Published in final edited form as:

Cell Motil Cytoskeleton. 2009 September ; 66(9): 679–692. doi:10.1002/cm.20398.

Structure and Dynamics of an Arp2/3 Complex-independent Component of the Lamellipodial Actin Network

John H. Henson^{1,2,3}, David Cheung^{1,3}, Christopher A. Fried^{1,2,3}, Charles B. Shuster^{2,4}, Mary K. McClellan^{1,3}, Meagen K. Voss^{1,3}, John T. Sheridan¹, and Rudolf Oldenbourg²

¹Department of Biology, Dickinson College, Carlisle, Pennsylvania 17013

²Marine Biological Laboratory, Woods Hole, Massachusetts 02543

³Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672

⁴Department of Biology, New Mexico State University, Las Cruces, NM 88003

Abstract

Sea urchin coelomocytes contain an unusually broad lamellipodial region and have served as a useful model experimental system for studying the process of actin-based retrograde/centripetal flow. In the current study the small molecule drug 2,3-butanedione monoxime (BDM) was employed as a means of delocalizing the Arp2/3 complex from the cell edge in an effort to investigate the Arp2/3 complex-independent aspects of retrograde flow. Digitally-enhanced phase contrast, fluorescence and polarization light microscopy, along with rotary shadow TEM methods demonstrated that BDM treatment resulted in the centripetal displacement of the Arp2/3 complex and the associated dendritic lamellipodial (LP) actin network from the cell edge. In its wake there remained an array of elongate actin filaments organized into concave arcs that displayed retrograde flow at approximately one quarter the normal rate. Actin polymerization inhibitor experiments indicated that these arcs were generated by polymerization at the cell edge, while active myosin-based contraction in BDM treated cells was demonstrated by localization with anti-phospho-MRLC antibody, the retraction of the cytoskeleton in the presence of BDM, and the response of the BDM arcs to laser-based severing. The results suggest that BDM treatment reveals an Arp2/3 complex-independent actin structure in coelomocytes consisting of elongate filaments integrated into the LP network and that these filaments represent a potential connection between the LP network and the central cytoskeleton.

Keywords

Actin; Arp2/3 complex; retrograde flow; BDM; coelomocyte

INTRODUCTION

Actin-based retrograde/centripetal flow involves the movement of the cortical actin network and the overlying membrane from the cell edge towards the cell center. This process is present in many different cell types and has been suggested to be important in cell motility, guidance, and exploration (reviewed in Pollard and Borisy, 2003). A series of recent studies indicate that the cortical actin network is more complex than previously thought and appears to consist of two distinct and separable arrays with different molecular compositions,

structural organization and flow dynamics (DesMarais et al., 2002; Ponti et al., 2004; Gupton et al., 2005; reviewed in Danuser, 2005; Chhabra and Higgs, 2007). The term lamellipodia (LP) has been applied to the dynamic, dense, and dendritic network of branched actin filaments that undergoes Arp2/3 complex-facilitated polymerization and is restricted to the most distal region of the cell edge. This LP network overlaps with, or perhaps overlies (Ponti et al., 2004; Giannone et al., 2007), a sparser network of longer and less dynamic actin filaments and bundles termed the lamellum (LM) that displays a form of mDia/formin-dependent polymerization (Gupton et al., 2007) and is generally wider than the LP. In terms of retrograde flow dynamics, the LP network undergoes relatively rapid flow that is mediated by actin polymerization while the LM engages in a slower form of flow that is mediated by acto-myosin contraction (Ponti et al., 2004; Gupton et al., 2005). However, this clear distinction between the structure and underlying mechanisms of the LP and LM networks is not embraced by all investigators. Recent electron microscopy-based studies argue against the superposition of an LP network on the LM array (Koestler et al., 2008; Small et al., 2008) while recent loss of function studies suggest that formin/mDia and the Arp2/3 complex may work together in structuring and/or animating the LP (Gupton et al., 2007; Yang et al., 2007).

Sea urchin coelomocytes represent an excellent model system for studying actin-based retrograde flow given that these discoidal-shaped cells display an exaggerated form of this type of motility in an unusually broad actin and Arp2/3-complex rich LP region (Henson et al., 1999;2002;2003). Using immunolocalization and electron microscopy of coelomocyte cytoskeletons, we have previously demonstrated that the Arp2/3 complex is associated with the elaborate actin brushwork present at the cell edge and that myosin II is associated with an actin bundle termination zone in the perinuclear region (Henson et al., 1999,2002). From these studies, we have developed a model in which centripetal flow in coelomocytes is mediated by a two-part, interconnected mechanism: actin polymerization pushing at the cell edge and acto-myosin contraction pulling at the cell center (Henson et al., 1999). A useful analogy is a tandem bike in which the polymerization and acto-myosin contraction forces are represented by the two riders, each of which can contribute at various levels of effort to the overall forward movement of the bike. Similar two part models have been postulated for retrograde flow in migrating epithelial cells (Vallotton et al., 2004) and neuronal growth cones (Zhang et al., 2003;Medeiros et al., 2006).

In the course of our pharmacological-based dissection of the mechanisms underlying coelomocyte centripetal flow, we discovered that the small molecule drug 2,3-butanedione monoxime (BDM) induced an unusual phenotype in these cells. Upon BDM treatment, the normal dense cortical actin network retracted from the cell edge and was replaced with a loose array of elongate actin filaments (Henson et al., 1999). Early studies employed the drug BDM as a myosin ATPase inhibitor to suppress contraction in cardiac (Sambrano et al., 2002), skeletal (Fryer et al., 1988), and smooth (Österman et al., 1993) muscle cells. It gained popularity in the nonmuscle cytoskeletal field following a publication by Cramer and Mitchison (1995) that characterized the drug as a low affinity inhibitor of non-muscle myosin II. Numerous subsequent studies utilized BDM in this manner, however a series of other investigations indicated that BDM did not inhibit non-muscle myosins (McCurdy, 1999; Cheung et al., 2001; Ostap, 2002; Danilchik et al., 2006). In addition a paper by Yarrow et al. (2003) showed that BDM treatment led to the delocalization of actin leading edge components (Arp2/3 complex, WAVE and VASP) and that this effect could mimic myosin inhibition. At present BDM continues to be employed in various cell types as either a myosin inhibitor [see for example Durán et al., 2003; Zhou et al., 2003; Funaki et al., 2004; Forer and Fabian, 2005] or simply as a drug that produces unusual and insightful actin cytoskeletal-related effects (Valentijn et al., 2001; Shaner et al, 2005; Danilchik et al., 2006).

