Actin turnover is required to prevent axon retraction driven by endogenous actomyosin contractility

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Growth cone motility and guidance depend on the dynamic reorganization of filamentous actin (F-actin). In the growth cone, F-actin undergoes turnover, which is the exchange of actin subunits from existing filaments. However, the function of F-actin turnover is not clear. We used jasplakinolide (jasp), a cell-permeable macrocyclic peptide that inhibits F-actin turnover, to study the role of F-actin turnover in axon extension. Treatment with jasp caused axon retraction, demonstrating that axon extension requires F-actin turnover. The retraction of axons in response to the inhibition of F-actin turnover was dependent on myosin activity and regulated by RhoA and myosin light chain kinase. Significantly, the endogenous myosin-based contractility was sufficient to cause axon retraction, because jasp did not alter myosin activity. Based on these observations, we asked whether guidance cues that cause axon retraction (ephrin-A2) inhibit F-actin turnover. Axon retraction in response to ephrin-A2 correlated with decreased F-actin turnover and required RhoA activity. These observations demonstrate that axon extension depends on an interaction between endogenous myosin-driven contractility and F-actin turnover, and that guidance cues that cause axon retraction inhibit F-actin turnover.

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Introduction

The extension and guidance of axons depends on the activity of the growth cone. Growth cones are highly motile, actively extending and retracting filopodia and lamellipodia. The protrusion of lamellipodia and filopodia requires the polymerization of filamentous actin (F-actin)* at the leading edge of the growth cone (for review see Suter and Forscher, 2000). After polymerization, F-actin is retrogradely transported toward the center of the growth cone by a myosin-based mechanism and is subsequently depolymerized (Lin et al., 1996). In the growth cone, F-actin is dynamic and undergoes turnover (Okabe and Hirokawa, 1990; Mallavarapu and Mitchison, 1999). F-actin turnover is the result of polymerization at the barbed ends of filaments in conjunction with depolymerization at the pointed ends, resulting in filament "treadmilling" (Pollard et al., 2000). Estimates of the rate of F-actin turnover in the lamellipodia of growth cones indicate that F-actin is completely recycled by turnover within 3–5 min, whereas filopodial actin bundles are more stable (Mallavarapu and Mitchison, 1999). However, although the role of F-actin polymerization in driving leading-edge protrusion is well established, the significance of F-actin turnover after polymerization is not known.

Growth cones "pull" by generating contractile forces (Lamoureux et al., 1989), and axon tension is an important component of axon extension (Heidemann and Buxbaum, 1994). The generation of cellular contractile forces often depends on actomyosin-based contractility. Antisense-mediated down-regulation of myosin IIA and IIB has been shown to inhibit neurite extension in neuro-2A cells (Wylie and Chantler, 2001), and myosins are involved in the protrusion of filopodia and lamellipodia (Jay, 2000; Bridgman et al., 2001). Importantly, the retrograde transport of F-actin in growth cones is driven by myosin motors (Lin et al., 1996). These reports indicate that myosins are required for axon extension.

Although myosins have been shown to be required for axon extension, myosin-based contractility also has been associated with the process of axon retraction. Myosin activity drives the retraction of axons in response to the perturbation of microtubule motor proteins (Ahmad et al., 2000). Similarly, the activity of the GTPase RhoA and its downstream kinase ROCK, which drive actomyosin-based contractility, cause

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*Abbreviations used in this paper: BDM, 2,3-butanedione monoxime; CaRhoA, constitutively active RhoA; DRG, dorsal root ganglion; jasp, jasplakinolide; lat-A, latrunculin-A; MLCK, myosin light chain kinase; mRLC, myosin regulatory light chains; RGC, retinal ganglion cell; skMyosin II, skeletal muscle myosin II.

