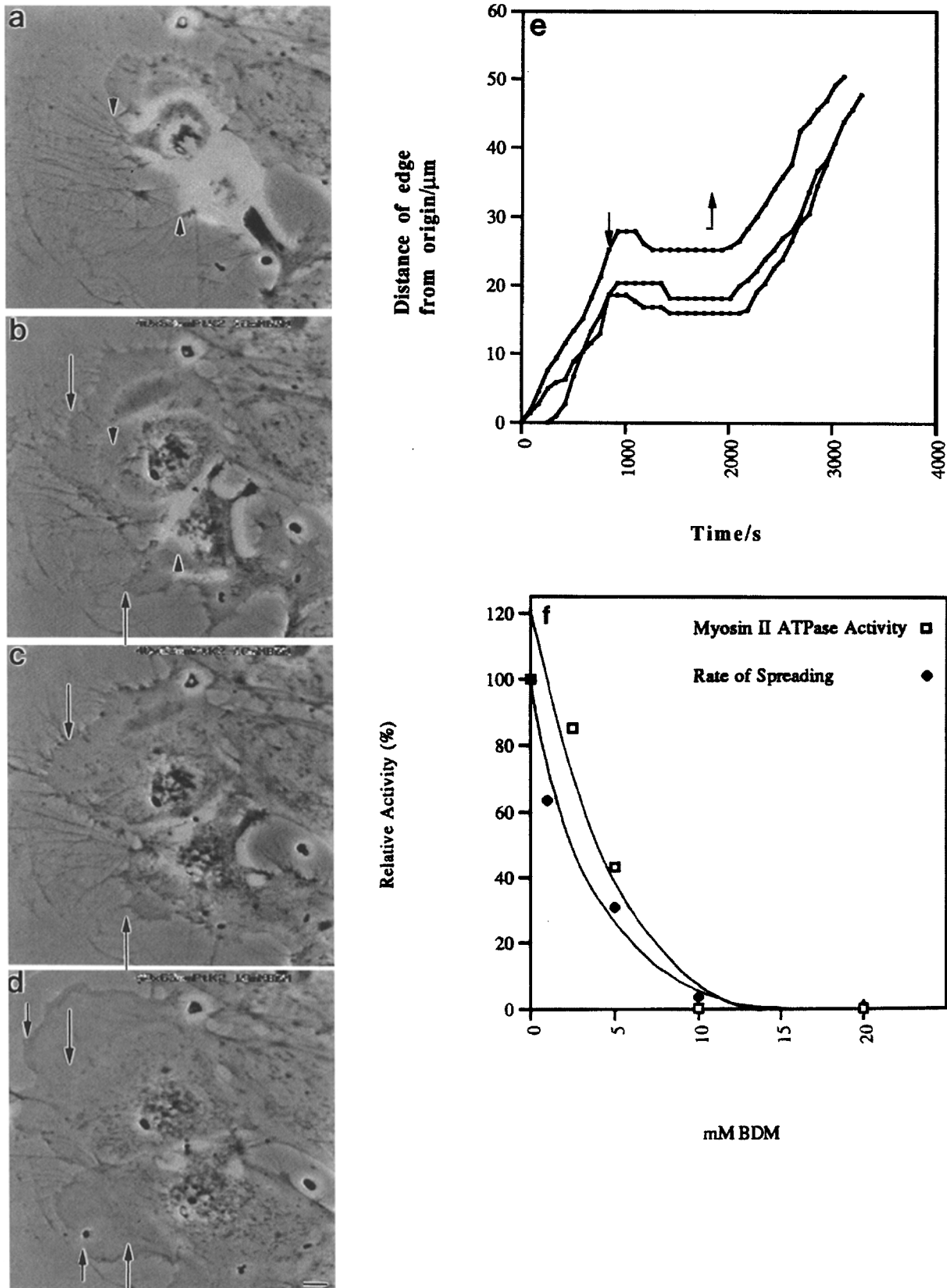
Figure 3. BDM reversibly inhibits spreading of postmitotic PtK2 cells. Arrowheads in *a* and *b* indicate the position of the spreading edge in *a*. (*a*) A spreading edge before addition of BDM. (*b*) 980 s later (last 140 s in 10 mM BDM), the edge has spread to the arrows. (*c*) The edge does not spread in the next 760 s of observation in BDM (*arrows* in *c* point to the extent of spread in *b*). (*d*) BDM is washed out and the cell starts spreading again to the short arrows (next 980 s) from its position in *c* (*long arrows*). (*e*) Distance versus time plots of three spreading edges before and during BDM (added at the first *arrow*) and after (from the second *arrow*) washout (*bar*). (*f*) Comparison of the titration curves for inhibition of spreading and platelet nonmuscle myosin II ATPase activity by BDM. Relative rate of spreading is the average rate in the presence of BDM as a percentage of the rate before addition of BDM in the same cell. Relative ATPase activity is the average activity in the presence of BDM as a percentage of the activity in the absence of BDM. Bar, 10 μm.