In the present study we have tested the hypothesis that BDM treatment of coelomocytes uncouples the Arp2/3 complex-dependent and -independent components of the cortical actin network. Using transmitted light, fluorescence, polarization and electron microscopy we report here that BDM displaces the Arp2/3 complex-dependent LP network from the cell edge and therefore exposes a population of elongate actin filaments oriented parallel to the cell edge that are generated by actin polymerization and undergo myosin II-dependent retrograde flow. These actin arcs are analyzed in terms of their structural and kinematic properties. We discuss how the BDM actin phenotype can be interpreted as either the coelomocyte LM or an integral component of the LP. From these studies, we propose a working model whereby the actin arcs revealed by BDM treatment are intermeshed with the LP and form the basis for the connection between the cortical and central cytoskeletons in coelomocytes.

MATERIALS AND METHODS

Animals, Cell Preparation, Antibodies, and Reagents

Sea urchins of the species *Strongylocentrotus droebachiensis* were either collected from the near shore waters surrounding the Mount Desert Island Biological Laboratory or obtained from the MBL Marine Resources Center and kept in either running sea water or closed artificial sea water systems at 15°C. Coelomocytes were isolated and maintained as described in Henson et al. (1992) with the coelomocyte culture media (CCM) consisting of 0.5 M NaCl, 5 mM MgCl₂, 1 mM EGTA, and 20 mM HEPES, pH 7.2. Typically the cells were used within 2–8 h of isolation. For hypotonic shock treatment of coelomocytes, CCM was diluted 1:1 with a buffer containing 5 mM MgCl₂, 1 mM EGTA, and 20 mM HEPES, pH 7.2. A monoclonal anti-actin antibody (clone C4) was obtained from ICN/MP Biomedicals (Costa Mesa, CA), anti-sea urchin fascin was a gift from Dr. Joann Otto (Western Washington University), fluorescent phalloidin and latrunculin A were purchased from Molecular Probes/Invitrogen (Eugene, OR), rabbit anti-phospho-Ser19 myosin regulatory light chain was purchased from Cell Signaling (Beverly, MA), TEM supplies and reagents were obtained from Electron Microscopy Sciences (Hatfield, PA), and all other reagents (including 2,3-butanedione monoxime and Cytochalasin D) and antibodies (including anti-Arp3) were purchased from Sigma/Aldrich Chemical Co. (St. Louis, MO).

Pharmacological Treatments

For pharmacological treatments, cells being processed for immunolocalization or electron microscopy were treated in 35-mm Petri dishes while cells prepared for live cell imaging were treated in simple perfusion chambers. These chambers were created by placing the coverslip containing cells on top of shims made of cut coverslips attached to a slide via petroleum jelly. Alternatively coverslips were attached to shims made of double stick adhesive tape. Solutions containing a drug were perfused through the chamber using the capillary action of a piece of filter paper. BDM was made up as a stock of 40 mM in CCM and typically used at a concentration of 20–30 mM. Cytochalasin D and latrunculin A were made up as 1mM stocks in DMSO and used at a concentration of 500 nM - 1 µM.

Live Cell Imaging and Laser Severing

Coelomocytes were settled onto untreated glass coverslips that were then mounted in perfusion chambers and viewed on a Nikon E600 microscope using a 60X 1.4 NA phase contrast planapochromatic objective lens. Digitally-enhanced images were obtained with a Hitachi CCD camera coupled to a Hamamatsu Argus-10 real time digital image processor. Frame averaged, background subtracted, and contrast enhanced images were imported into NIH Image running on a Macintosh G4 computer where additional digital contrast enhancement was performed.

For axis-independent polarization microscopy, cells in perfusion chambers were viewed on an LC PolScope (Cambridge Research and Instrumentation, Woburn, MA) using a strain free 60X 1.4 NA DIC planapochromatic objective lens mounted on a Nikon Microphot SA microscope and images captured using a Q-Imaging (Surrey, BC) Retiga camera and processed using PSj software (an Image J-based image acquisition and processing system).

For laser-based severing experiments, cells in perfusion chambers were viewed on a Zeiss Axiovert microscope using a 100X 1.3 NA phase contrast objective lens. A Teem Photonics (Grenoble, France) 532 nm laser capable of 1 ns pulses at 3 microJoule per pulse was routed through the epi port of the microscope and brought to a diffraction limited focal spot in the specimen. Phase contrast images were collected on a Q-Imaging Retiga camera and processed using PSj software.

Fluorescent Localization

For fluorescent localization of filamentous actin with phalloidin, coelomocytes were fixed with 0.25% glutaraldehyde plus 0.1% Triton X-100 in buffer A (75 mM KCl, 2 mM MgCl₂, 320 mM sucrose, 20 mM EGTA, 20 mM PIPES, pH 7.0) for 10 min, rinsed in PBS, and then stained with AlexaFluor 488 or 568 conjugated phalloidin for 20 min. For immunolocalization of Arp3, fascin and actin, coelomocytes were prefixed in 0.0001% glutaraldehyde in CCM for 5 min, fixed in 1% formaldehyde and 0.5% Triton X-100 in buffer A for 10 min, and then postfixed in 100% methanol at -20°C for 10 min. Following a rinse with PBS, cells were blocked with PBS plus 1% BSA and 2% goat serum, and stained with primary antibodies followed by the appropriate fluorescently labeled secondary antibodies. Cells were mounted in Prolong antiphotobleach (Molecular Probes/Invitrogen, Eugene, OR) and imaged using a Nikon E600 microscope equipped with a 60X 1.4 NA planapochromatic phase contrast objective lens and digital images were captured using a Photometrics CoolSnap Cf camera (Roper Scientific, Tuscon, AZ).

Critical Point-dry and Rotary-shadow Transmission Electron Microscopy (TEM)

TEM imaging of critical point-dried and rotary-shadowed replicas of coelomocyte cytoskeletons followed methods described in Henson et al. (1999) and Svitkina (2007). Briefly, coelomocytes settled onto naked glass coverslips were prefixed in 0.001% glutaraldehyde in CCM and then extracted in 0.5% Triton X-100 in buffer A. After extraction, the cells were then fixed in 2.5% glutaraldehyde in the same buffer and then treated with aqueous tannic acid followed by aqueous uranyl acetate. Cells were then dehydrated in a graded ethanol series, critical point dried, and rotary shadowed with platinum and carbon. Metal replicas were separated from glass coverslips using hydrofluoric acid, mounted on Formvar-coated grids, and imaged using either a JEOL 100S or Zeiss EM-10 TEM operating at 80 kV.