Key words: jasplakinolide; RhoA; ephrin; cytoskeleton; myosin

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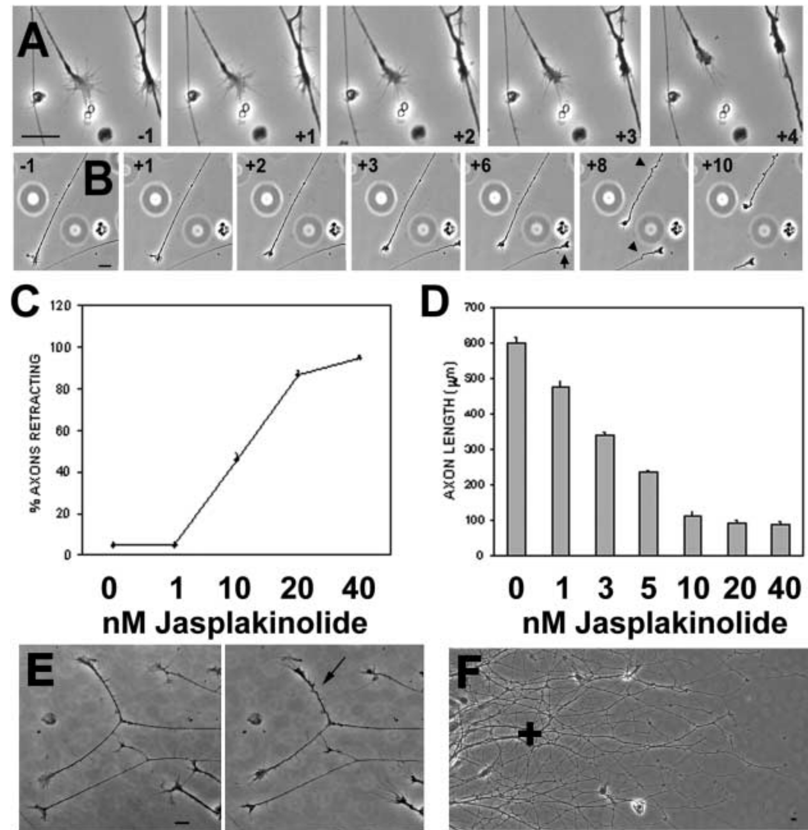

Figure 1. **jasp induces axon retraction.** Numbers in individual panels indicate minutes relative to the addition of jasp (40 nM at 0 min). The bars in the leftmost panels denote 10 µm. (A) Within 2–3 min after treatment with jasp, growth cones became quiescent and underwent contraction followed by axonal retraction. (B) Axons began retracting 4–6 min after treatment with jasp. Axon retraction often resulted in the formation of axonal bends (indicated by arrowheads at 8 min). Note that at 6 min after treatment, an additional retracting growth cone becomes visible (arrow). (C) jasp caused axon retraction during a 20-min observation period in a concentration-dependent manner ($n \geq 37$ axons at each concentration). (D) Overnight treatment of DRG explant cultures with jasp caused a dose-dependent decrease in the length of axon outgrowth ($n > 8$ explants per concentration). (E) Localized application of jasp to growth cones causes contraction and axon retraction. Bends in axons form distally after growth cone contraction (arrow). (F) Application of jasp to axons does not cause the formation of axon bends at the site of application, but after prolonged application and diffusion of jasp, axons retract with bends first forming at the distal end of axons. The pipette was removed before acquisition of the images from which the montage is created. The pipette position is denoted by the + symbol. Bars, 10 µm.

neurite retraction (Kozma et al., 1997; Katoh et al., 1998). Furthermore, axon guidance cues that cause retraction act through a RhoA-dependent mechanism (Katoh et al., 1996; Kranenburg et al., 1999; Wahl et al., 2000). Negative regulation of RhoA or ROCK activity prevents the retraction of established axons in vivo (Billuart et al., 2001). Collectively, these studies demonstrate that myosin activity is required for both axon extension and retraction. Thus, how myosin-based contractility is regulated to contribute to axon extension versus retraction is an important issue in the biology of axon extension and guidance.