filaments. Treated cells have reduced myosin II staining on stress fibers and circumferential rings (compare Fig. 2, a and b). Also, myosin II staining becomes diffuse in the cytoplasm in treated cells (Fig. 2 b, arrowheads). We think these effects may be a consequence of inactivating myosin II ATPase activity, and they are reversible (not shown). The BDM-induced disruption of myosin II is not because BDM directly disrupts actin filaments; actin filaments are not affected after 5 min in BDM (compare Fig. 2, c and d). We estimate that, after 5 min in BDM, >80% of cells have reduced myosin II stress fiber and circumferential ring staining, whereas actin is affected in only 1% of cells. With longer incubations in BDM, stress fibers get shorter and stain less intensely for actin (10% of cells are affected at 50–60 min), but this effect on actin considerably lags behind that of myosin II (>90% of cells affected at 50–60 min) and is presumably due to general destabilization of stress fibers through loss of myosin. Also, 10 mM BDM disrupts cytokinesis in PtK2 cells as expected (cytokinesis is either inhibited or cells do not form normal furrows), but not microtubules nor the process of mitosis (not shown).

BDM Reversibly Inhibits Spreading of Postmitotic PtK2 Cells

Within 1–3 min after addition of 10 mM BDM to postmitotic cells, spreading edges stop moving forward. They then slightly retract toward the cell body or remain stationary (during 15–30 min of observation in BDM). A typical example is shown in Fig. 3. Edges resume spreading after washout of BDM at similar rates to before addition of inhibitor (Fig. 3 e). Cells spread normally in DMSO, which was used to dissolve BDM for some of the time-lapse runs (not shown). The rate of spreading at different concentrations of BDM is similar to the extent of inhibition of platelet nonmuscle myosin II ATPase (Fig. 3 f). Half maximal inhibition is at 2.5–4 mM BDM.

It has been reported that 10 mM BDM affects intracellular calcium in muscle (Horiuti et al., 1988), but our estimates from data presented in other reports on muscle tissue show that 10 mM BDM has little (Blanchard et al., 1990; Osterman et al., 1993) or no effect on intracellular calcium (Spurgeon et al., 1992). Inhibition of postmitotic cell spreading is unlikely to be due to any affect of BDM on intracellular calcium because cells spread normally in the presence of the calcium ionophore A23187 with or without EGTA in the media (not shown).

BDM Does Not Inhibit Propulsion of L. monocytogenes in PtK2 Cells

An alternative potential mechanism for forward movement of cell margins is force from actin polymerization. To test whether BDM affects this type of motility, we assayed its effect on L. monocytogenes propulsion, a type of forward movement thought to be driven at least in part by actin polymerization (Sanger et al., 1992; Theriot and Mitchison, 1992). Fig. 4 shows that 10 mM BDM does not significantly change the rate of Listeria propulsion in PtK2 cells. When the same Listerium was tracked before and after the addition of BDM, no difference in bacterial behavior was observed.

Spatial Organization and Polarity of Actin Filaments in Postmitotic Spreading Cells

We assayed the spatial organization and polarity of actin filaments in spreading edges and retraction fibers by decorating filaments with S1 myosin. Actin filaments in retraction fibers and overlapping actin polymer in spreading edges are bundled and lie perpendicular to the cell edge (Fig. 5 a, thin and thick arrows). 90% of this actin is oriented with barbed ends towards the distal end of retraction fibers (away from the cell body, in the direction of spreading) (Fig. 5 b, right panel, arrows). Individual retraction fiber actin filaments that had their pointed ends facing outward (10%) were located randomly within the retraction fiber and were not detected to run the length of the fiber. In contrast, other actin in the spreading edge is more randomly oriented and spatially arranged (Fig. 5, a, large arrowheads, short arrows, small arrowheads, and c, short arrows, arrowheads).