RESULTS

Displacement of the LP Actin Network Reveals Elongate Actin Arcs

Digitally enhanced phase contrast microscopy revealed that discoidal-shaped coelomocytes treated with 20-30 mM BDM displayed a characteristic phenotype (initially described in Henson et al., 1999) in which the dense cortical actin network retracted from the cell edge and was replaced by an array of elongate bundles/arcs, oriented roughly parallel to the plasma membrane (Fig. 1). The arcs stained for F-actin using fluorescent phalloidin (Fig. 1E) and were clearly visible using axis-independent polarization optics where these structures exhibited birefringence consistent with small filamentous bundles (Fig. 1D). These actin arcs displayed retrograde flow in which they appeared to “peel off” of the cytoplasmic face of the plasma membrane (Supplemental Movies 1 and 2) and the ends of

these bundles often collected around the distal tips of radial stripes that extended out from the perinuclear region of the central, retracted cytoskeleton (Figs. 1, 5). Double labeling for actin and Arp3 shows that the dense actin and Arp2/3 complex network present at the edge of control cells (Figs. 1F-H) displaced centripetally in cells treated with BDM (Figs. 1I-K) and that the Arp2/3 complex was not associated with the BDM-induced actin arcs. Note that the BDM phenotype did not appear in control experiments in which coelomocytes were treated with CCM supplemented with either 20-30 mM mannitol or urea in order to mimic the hypertonic effect of the BDM treatment (not shown). In addition, preliminary pixel intensity profiles from actin stained cells suggested that there was a general loss of actin at the cell edge with BDM treatment and the associated retraction of the Arp2/3 complex network and the exposure of the actin arcs (not shown).

It is possible that actin arcs induced by BDM treatment of coelomocytes were formed in a manner similar to that seen with filopodial core bundles. Coelomocytes have been shown to form elaborate arrays of filopodia in response to hypotonic shock or elevation of intracellular calcium (Edds, 1979; Henson and Schatten, 1983), and in mammalian cells filopodial cores have been demonstrated to contribute to actin bundles present in the LM (Nemethova et al., 2008). However, localization of the actin filament crosslinking protein fascin shows that while it was clearly associated with hypotonic shock-induced filopodia (as originally reported by Otto et al., 1979), fascin was not present in the cortical actin bundles resulting from BDM treatment (Fig. 2).

The dramatic alteration in cortical actin structural organization caused by BDM can be observed at higher resolution in cells processed for critical point dry and rotary shadow TEM (Fig. 3). In control cells a LP-like network of actin filaments fills the cortical region of the cell (Figs. 3A, C). Cells fixed following the application of BDM revealed that the LP dense network was centripetally displaced and in its wake appeared actin arcs oriented parallel to the cell margin (Figs. 3B, D). The actin arcs consisted of loose bundles of elongate and curved actin filaments that often feed into in regions associated with radial bundles of actin extending out from the cell center. Interestingly, careful examination of control cell LP networks revealed the presence of elongate actin filaments similar to those seen in the BDM-induced actin arcs (arrows in Fig. 3C) suggesting that these actin elements are present in control cells but masked by the density of the LP actin network.

The BDM effect was reversible, and when the drug was washed out, an LP-like actin network containing the Arp2/3 complex once again assembled at the cell edge. The re-initiation of an Arp2/3 complex-based actin network was clearly seen at the light microscopic level in cells labeled for actin and Arp3 (Figs. 4A-C). At the TEM level (Fig. 4D) the newly forming network following BDM wash-out displayed the dendritic array of filaments characteristic of Arp2/3-nucleated actin polymerization, although filament branching was not as obvious as reported in other cell types (Svitkina and Borisy, 1999) and many elongate, apparently unbranched filaments were also present. Arc-like, elongate, and curved actin filaments were particularly prominent in these nascent network regions, with many of these filaments extending into the cleared zone behind the dense network corresponding to the region of actin structure revealed by BDM treatment (Fig. 4D).

Actin Arcs are Generated by Actin Polymerization

Digital video phase contrast microscopy indicated that the actin arcs in BDM treated cells displayed retrograde/centripetal flow (Fig. 5, Supplemental Movies 1 and 2). Arcs appeared to originate at a point of contact with the membrane at the cell edge and then flowed inwards, eventually accumulating onto the retracted remnant of the LP network (Fig. 5A-D). Over time it was clear that new actin arcs were being created at the cell margin. The rate of centripetal movement averaged roughly 1 micron/min (n =10 cells), approximately four-fold

slower than the average rate of approximately 4 microns/min seen in control cell centripetal flow (Figure 5G). Note that the difference in mean rates of flow between control and BDM treated cells was statistically significant at the $P < 0.01$ level based on the results of a T-test.

To determine whether actin arc generation in BDM-treated cells was dependent on actin polymerization, cells under the influence of BDM were exposed to the actin polymerization inhibitors cytochalasin D (CytoD) or latrunculin A (LatA). Both of these inhibitory drugs resulted in a cessation of the generation of arcs from the cell margin and a finite retraction of the cytoskeleton (Fig. 6). Digital video microscopy of living cells indicated that perfusion of CytoD resulted in the appearance of phase dense spots at the cell margin that elongated into tracks suggestive of actin polymerization sites that are being clogged due to the CytoD blockage of actin monomer addition at the barbed end (Figs. 6A-D, 6I; Supplemental Movie 3). Eventually these spots broke free of the plasma membrane and tracked back with the actin arcs in concert with cytoskeletal retraction, leaving a cleared cortical margin devoid of actin filaments. Similar spots were not as pronounced in cells treated with LatA (Figs. 6E-H, 6J; Supplemental Movie 4) and also appeared in the cleared zone in the wake of the retracted cytoskeleton. Similar to CytoD, LatA stopped actin arc generation and resulted in a cytoskeletal retraction and a cleared margin. Actin labeling of cells treated with CytoD and LatA confirmed the retraction of the BDM-induced arcs and the actin-free nature of the cleared margin, while at the same time it showed a thin rim of punctate actin staining remaining associated with the cell edge (Fig 6I, 6J).

Actin Arcs and Myosin II Activity

BDM has been described as an inhibitor of nonmuscle myosin II (Cramer and Mitchison, 1995; Forer and Fabian, 2005), and we performed a series of experiments aimed at determining whether BDM was inhibiting myosin II contractile activity in coelomocytes. Two lines of evidence suggested that the BDM-induced actin arc phenotype in coelomocytes was not the result of myosin II inhibition. First, the retraction of the cytoskeleton in BDM treated cells subjected to inhibition of actin polymerization (Fig. 6) is indicative of a residual actomyosin-based contractile tension on the cytoskeleton as first hypothesized by Forscher and Smith (1988) in experiments involving the retraction of the actin cytoskeleton in CytoD treated *Aplysia* growth cones. We have previously shown in coelomocytes that the post-CytoD retraction in control cells will not take place following pretreatment with myosin light chain kinase inhibitors that inhibit acto-myosin contraction (Henson et al., 2002), further reinforcing this relationship between acto-myosin contraction and cytoskeletal retraction. Second, immunolocalization of the active/phosphorylated (Ser19-P) form of the myosin regulatory light chain demonstrated that active myosin II was associated with the central cytoskeleton of BDM-treated cells (Fig. 7B), particularly in the regions corresponding to the radial bundles which serve as collection sites for the actin arcs. Note that in control cells active phosphorylated myosin II was associated with the perinuclear array of myosin II (Fig. 7A) that forms the basis of the retrograde flow-producing pulling force (Henson et al., 1999; 2003).