In this paper, we demonstrate that pharmacological inhibition of F-actin turnover causes myosin-dependent axon retraction and inhibition of axon extension. Additionally, we provide evidence that the repellent guidance cue (ephrin-A2) decreases the turnover of F-actin in retinal axons and causes RhoA-dependent axon retraction. These data reveal for the first time that axon extension and retraction depend on an interaction between F-actin turnover and myosin-based contractility.

Results

Jasplakinolide (jasp) causes growth cone contraction and axon retraction

To determine the response of growth cones to the inhibition of F-actin turnover, we investigated the effects of jasp on retinal and dorsal root ganglion (DRG) neuron cultures. jasp is a cell-permeable macrocyclic peptide that binds to F-actin and inhibits depolymerization at the pointed ends of filaments, thereby inhibiting filament turnover. jasp has been used to investigate actin turnover in a number of cell types (Lee et al., 1998; Cramer, 1999; Ayscough, 2000; Lautermilch and Spitzer, 2000; Watanabe and Mitchison, 2002).

Videomicroscopic observations of both retinal and DRG axons demonstrated that after treatment with jasp, growth cone motility, defined as lamellipodial and filopodial extension, stopped and centripetal contraction of the peripheral domain begun within 3–5 min after treatment (Fig. 1 A). After growth cone contraction, axons started to retract 4–6 min after treatment with jasp (Fig. 1 B). The percentage of axons retracting was maximal at 40 nM jasp (Fig. 1 C). During retraction, in response to 40 nM jasp, 73% of axons became deformed and bent (Fig. 1 B). The response of growth cones and axons to jasp was the same in embryonic chick DRG cells and retinal ganglion cells (RGC), and embryonic mouse–cerebellar granule cells (unpublished data). Additionally, axons retracted in response to jasp when cultured on glass coated with either laminin or polylysine (unpublished data), indicating that the effects of jasp are not mediated by a disruption of integrin-based signaling. Treatment of cultures with phalloidin, a peptide that stabilizes F-actin through a mechanism similar to that of jasp, also caused axon retraction (unpublished data).

We tested the effects of jasp treatment on long-term axon growth. DRG explants were cultured for 24 h in the presence of 1–40 nM jasp, and the length of axons was compared with vehicle-treated controls. jasp inhibited DRG axon extension (Fig. 1 D). In addition to the dose-dependent inhibition of axonal length by jasp, an inhibitory effect on the number of axons extended from explants also became evident at 10 nM (unpublished data). Cultures treated with