Localization of Myosin II in Mitotic and Postmitotic Cells

To begin to investigate candidate myosins that could have a role in postmitotic cell spreading, we fixed and stained cells for myosin II and actin filaments. In mitotic cells, cortical specializations (Mitchison, 1992) at the retraction fiber–cell body junction contain the majority of myosin II associated with actin filaments (Fig. 6 b, short arrow, yellow staining). Retraction fibers contain actin filaments (red staining), but not myosin II along their length (Fig. 6

Time/s
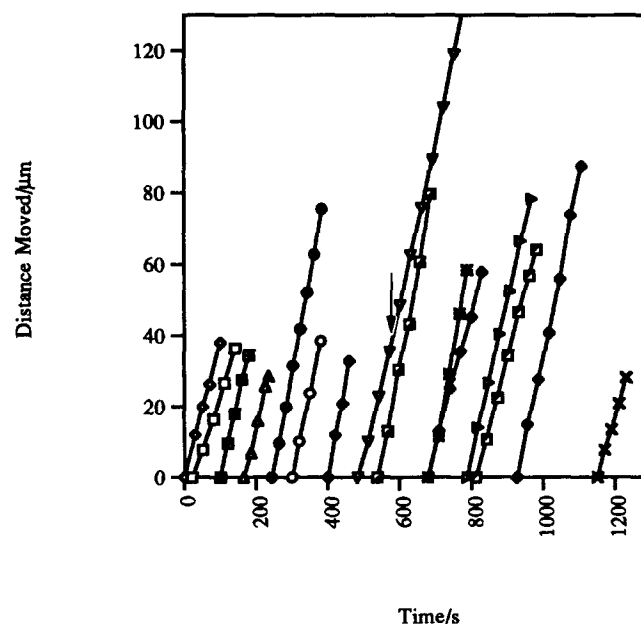
Figure 4. BDM does not inhibit propulsion of L. monocytogenes in Ptk2 cells. A selection of typical distance versus time plots of the 39 observed Listeria in eight infected cells before and after treatment with 10 mM BDM (added at the arrow; average rate of movement in BDM is 107 ± 1% of control Listeria). Each line represents a different Listerium tracked during the course of the experiment. Any Listeria tracked after 20 min were plotted below 1,200 s so that a comparison to control Listeria would be meaningful.
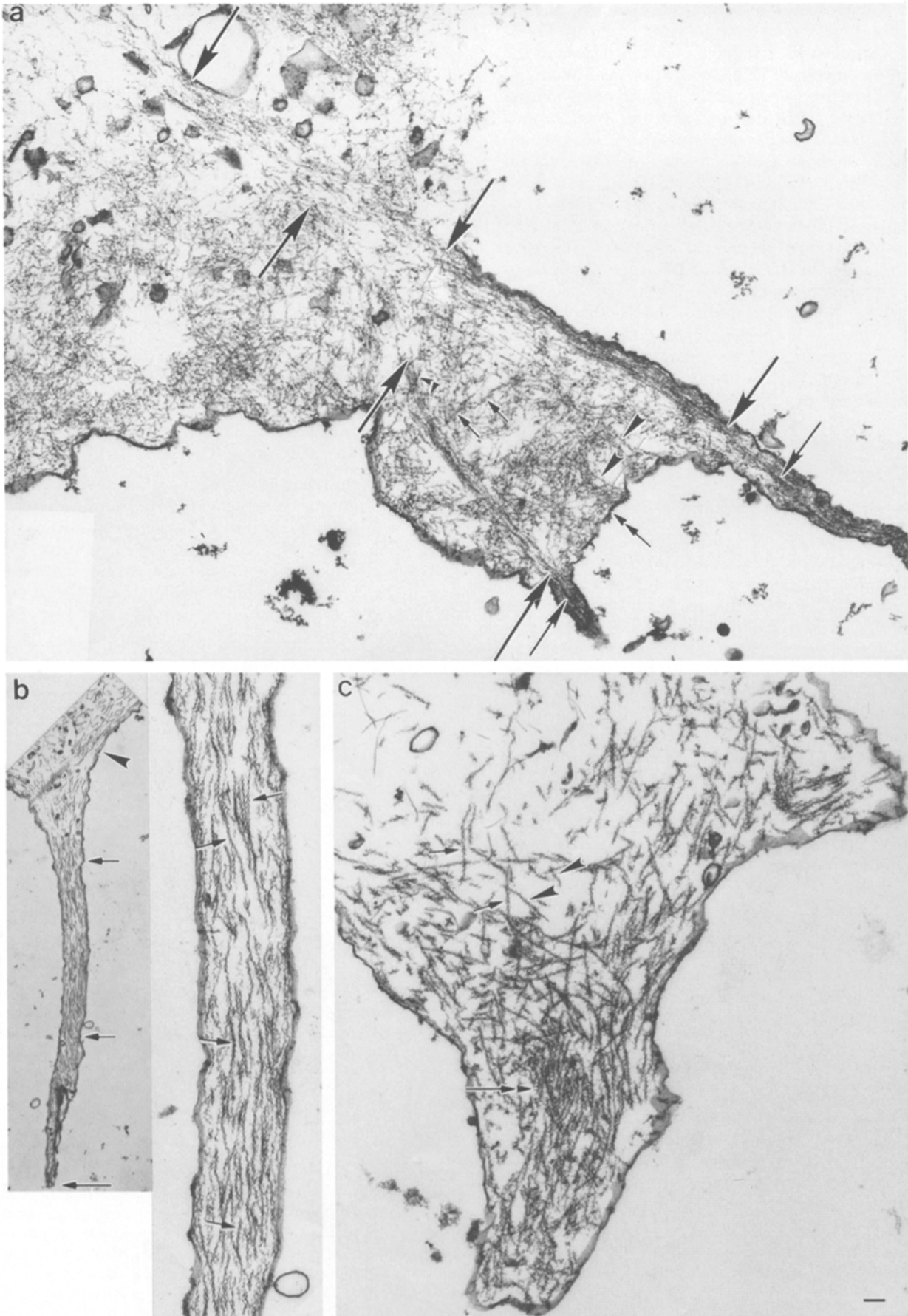
Figure 5. Spatial orientation and polarity of actin filaments in postmitotic spreading cells. Extracted PtK2 cells were incubated with S1 myosin, embedded, and sectioned for EM. (*a*) Actin filaments in retraction fibers are bundled and lie perpendicular (*thin, longer arrows*) to the cell margin (*doubleheaded arrow*) ($n = 832/962$ filaments $= 87\%$ in seven daughters). These or overlapping filaments main-