Laser-based Severing of Actin Arcs

In an effort to further explore the forces acting on the BDM-induced actin arcs we used a pulsed 532 nm laser to sever these filament bundles in living cells. Laser severing caused actin arcs to lose their curved structure indicative of a reduction in inherent tension (Fig. 8). In some experiments the end of the severed arc closest to the cell center (marked with an * in Figs 8C-E) continued to undergo centripetal flow suggestive of the maintenance of a “pulling” force based on acto-myosin contraction (Figs. 8A-E) and further supporting our notion that BDM was not inhibiting myosin II contractile activity. In previous studies using laser severing of actin stress fibers (Colombelli et al., 2005; Kumar et al., 2007) both ends of

these severed structures underwent persistent retraction consistent with the integration of myosin II contractile activity along the length of the fiber. Our localization studies indicate that myosin II was not present on the BDM-induced actin arcs (not shown) and we did not see evidence of the retraction of both ends of the severed bundles. In other experiments on coelomocytes severing of an actin arc led to abrupt alterations in actin structure in adjacent regions (Figs. 8F-J) underscoring the interconnected nature of the overall cortical actin cytoskeleton. To our knowledge this is one of the initial demonstrations of the laser-based severing of an actin filament bundle inside a cell that is not a stress fiber.

DISCUSSION

Yarrow et al. (2003) previously demonstrated that BDM treatment of mammalian cells resulted in a de-localization of the LP leading edge components Arp2/3 complex, WAVE and VASP. Our present study on coelomocytes extends this work through the use of pharmacological treatments and light and electron microscopic methods to analyze the structure and dynamics of the BDM-induced phenotype. The chief feature of the actin organization remaining following the BDM-based displacement of the Arp2/3 complex-containing LP network in coelomocytes is an array of loose bundles of elongate actin filaments in the form of concave arcs oriented parallel to the plasma membrane (Figs. 1-8). These actin arcs were generated by polymerization at the cell edge (Figs. 5, 6) and underwent retrograde flow (Fig. 8) in which they formed at the cytoplasmic face of the membrane and moved centripetally at roughly one fourth the rate seen in control cells (Fig. 5). The elongate nature of the actin filaments in the BDM-induced arcs and their relatively slow rate of myosin II-dependent flow suggest that this actin array may correspond to the coelomocyte equivalent of the LM. A number of studies suggest that the LM and LP are autonomous actin networks with the LP lying over top of the LM [reviewed in Chhabra and Higgs, 2007]. However, our TEM images of coelomocyte cortical actin (Figs. 3, 4) suggest that the elongate actin filaments present in the BDM-induced arcs are actually intermeshed with, instead of separate from the Arp2/3 complex dendritic array. This is particularly evident in TEM images of coelomocytes undergoing recovery from BDM treatment in which the Arp2/3 complex-based network formation was reinitiated at the cell edge and the elongate filaments from the BDM zone are clearly intercalated into this network (Fig. 4). It is possible that unlike some other cell types, the coelomocyte LM array is integrated into the LP. A recent study by Koestler et al. (2008) using correlative light and electron microscopy of B16 cells indicated that the LP network is not superimposed on another actin array and that this network contained numerous long filaments. One possible function of elongate filaments in the LP would be for them to serve as a connection between this structure and the central cytoskeleton [as suggested by Yang et al., 2007]. Evidence of a physical connection between the LP and the central cytoskeleton in coelomocytes was seen in the myosin-based retraction that the entire actin cytoskeleton undergoes following inhibition of actin polymerization (Fig. 6) [see Henson et al., 1999; 2002]. This retraction initiates at the cell edge suggesting that the Arp2/3 complex-dependent LP is physically continuous with the central actin cytoskeleton. We speculate that the elongate actin filaments revealed by BDM treatment may form the basis for this type of cytoskeletal connectivity.

In terms of relating the actin structures present during BDM treatment to those seen in other cells, elongate actin filaments intermeshed into the cortical network and oriented at low angles to the plane of the membrane are evident in TEM images of the LP regions of a number of cell types (see Small et al., 1995; Svitkina and Borisy, 1999; Svitkina et al., 2003; Verkhovsky et al., 2003; Svitkina, 2007; Yang et al., 2007). This suggests that these elongate actin filaments are a common feature of the cortical actin array. In addition Koestler et al. (2008) recently reported that elongate actin filaments parallel to the cell edge are particularly prominent in areas not undergoing active protrusion. They demonstrated that

changes from protrusion to pause at the cell edge are associated with an increase in filaments oriented more parallel with the cell margin. The presence of extensive parallel actin filaments in coelomocytes is consistent with the non-protrusive nature of this cell type. Our current hypothesis is that the BDM-induced and Arp2/3 complex-independent actin filaments in coelomocytes derive from the activity of membrane-associated formin/mDia proteins given the ability of these proteins to favor the nucleation and growth of elongate and unbranched actin filaments and to resist the action of the barbed end capping proteins that limit actin filament length (reviewed by Evangelista et al., 2003; Faix and Grosse, 2006; Chhabra and Higgs, 2007). In other cell types formin/mDia has been shown to localize to the cell edge (Gupton et al., 2007; Yang et al., 2007) and manipulation of formin/mDia activity has been associated with alterations in the structure and behavior of the LP (Gupton et al., 2007; Yang et al., 2007). Importantly, Yang et al. (2007) has demonstrated using mammalian cells that siRNA knock down of mDia2 resulted in a decrease in long filaments in the LP while constitutively active mDia2 led to an increase in LP elongate filaments. It is possible that the BDM-induced actin arcs correspond to formin/mDia induced “mother” filaments hypothesized by Yang et al. (2007) and Gupton et al. (2007) to exist at the cell edge in order to provide a stable platform/scaffold for the Arp2/3 complex-dependent nucleation of the dendritic LP network. Confirmation of the role for formin/mDia proteins in the generation of elongate actin filaments in coelomocytes awaits the success of our pursuit of specific probes for the sea urchin formin/mDia homologues.