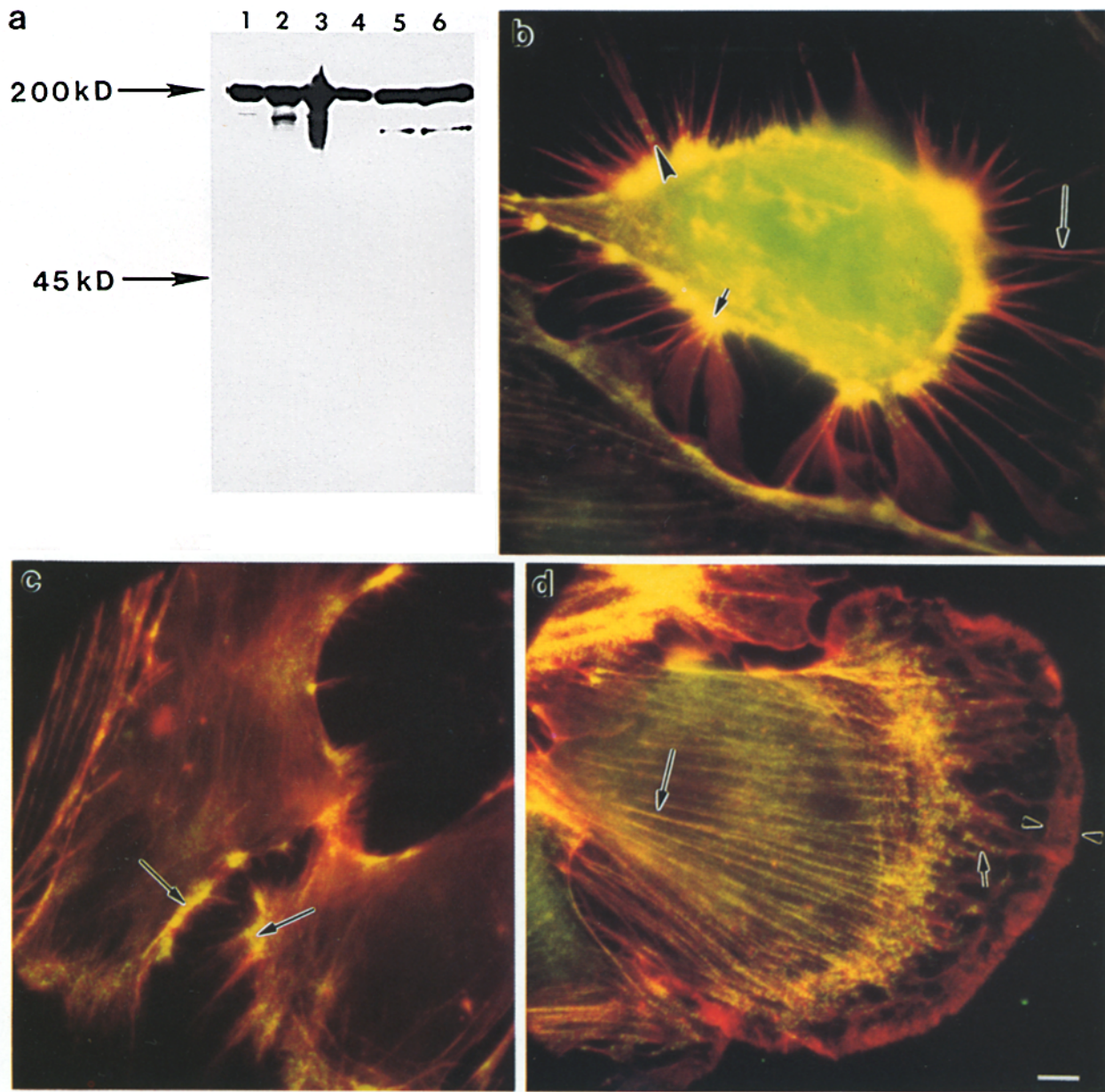
Figure 6. Localization of myosin II in mitotic and postmitotic cells. PtK2 cells were briefly extracted, fixed, permeabilized, and co-stained for myosin II (*green*) and actin filaments (*red*). Myosin II colocalizing with actin filaments appears yellow or yellow-green. (*a*) The antimyosin II antibody we used in *b–d* recognizes a single major band of 200 kD in PtK2 cell homogenates (∼25 μg [lane *1*], 50 μg [lane *2*], and 100 μg [lane *3*] protein loaded), which comigrates with purified human platelet nonmuscle myosin II (∼0.2 μg [lane *4*], 0.4 μg [lane *5*], and 0.6 μg [lane *6*] protein loaded) by Western blot. (*b*) A rounded-up mitotic cell. Myosin II and actin filaments colocalize at the mitotic cell periphery in cortical specializations (*short arrow*). Retraction fibers contain actin filaments, but do not stain for myosin II (*long arrow*) except at the top of some retraction fibers (*arrowhead*). (*c*) Spreading edges of postmitotic cells. Myosin II and actin filaments colocalize at the spreading edge (*arrows*). (*d*) End of spreading. Myosin II is found on stress fibers (*long arrow*) and on actin filaments in the lamella (*short arrow*), but not in the lamellipodium (between the *arrowheads*). Bar, 10 μm.