A possible alternative explanation for the elongate filaments is via association with tropomyosin, an actin filament stabilizing protein. Studies have shown that tropomyosin is associated with long actin filaments in the LM (DesMarais et al., 2002; Gupton et al., 2005), can protect actin filaments from severing (Bernstein and Bamburg, 1982) and depolymerization (Broschat, 1990), can interfere with Arp2/3 complex-mediated branching (Blanchoin et al., 2001), and, when present in excess, inhibit functional LP formation (Gupton et al., 2005). Given that tropomyosin is restricted to the LM zone away from the cell edge in other cells (DesMarais et al., 2002; Gupton et al., 2005), we do not believe it provides the basis for the elongate LP filaments in coelomocytes revealed by BDM treatment.

The specific mechanisms underlying BDM's influence on actin organization in coelomocytes are difficult to determine. We are confident that the actin arcs do not derive from an inhibition of myosin II's ability to form contractile interactions with actin. We present evidence of the retention of myosin II activity under the influence of BDM in the form of the retraction of the central cytoskeletal in BDM treated cells exposed to actin polymerization inhibitors (Fig. 6), the localization of active phospho-myosin II in BDM treated cells (Fig. 7), and the tension release and centripetal motion of the proximal ends of arcs severed by laser pulses (Fig. 8). These results are in agreement with previous biochemical studies that indicate that BDM does not inhibit the function of several types of nonmuscle myosins (Cheung et al., 2001; Ostap, 2002). Given BDM's ability to impact the distribution of the Arp2/3 complex-dependent LP network, it is possible that this drug directly impacts Arp2/3 complex-facilitated polymerization. Recent work by LeClaire et al. (2008) suggests that phosphorylation of the Arp2/3 complex is necessary for nucleation function and the chemical phosphatase action of BDM could be impacting the phosphorylation status of the complex. Alternatively, the ATPase inhibitor activity of BDM may be interfering with ATP hydrolysis by either actin itself or the Arp2/3 complex, although ATP hydrolysis by the Arp2/3 complex appears to be associated with but not essential for actin polymerization (Pollard, 2007). However, Yarrow et al. (2003) indicate that BDM does not inhibit either Arp2/3 complex-mediated actin polymerization in vitro and or the in vivo Arp2/3-dependent intracellular motility of *Listeria* bacteria. Perhaps BDM treatment is inhibiting cofilin/ADF-based actin depolymerization which is thought to be

important in the structuring of the LP network (Ichetovkin et al., 2002). Shaner et al. (2005) have indicated that BDM treatment does inhibit actin depolymerization in the actin rich pedestals induced by Enteropathogenic *E. coli* (EPEC) interaction with cultured PtK2 cells. Another possibility is that BDM is interfering with capping protein activity and the resulting elongate actin filaments are helping displace the LP network. Two studies employing *Drosophila* S2 cells (Rogers et al., 2003; Iwasa and Mullins, 2007) have shown that RNAi-based depletion of capping protein resulted in displacement of the LP and Arp2/3 complex away from the cell edge and the accumulation of elongate cortical actin filaments - sometimes in the form of curved marginal actin bundles (Iwasa and Mullins, 2007) reminiscent of BDM-induced arcs in coelomocytes. Still another attractive possibility is that BDM is down regulating the lamellipodial-favoring activity of Rac/Cdc42 perhaps through a stimulation of Rho activation (as suggested by Danilchick et al., 2006). However our preliminary, unpublished studies show a lack of apparent association between BDM treatment and Rho activation and that the BDM phenotype is present in cells pretreated with Rho/ROCK inhibitors. Finally, BDM treatment has also been reported to alter aspects of calcium transport and release in cells (Gwathmey et al., 1991; Watanabe et al., 2001; Turvey et al., 2003) and elevations in intracellular calcium have been shown to transform coelomocytes from a lamellipodial to filopodial form (Henson and Schatten, 1983). However in our preliminary, unpublished studies we have not been able to reproduce the BDM phenotype in coelomocytes in which intracellular calcium levels are altered via calcium ionophore treatment. Clearly elucidation of BDM's precise mechanism will require additional experimentation although at present we favor the hypothesis that this drug is down regulating the ability of the Arp2/3 complex to facilitate actin polymerization.

It could be argued that not knowing the exact mechanism and specificity of BDM makes this drug useless as a cell biological tool, however there are a number of counter arguments in favor of continuing the characterization of its effects. First of all, the drug continues to be used as a nonmuscle myosin II inhibitor in a number of cell types [examples of recent papers include Lu et al., 2008; Esseling-Ozdoba et al., 2008; Lee and Song, 2007]. We agree with Yarrow et al. (2003) that many of the effects of BDM ascribed to myosin II inhibition can be explained based on the drug's ability to interfere with canonical Arp2/3 complex-based actin polymerization mechanisms. Second, BDM does produce interesting phenotypes in experimental systems that have led to significant insights such as the BDM-induced cortical rotation in *Xenopus* eggs (Danilchik et al., 2006) and alterations in the EHEC pedestals on PtK2 cells (Shaner et al., 2005). Third, unlike cytochalasins and latrunculins, BDM can be washed out quickly which allows for rapid recovery post treatment (Fig. 4) (Henson et al., 1999; Danilchik et al., 2006). Fourth, BDM has potential biomedical importance given that it has shown promise in promoting the viability of isolated cardiomyocytes and explanted hearts (Thum and Barlak, 2001a,b; Sambrano et al., 2002) as well as in improving the functioning of transplanted hearts (Warnecke et al., 2002).

CONCLUSIONS

In summary, we have demonstrated that BDM treatment of sea urchin coelomocytes results in the centripetal displacement of the Arp2/3 complex-dependent LP actin network and the revelation of an array of elongate actin filament bundles oriented parallel to the cell edge. These actin arcs are generated at the cell edge via actin polymerization and undergo an apparent myosin II-dependent form of retrograde flow. Rotary shadow TEM images suggest that these elongate filaments are an Arp2/3 complex-independent component of the actin LP array and we speculate that they may provide a connection between the LP and the central actin cytoskeleton. Our current hypothesis is that these filaments are generated at the cell edge by the action of formin/mDia proteins that help nucleate and promote the growth of elongate actin filaments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Special thanks are extended to Dr. Tanya Svitkina (University of Pennsylvania) for hosting several visits to her laboratory and for her expert and invaluable assistance with preparation of critical point dry and rotary shadow TEM replicas. Thanks are also extended to Dr. Joann Otto (Western Washington University) for her gift of sea urchin fascin antibody, Dr. John Cooper (Washington University) for helpful discussions, Grant Harris (MBL) for technical support of the LC-PolScope and laser ablation system, and Dr. Rob Jinks (Franklin and Marshall College) and Louie Kerr (MBL) for help in using their TEMs. In addition Dr. Robert Morris (Wheaton College) provided productive interactions as well as assistance with experiments. This research was supported by NIH grant GM60925 and Laura and Arthur Colwin Sabbatical and Summer Research Fellowships from the MBL to JHH, NIH grant EB002583 to RO, and the MBL Laura and Arthur Colwin Summer Research Fellowship from the MBL and NIH grant GM08136 to CBS.

REFERENCES

- Bernstein BW, Bamburg JR. Tropomyosin binding to F-actin protects the F-actin from disassembly by brain actin-depolymerizing factor (ADF). *Cell Motil.* 1982; 2:1–8. [PubMed: 6890875]
- Blanchoin L, Pollard TD, Hitchcock-DeGregori S. Inhibition of the Apr2/3 complex nucleated actin polymerization and branch formation by tropomyosin. *Curr Biol.* 2001; 11:1300–1304. [PubMed: 11525747]
- Broschat KO. Tropomyosin prevents depolymerization of actin filaments from the pointed end. *J Biol Chem.* 1990; 265:21323–21329. [PubMed: 2250026]
- Cheung A, Dantzig JA, Hollingworth S, Baylor SM, Goldman YE, Mitchison TJ, Straight AF. A small-molecule inhibitor of skeletal muscle myosin II. *Nature Cell Biol.* 2002; 4:83–88. [PubMed: 11744924]
- Chhabra ES, Higgs HN. The many faces of actin: matching assembly factors with cellular structures. *Nature Cell Biol.* 2007; 9:1110–1121. [PubMed: 17909522]
- Colombelli J, Reynard EG, Rietdorf J, Pepperkok R, Stelzer EHK. In vivo selective cytoskeletal dynamics quantification in interphase cells induced by pulsed ultraviolet laser nanosurgery. *Traffic.* 2005; 6:1093–1102. [PubMed: 16262721]
- Cramer LP, Mitchison TJ. Myosin is involved in postmitotic cell spreading. *J Cell Biol.* 1995; 131:179–189. [PubMed: 7559774]
- Danilchik MV, Brown EE, Riegert K. Intrinsic chiral properties of the *Xenopus* egg cortex: an early indicator of left-right asymmetry? *Development.* 2006; 113:4517–4526. [PubMed: 17050623]
- Danuser G. Coupling the dynamics of two actin networks - new views on the mechanics of cell protrusion. *British Soc Trans.* 2005; 33:1250–1253.
- DesMarais V, Ichetovkin I, Condeelis J, Hitchcock-Grepori SE. Spatial regulation of actin dynamics: a tropomyosin-free, actin-rich compartment at the leading edge. *J Cell Sci.* 2002; 115:4649–4660. [PubMed: 12415009]
- Durán JM, Valderrama F, Castel S, Magdalena J, Tomas M, Hosoya H, Renau-Piqueras J, Malhotra V, Egea G. Myosin motors and not actin comets are mediators of the actin-based golgi-to-endoplasmic reticulum protein transport. *Mol Biol Cell.* 2003; 14:445–459. [PubMed: 12589046]
- Edds K. Isolation and characterization of two forms of a cytoskeleton. *J Cell Biol.* 1979; 83:109–115. [PubMed: 574512]
- Esseling-Ozdoba A, Houtman D, Van Lammeren AA, Eisner E, Emons AM. Hydrodynamic flow in the cytoplasm of plant cells. *J Microsc.* 2008; 231:274–283. [PubMed: 18778425]
- Evangelista M, Zigmond S, Boone C. Formins: signaling effectors for assembly and polarization of actin filaments. *J Cell Sci.* 2003; 116:2603–2611. [PubMed: 12775772]
- Faix J, Grosse R. Staying in shape with formins. *Devel Cell.* 2006; 10:693–706. [PubMed: 16740473]
- Forscher P, Smith SJ. Actions of cytochalasins and colcemid on the organization of actin filaments and microtubules in a neuronal growth cone. *J Cell Biol.* 1988; 107:1505–1516. [PubMed: 3170637]

- Forer A, Fabian L. Focus on cellular biochemistry. Does 2,3-butanedione monoxime inhibit nonmuscle myosin? *Protoplasma*. 2005; 225:1–4. [PubMed: 15868207]
- Fryer M, Neering I, Stephenson D. Effects of 2,3-butanedione monoxime on the contractile activation properties of fast- and slow-twitch muscle fibers. *J Physiol (Lond)*. 1988; 415:601–624.
- Funaki K, Nagata A, Akimoto Y, Shimada D, Ito K, Yamamoto K. The motility of *Chara corallina* myosin was inhibited reversibly by 2,3-butanedione monoxime (BDM). *Plant Cell Physiol*. 2004; 45:1342–1345. [PubMed: 15509860]
- Giannone G, Dubin-Thaler BJ, DÖbereiner H-G, Kieffer N, Bresnick AR, Sheetz MP. Periodic lamellipodial contractions correlate with rearward actin waves. *Cell*. 2004; 116:431–443. [PubMed: 15016377]
- Gupton SL, Anderson KL, Kole TP, Fischer RS, Ponti A, Hitchcock-DeGregori SE, Danuser G, Fowler VM, Wirtz D, Hanein D, Waterman-Storer CM. Cell migration without a lamellipodium: translation of actin dynamics into cell movement mediated by tropomyosin. *J Cell Biol*. 2005; 168:619–631. [PubMed: 15716379]
- Gupton SL, Eisenman K, Alberts A, Waterman-Storer CM. mDia2 regulates actin and focal adhesion dynamics and organization in the lamella for efficient cell migration. *J Cell Sci*. 2007; 120:3475–3487. [PubMed: 17855386]
- Gwathmey JK, Hajjar RJ, Solaro RJ. Contractile deactivation and uncoupling of crossbridges. Effects of 2,3-butanedione monoxime on mammalian myocardium. *Circ Res*. 1991; 69:1280–1292. [PubMed: 1934358]
- Henson JH, Schatten G. Calcium regulation of the actin mediated cytoskeletal transformation of the sea urchin coelomocyte. *Cell Motil*. 1983; 3:525–534. [PubMed: 6420068]
- Henson JH, Nesbitt D, Wright BD, Scholey JS. Immunolocalization of kinesin in sea urchin coelomocytes: associations of kinesin with intracellular organelles. *J Cell Sci*. 1992; 103:309–320. [PubMed: 1478935]
- Henson JH, Svitkina T, Burns AR, Hughes HE, McPartland KJ, Nazarian R, Borisy GG. Two components of actin-based retrograde flow in sea urchin coelomocytes. *Mol Biol Cell*. 1999; 10:4075–4090. [PubMed: 10588644]
- Henson JH, Nazarian R, Schulberg KL, Trabosh VA, Kolnik SE, Burns AR, McPartland KJ. Wound healing in the lamellipodia of single cells: mediation by actin polymerization in the absence of an actomyosin purse string. *Mol Biol Cell*. 2002; 13:1015–1029. [PubMed: 11907279]
- Henson JH, Kolnik S, Fried C, Nazarian R, McGreevy J, Schulberg KL, Detweiler M, Trabosh VA. Actin-based centripetal flow: phosphatase inhibition by Calyculin A alters flow pattern, actin organization and actomyosin distribution. *Cell Motil Cytoskeleton*. 2003; 56:252–266. [PubMed: 14584028]
- Iwasa JH, Mullins RD. Spatial and temporal relationships between actin-filament nucleation, capping, and disassembly. *Current Biol*. 2007; 17:395–406.
- Koestler SA, Auinger S, Vinzenz M, Rottner K, Small JV. Differentially oriented populations of actin filaments generated in lamellipodia collaborate in pushing and pausing at the cell front. *Nature Cell Biol*. 2008; 10:306–313. [PubMed: 18278037]
- Kumar S, Maxwell IZ, Heisterkamp A, Polte TR, Lele TP, Salanga M, Mazur E, Ingber DE. Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization and extracellular matrix mechanics. *Biophys J*. 2006; 90:3762–3773. [PubMed: 16500961]
- LeClaire LL, Baumgartner M, Iwasa JH, Mullins RD, Barber DL. Phosphorylation of the Arp2/3 complex is necessary to nucleate actin filaments. *J Cell Biol*. 2008; 182:647–654. [PubMed: 18725535]
- Lee K, Song K. Actin dysfunction activates ERK1/2 and delays entry into mitosis in mammalian cells. *Cell Cycle*. 2007; 6:1487–1495. [PubMed: 17582224]
- Lu L, Feng Y, Hucker WJ, Oswald SJ, Longmore GD, Yin FC. Actin stress fiber pre-extension in human aortic endothelial cells. *Cell Motil Cytoskel*. 2008; 65:281–294.
- Medeiros NA, Burnette DT, Forscher P. Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nature Cell Biol*. 2006; 8:215–226. [PubMed: 16501565]