tain this bundled organization across the spreading edge (delineated by three *thick, longer arrows* behind both retraction fibers). Other actin filaments in the spreading edge are more randomly oriented: 325/1,200 filaments = 27% lie perpendicular (*large arrowheads*), 527/1,200 filaments = 44% lie parallel (*short arrows*), and 348/1,200 filaments = 29% lie oblique (*small arrowheads*) to the cell margin in five daughters. (*b*) Actin in the retraction fiber and spreading edge bundles is oriented with barbed ends towards the distal end of retraction fibers (392/436 filaments = 90% in seven daughters, e.g., *right panel, arrows*). The right panel is an enlarged version of the area between the short arrows in the left panel. An arrowhead denotes the spreading edge and a long arrow the distal end of the retraction fiber. (*c*) 61 and 39% of actin filaments in spreading edges that are not in bundles are oriented with their barbed (e.g., *arrows*) and pointed (e.g., *arrowheads*) ends, respectively, toward the spreading margin (*n* = 385 filaments that have a bias toward the cell edge in five daughters). The doubleheaded arrow points to retraction fiber actin. Bar: (*a*) 145 nm; (*b, left panel*) 500 nm; (*b, right panel*) 125 nm; (*c*) 135 nm.
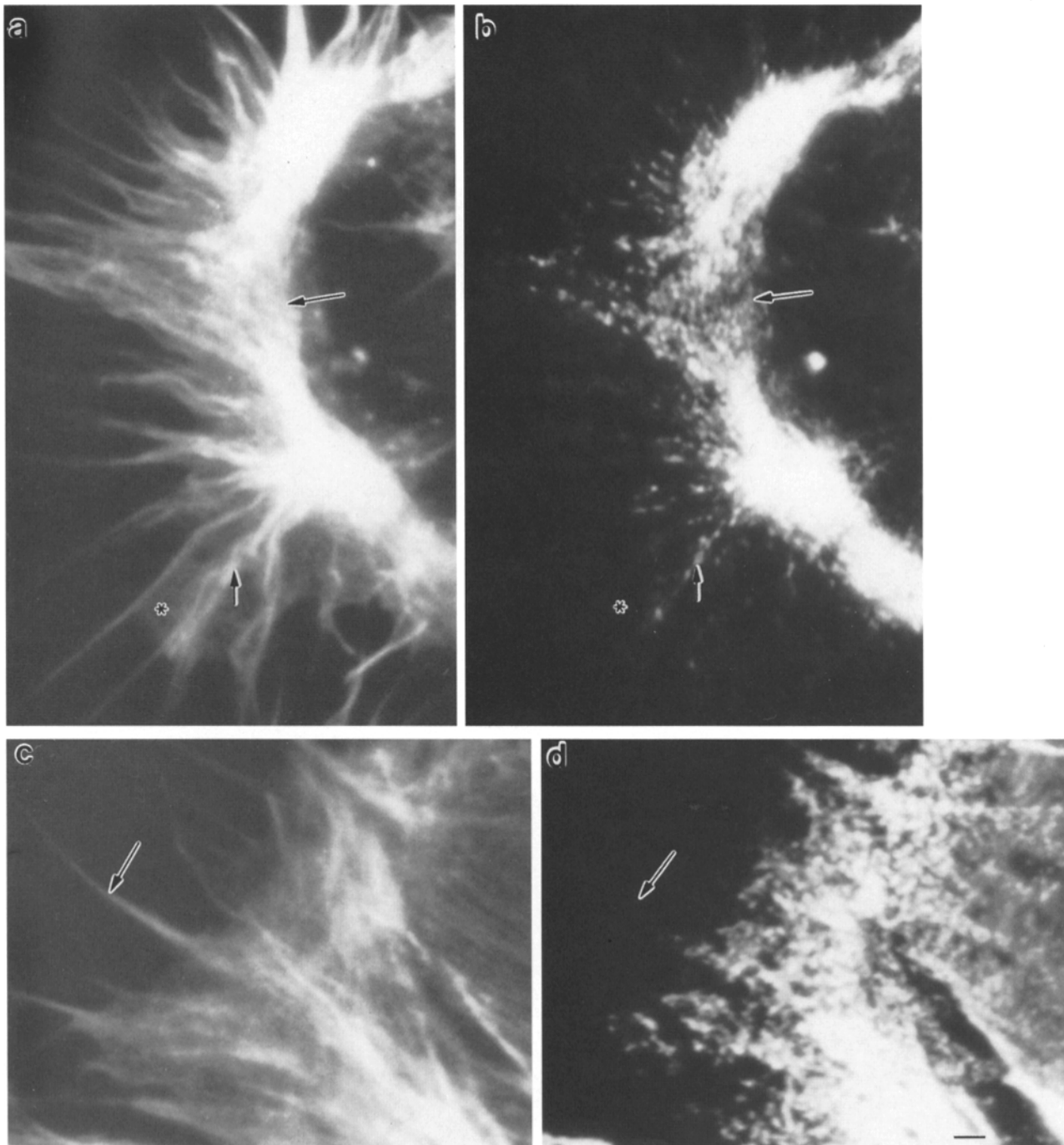
Figure 7. Detailed distribution of myosin II in postmitotic spreading edges. Paired images of actin (*a* and *c*) and myosin II (*b* and *d*) in two spreading edges. Within a spreading edge, myosin II is found on actin filaments that are colinear with actin in retraction fibers (*a* and *b, short arrows*) and on other actin fibers in the edge (*a* and *b, long arrows*), but mostly not detected on retraction fiber actin ahead of spreading edges (*c* and *d, arrows*) nor on thin regions of actin polymer (*a* and *b, asterisks*). Bar, 1.5 μm.