- McCurdy DW. Is 2,3-butanedione monoxime an effective inhibitor of myosin-based activities in plant cells? *Protoplasma*. 1999; 209:120–125. [PubMed: 18987800]
- Nemethova M, Auinger S, Small JV. Building the actin cytoskeleton: filopodia contribute to the construction of contractile bundles in the lamellum. *J Cell Biol*. 2008; 180:1233–1244. [PubMed: 18362182]
- Ostap EM. 2,3-Butanedione monoxime (BDM) as a myosin inhibitor. 2002. *J Muscle Res Cell Motil*. 2002; 23:305–308. [PubMed: 12630704]
- Österman A, Arner A, Malmqvist U. Effects of 2,3-butanedione monoxime on activation of contraction and crossbridge kinetics in intact and chemically skinned smooth muscle fibers from guinea pig taenia coli. *J Muscle Res Cell Motil*. 1993; 14:186–194. [PubMed: 8315022]
- Otto JJ, Kane RE, Bryan J. Formation of filopodia in coelomocytes: localization of fascin, a 58,000 dalton actin cross-linking protein. *Cell*. 1979; 17:285–293. [PubMed: 378407]
- Pollard TD. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annu Rev Biophys Biomol Struct*. 2007; 36:451–477. [PubMed: 17477841]
- Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 2003; 112:453–465. [PubMed: 12600310]
- Ponti A, Machacek M, Gupton SL, Waterman-Storer CM, Danuser G. Two distinct actin networks drive the protrusion of migrating cells. *Science*. 2004; 305:1782–1786. [PubMed: 15375270]
- Rogers SL, Wiedemann U, Stuurman N, Vale RD. Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J Cell Biol*. 2003; 162:1079–1088. [PubMed: 12975351]
- Sambrano GR, Fraser I, Han H, Ni Y, O'Connell T, Yan Z, Stull JT. Navigating the signaling network in mouse cardiac myocytes. *Nature*. 2002; 420:712–714. [PubMed: 12478303]
- Shaner NC, Sanger JW, Sanger JM. Actin and alpha-actinin dynamics in the adhesion and motility of EPEC and EHEC on host cells. *Cell Motil Cytoskel*. 2005; 60:104–120.
- Small JV, Herzog M, Anderson K. Actin filament organization in the fish keratocyte lamellipodium. *J Cell Biol*. 1995; 129:1275–1286. [PubMed: 7775574]
- Small JV, Auinger S, Nemethova M, Koestler S, Goldie KN, Hoenger A, Resch GP. Unravelling the structure of the lamellipodium. *J Microsc*. 2008; 231:479–485.
- Svitkina T. Electron microscopic analysis of the leading edge of cells. *Meth Cell Biol*. 2007; 79:295–319.
- Svitkina TM, Borisy GG. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J Cell Biol*. 1999; 145:1009–1026. [PubMed: 10352018]
- Svitkina TM, Bulanova EA, Chaga OY, Vignjevic DM, Kojima S, Vasiliev JM, Borisy GG. Mechanism of filopodial initiation by reorganization of a dendritic network. *J Cell Biol*. 2003; 160:409–421. [PubMed: 12566431]
- Thum T, Borlak J. Butanedione monoxime increases the viability and yield of adult cardiomyocytes in primary culture. *Cardiovasc Toxicol*. 2001A; 1:61–72. [PubMed: 12213998]
- Thum T, Borlak J. Reprogramming of gene expression in cultured cardiomyocytes and in explanted hearts by the myosin ATPase inhibitor butanedione monoxime. *Transplantation*. 2001B; 71:543–552.
- Turvey MR, Laude AJ, Ives EO, Seager WH, Taylor CW, Thorn P. Modulation of IP₃-sensitive Ca²⁺ release by 2,3-butanedione monoxime. *Pflugers Arch*. 2003; 445:614–621. [PubMed: 12634934]
- Vallotton P, Gupton SL, Waterman-Storer CM, Danuser G. Simultaneous mapping of filamentous actin flow and turnover in migrating cells by quantitative fluorescent speckle microscopy. *Proc Natl Acad Sci USA*. 2004; 101:9660–9665. [PubMed: 15210979]
- Verkhosky AB, Chaga OY, Schaub S, Svitkina TM, Meister JJ, Borisy GG. Orientational order of the lamellipodial actin network as demonstrated in living cells. *Mol Biol Cell*. 2003; 14:4667–4675. [PubMed: 13679520]
- Warnecke Y, Schulze B, Hagl C, Haverich A, Klima U. Improved right heart function after myocardial preservation with 2,3-butanedione monoxime in a porcine model of allogeneic heart transplantation. *J Thorac Cardiovasc Surg*. 2002; 123:81–88. [PubMed: 11782759]