b, long arrow). Myosin II is also sometimes observed on short stretches of retraction fibers just below cortical specializations (Fig. 6 *b, arrowhead, yellow dots*). In postmitotic spreading cells, the majority of myosin II bound to actin filaments is concentrated at the spreading edge (Fig. 6 *c, arrows, yellow* or *yellow-green staining*). Myosin II staining is often greater in regions of the cell edge that join clusters of retraction fibers (e.g., Fig. 6 *c, right arrow*) than

in regions of the cell edge each side of the cluster. This pattern correlates with faster spreading where retraction fibers are clustered (Cramer and Mitchison, 1993). Myosin that is not bound to actin is only found in the cell body and not in cell edges (e.g., Fig. 6 *b, green staining*). This is probably nonfilamentous because it is readily extracted with increasing time in detergent (not shown). Toward the end of spreading, lamellipodia protrude from the spreading edge,

and the postmitotic cell regains interphase morphology. Myosin II is found on actin filaments in the lamella (Fig. 6 *d, short arrow*) and on stress fibers (Fig. 6 *d, long arrow*), but not in the lamellipodium (Fig. 6 *d, between the arrowheads*). The localization of myosin II at the edges of postmitotic spreading cells supports the possibility that it could play some role in postmitotic cell spreading. Although it is beyond the scope of this article to compare the distribution of all known myosins in postmitotic spreading cells, we found that an antibody to brush border myosin I (gift from Paul Matsudaira, Department of Biology, Massachusetts Institute of Technology, Whitehead Institute for Biomedical Research, Cambridge, MA) that recognizes an unknown myosin I isotype in PtK2 cells does not stain spreading edges (not shown).

Fig. 7 illustrates the localization of myosin II and actin filaments in more detail in two typical spreading edges fixed in the middle of spreading before lamellipodia protrusion starts. Within spreading edges, myosin II is readily detected on actin filaments, which are colinear with actin in retraction fibers (Fig. 7, *a* and *b, short arrows*) and on other actin filaments (Fig. 7, *a* and *b, long arrows*) in short regular stretches of punctate staining, which may represent individual myosin II filaments (Yonemura and Pollard, 1992). Myosin II is not significantly detected on actin in retraction fibers themselves (Fig. 7, *c* and *d, arrows*).

Discussion

Use of BDM as an Inhibitor of Myosin

Progress on understanding the role of myosins in cell motility has been hampered by the absence of pharmacological probes. The best-characterized agent that directly targets myosin is BDM. Several laboratories working on muscle contraction concluded that this agent directly inhibits actin–myosin interaction (Higuchi and Takemori, 1989; Yagi et al., 1992) by inhibiting myosin ATPase activity with low affinity (Higuchi and Takemori, 1989). Some reports did not take into account the antagonistic effect of imidazole on BDM, and others have suggested additional effects of this drug. Calcium levels may be affected in skeletal muscle (e.g., Horiuti et al., 1988), though it is not clear if this is a direct effect of the drug, and myosin light chain phosphorylation is reduced in smooth muscle (Siegman et al., 1994). Calcium levels probably do not directly regulate spreading in our system, but we performed additional controls for the specificity of BDM as an antimyosin agent in tissue culture cells. In vitro, we found that 10 mM BDM inhibited nonmuscle myosin II and V ATPase activity, and under certain conditions, actin filament motility on muscle heavy meromyosin is inhibited to <30% of control, whereas kinesin ATPase activity and actin polymerization were unaffected (Fig. 1, and Sabry, J., and L. P. Cramer, unpublished observations). In vivo, in PtK2 cells we found that 10 mM BDM disrupts cytokinesis and myosin II localization in stress fibers, but not actin filaments. 10 mM BDM also increased the time between contractions of the contractile vacuole of *Acanthamoeba* more than twofold (Cramer, L. P., and T. J. Mitchison, unpublished observations), a process that involves the activity of myosin 1C (Doberstein et al., 1993). In contrast, mitosis and *Listeria*

motility in PtK2 cells were unaffected by 10–20 mM BDM, arguing against nonspecific effects of BDM on general metabolism and many ATPases. Taken together, we believe that 10 mM BDM is a useful specific inhibitor of some myosins in PtK2 cells. It may be a general myosin inhibitor, though its effects on other myosins, particularly myosin Is, still need to be tested.

A Role for Myosin in Postmitotic Cell Spreading

We previously showed that there are two dynamic populations of actin filaments in spreading cells differentiated by their location and dynamic behavior; one is stationary with respect to substrate and is found in retraction fibers and at a low level in spreading edges. The other moves forward in spreading edges at the same rate as the edge (Cramer and Mitchison, 1993). From this and other data we proposed a model in which the activity of a myosin pulls actin filaments in the spreading edge forward as it walks outward along retraction fiber actin. In this work, we have used
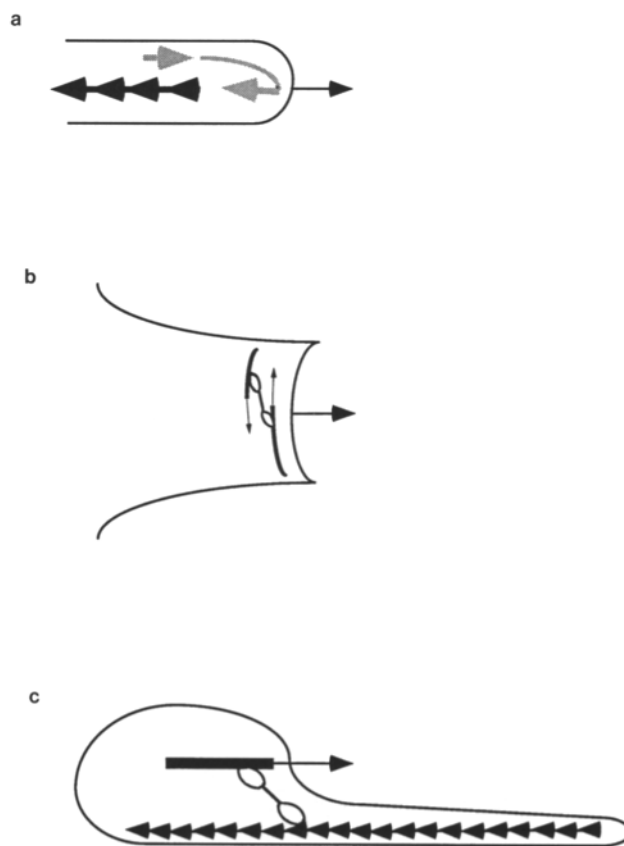
Figure 8. Actin-dependent forward movement in different types of motile systems. (*a*) Forward movement of the cell edge is driven by the polymerization of actin monomer (*single arrowheads*) into filaments (*arrowheads*) (e.g., lamellipodia protrusion). (*b*) Forward movement of the cell edge is driven by actomyosin contraction (e.g., healing of certain kinds of wounds). (*c*) Forward movement of the cell edge is driven by myosin (O–O) pulling actin filaments forward (*solid black bar*) over other immobilized actin filaments (*arrowheads*). Paradoxically, this is a kind of contraction except the geometry is different than *b*, and some of the actin does not move (e.g., postmitotic cell spreading).

drug treatments and cytology to test whether myosin is involved in spreading.