- Watanabe Y, Iwamoto T, Matsuoka I, Ohkubo S, Ono T, Watano T, Shigekawa M, Kimura J. Inhibitory effect of 2,3-butanedione monoxime (BDM) on $\text{Na}^+/\text{Ca}^{2+}$ exchange current in guinea-pig cardiac ventricular myocytes. *British J Pharm.* 2001; 132:1317–1325.
- Yang C, Czech L, Gerboth S, Shin-ichiro K, Scita G, Svitkina T. Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. *PLoS Biol.* 2007; 5:2624–2645.
- Yarrow JC, Lechler T, Li R, Mitchison TJ. Rapid de-localization of actin leading edge components with BDM treatment. *BMC Cell Biol.* 2003; 4:5. [PubMed: 12783627]
- Zhang XF, Schaefer AW, Burnette DT, Schoonderwoert VT, Forscher P. Rho-dependent contractile responses in the neuronal growth cone are independent of classical peripheral retrograde actin flow. *Neuron.* 2003; 40:931–944. [PubMed: 14659092]
- Zhou R, Watson C, Fu C, Yao X, Forte JG. Myosin II is present in gastric parietal cells and required for lamellipodial dynamics associated with cell activation. *Am J Physiol.* 2003; 285:662–673.

Figure 1.

BDM treatment displaces the Arp2/3 complex-based actin LP network. Phase contrast (A-C) and LC-PolScope (D) images illustrate that BDM treatment (minutes post treatment appear at the top of panels A-C) caused the centripetal retraction of the dense actin meshwork present at the edge of control cells (A) and induced formation of a series of elongate, concave, and birefringent arcs oriented parallel to the cell edge (B-D). Staining with fluorescent phalloidin indicated that these arcs consist of bundles of F-actin (E). Immunolocalization of actin (F, I) and Arp3 (G, J) in control (F-H) and BDM-treated (I-K, treatment time 10 min) cells show that BDM-treatment induced centripetal displacement of the Arp3 labeling and that the cortical actin arcs appeared devoid of Arp3 staining. Bar for A-C and F-K ; D and E = 10 microns.

Figure 2.

BDM-induced actin arcs do not contain the actin bundling protein fascin. Staining of actin (A) and fascin (B) in cells treated with BDM (A, B) showed that fascin was not associated with the actin arcs present at the cell edge (note that the brightness and contrast in these images was increased to make the arcs more visible). This contrasted with cells transformed to a filopodial form by treatment with hypotonic shock (C, D) in which the filopodial core actin bundles (C) were heavily stained for fascin (D). Bar for A-D = 10 microns.

Figure 3.

TEM of rotary shadow platinum replicas of control (A, C) and BDM-treated (B, D, treatment time = 10 min) cells illustrated the dense actin meshwork present in control cells and the elongate actin arcs oriented parallel to the edge that result from BDM treatment. Close examination of cortical actin structure in control cells revealed that there are elongate actin filaments intermeshed into the LP network (highlighted by arrows in panel C) resembling the filaments that make up the BDM-induced bundles. Bar in A and B = 10 microns; C and D = 1 micron.

Figure 4.

BDM removal reinitiates formation of the Arp2/3-based LP actin network.

Immunolocalization of actin (A) and Arp3 (B) in a cell 1 min following BDM wash out demonstrated the immediate initiation of Arp2/3 complex-based actin meshwork formation at the cell edge (A-C). TEM of the leading edge 1 min after BDM wash out (D) revealed a newly forming dendritic actin meshwork at the cell edge and an array of elongate actin filaments extending centripetally from the nascent network at the cell edge into the remnant of the arcs present in the BDM-induced zone. Bar for A-C = 10 microns; in D = 1 micron.

Figure 5.

BDM-induced arcs exhibit retrograde flow at a rate slower than control cells. Digitally enhanced phase contrast imaging of a BDM treated cell (A-F, time post treatment in min:sec appears at the top of each panel) showing the retraction of the central cytoskeleton and the appearance of the characteristic actin arcs. These arcs peeled away from the cytoplasmic face of the plasma membrane and moved centripetally. The ends of arcs appeared to coalesce around regions of the central cytoskeleton that contain radially oriented phase dense ribs (arrow in F) suggesting that tension is being generated in this area. The graph in G shows that the rate of centripetal flow in control cells was some 4 fold higher than the flow of actin arcs in BDM treated cells. Bar for A-F = 10 microns.

Figure 6.

Actin polymerization is required for the generation of BDM-induced actin arcs. Phase contrast imaging of live cells (A-H) showed that actin arc generation ceased in BDM treated cells (A, E, BDM treatment time = 5 min) in which actin polymerization was inhibited by exposure to either CytoD (B-D) or LatA (E-H), and both inhibitors also resulted in the overall retraction of the actin cytoskeleton (time post exposure to the inhibitor appears at the top of panels B-D and F-H). The arrow in D denotes a microtubule that extended out from the cell center. Actin localization in cells treated with BDM for 5 min then CytoD for 10 min (I) or BDM for 5 min then LatA for 10 min (J) demonstrates the retracted central actin cytoskeleton containing the BDM-induced arcs, a cleared marginal zone free of actin and a thin rim of punctate actin labeling along the cell edge. Bar for A-J = 10 microns.

Figure 7. BDM treated cells contain active myosin II. Localization of actin (red) and phosphorylated MRLC (green) in control (A) and BDM treated (B) cells revealed that activated myosin II was concentrated in a perinuclear array (nucleus stained blue) under control conditions (A) and spread into the central cytoskeleton following treatment with BDM (B). In the BDM treated cell, the active myosin II concentrated in the radial ribs that serve as collection points for arc terminations (see Fig. 5F). Bar = 10 microns.

Figure 8.

Laser-mediated severing of BDM-induced actin arcs. BDM treated cells (A, F) were subjected to laser severing and followed by timelapse phase contrast microscopy. The boxes in A and F correspond to the magnified regions shown in B-E and G-J. In the top example (A-E) laser severing of an actin bundle (arrow in C) resulted in the loss of curvature and the continued centripetal movement of the proximal end. The arrow in E indicated that new actin arcs continue to be generated following laser severing (total elapsed time following laser firing = 10 mins). In the bottom example (F-J) the laser severing (arrow in I) of an arc undergoing centripetal flow (G-H) resulted in rapid deformation of the bundle with the upper cut end swinging to the right, resulting in the transmission of force within the connected array as demonstrated by the breakage of a linkage between two arcs (arrow in H). The lower end of the severed bundle continued to move centripetally (total time elapsed post laser firing = 4 min). Bar = 10 microns.