BDM rapidly and reversibly inhibits spreading (Fig. 3). Taking into account the caveats discussed above, the simplest interpretation of these data is that a myosin is involved in generating the force for spreading. All known myosins that have been tested move toward the barbed-end of actin filaments. We find that actin bundles in retraction fibers are continuous with bundles of actin filaments in the spreading edge, and that 90% of these actin filaments have their barbed ends in the direction of forward movement (Fig. 5). This actin (which is stationary, based on our previous data) is therefore of the correct polarity to support the movement of a myosin in the direction of spreading. The remaining actin filaments in spreading edges have a more mixed polarity and orientation. This less-organized actin accounts for the bulk of actin in the spreading edge. Given its more random orientation, it is more likely to be transported forward by the myosin.

Which type of myosin is likely responsible for spreading? The most abundant myosin in most cells is myosin II. We found that myosin II colocalizes with actin filaments in spreading edges (Figs. 6 and 7). Also, in fixed cells, myosin II appears to move coordinately forward with the spreading edge because the majority of myosin II is not observed on retraction fibers ahead of the edge (Fig. 7). Although localization data alone could not distinguish between myosin II providing force for spreading and simply being a passenger, we interpret these data in the light of our BDM experiments. BDM acts rapidly enough on myosin II to explain inhibition of spreading (Fig. 2), and a role for myosin II in spreading is consistent with pharmacological data that shows that the BDM titration curves for spreading and myosin II ATPase inhibition are very similar (Fig. 3 f). We believe that this drug and localization data taken together strengthen our argument that a myosin has a role in postmitotic cell spreading. Although we do not rule out the possibility that other myosins could also have a role in postmitotic cell spreading, we tested whether myosin II could have such a role by treating cells with high affinity inhibitors of myosin light chain kinase (KT5926 and kaempferol). Nonmuscle myosin II can be regulated by light chain phosphorylation, and certain inhibitors of myosin light chain kinase have been used to inactivate this myosin (Lamb et al., 1988). Although we do not yet know the basis for the regulation of spreading in PtK2 cells, we found that both myosin light chain kinase inhibitors disrupt the localization of myosin II in PtK2 cells and reduce the rate of postmitotic cell spreading about twofold (Cramer, L. P., and T. J. Mitchison, unpublished data).

Comparison of Postmitotic Cell Spreading to Other Types of Cell Motility

We argue that myosin is involved in postmitotic cell spreading. In contrast, two other types of cell motility, lamellipodia protrusion, and *Listeria* propulsion, are thought to be driven at least in part by forces derived from actin polymerization (Wang, 1985; Forscher and Smith, 1988; Theriot and Mitchison, 1991, 1992; Sanger et al., 1992) (Fig. 8 a). The BDM data are probably the strongest evidence to date that myosins are not required for *Listeria* motility

(Fig. 4), although we cannot rule out the possibility that other BDM-insensitive motors generate propulsive force. De novo lamellipodia protrusion is also not inhibited by 10 mM BDM in primary and tissue culture fibroblasts (Cramer, L. P., and T. J. Mitchison, unpublished results). Cell spreading is not the only type of edge motility that depends on myosin. Recently, a type of forward-moving cell edge distinct from spreading edges has been observed in wound healing and morphogenesis (Martin and Lewis, 1992; Bement et al., 1993; Young et al., 1993). Protrusion of this type of edge is probably driven by myosin II (Bement et al., 1993; Young et al., 1993). However, in these systems myosin II may act by its more familiar contractile mechanism (Fig. 8 b). In contrast, the proposed role for myosin II in postmitotic cell spreading is more of a transport motor (Fig. 8 c).

Forces derived from myosin activity and actin polymerization are likely to generate forward movement in different types of motile systems (Cramer et al., 1994). With further characterization, BDM may be a useful reagent for distinguishing between these forces, and we are currently analyzing the effects of BDM on a number of different types of motility. In complex motile cell systems, such as neuronal growth cones, and migrating cells we envisage that motile force is generated from both myosin activity and actin polymerization.

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