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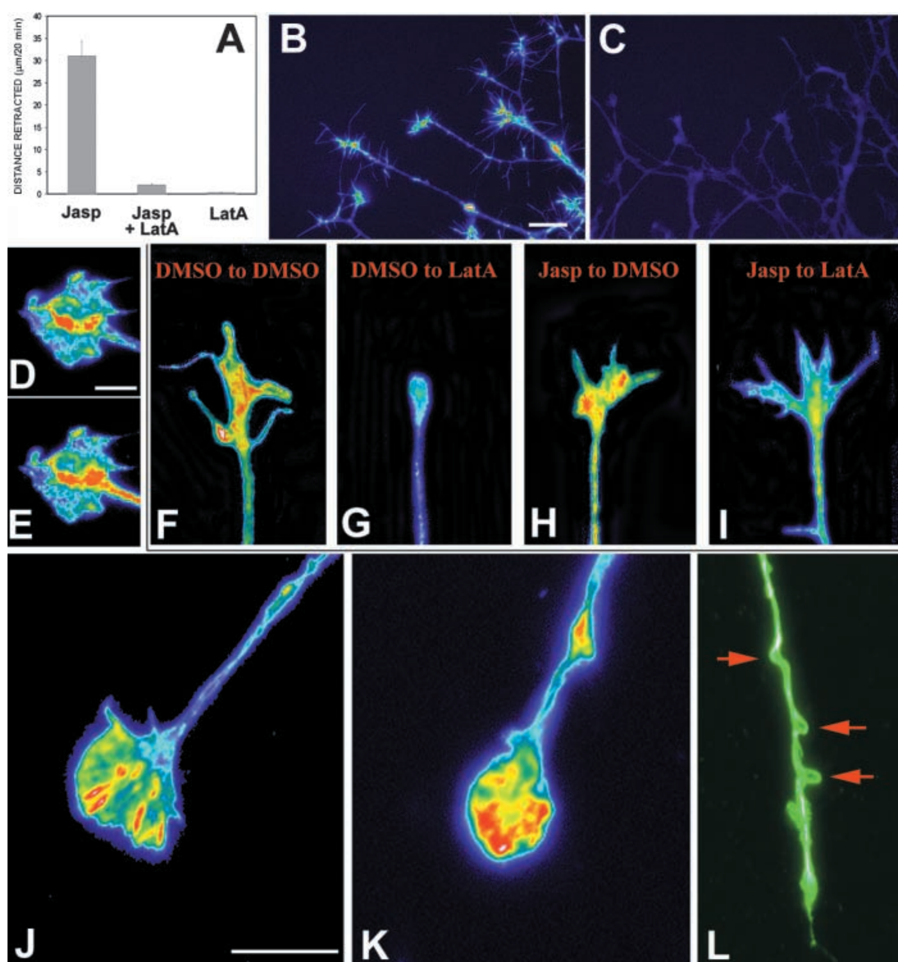

Figure 2. **jasp is rapidly internalized, binds to F-actin, and inhibits its turnover.** (A) Treatment of DRG cultures with 40 nM jasp caused axon retraction (μm/20 min). Treatment with lat-A (LatA) did not cause axon retraction, but blocked the effects of jasp ($n \geq 32$ axons per group). (B) Phalloidin staining of DRG axons treated with DMSO or 40 nM jasp (C) for 10 min. Bar (B) 10 μM. Note the largely diminished staining in jasp-treated axons. D and E are pseudocolored images of the same growth cone double stained with phalloidin and the antiactin antibody, respectively. Note the almost identical staining pattern demonstrating that using the combined fixation-extraction protocol, the antibody staining is reflective of F-actin in growth cones. (F–I) Actin antibody staining of growth cones treated for 4 min with either DMSO (F and G) or 40 nM jasp (H and I) and then for an additional 2 min with either DMSO (F and H) or 2 μM lat-A (LatA; G and I). Bars (D– I) 5 μM. Note that although lat-A causes the depolymerization of the majority of F-actin in DMSO-treated growth cones, it has only a partial effect on growth cones treated first with jasp. jasp causes the centripetal accumulation of growth cone F-actin as revealed by actin antibody staining. J and K are examples of growth cones treated for 10 min with DMSO or 40 nM jasp, respectively. Note the accumulation of F-actin as reflected by the presence of warmer colors (red and yellow) in the contracted growth cone treated with jasp. Bar (J), 10 μM. L is an example of the deformations (red arrows) of the axonal microtubule array that develop in axons undergoing jasp induced axon retraction (40 nM for 10 min).

≥10 nM jasp had only sparse axonal outgrowth. The difference in the concentration dependence of the inhibitory effects of jasp on axon number relative to the inhibition of axon length may be attributable to the observation that axons retract in response to the acute addition of jasp at concentrations ≥10 nM. Similar results were obtained using retinal explants (unpublished data).

To determine the site of action of jasp responsible for axon retraction, we investigated the effects of applying jasp locally to growth cones or axons. Localized delivery of jasp to growth cones caused them to contract and retract (Fig. 1 E). In these cases, bends first became evident along the distal most 20–40 μm behind the growth cone as it started to retract (Fig. 1 E, arrow). Conversely, localized application of jasp to axons did not cause the formation of bends. However, after prolonged delivery of jasp to axons, diffusion of jasp to the distal growth cones was sufficient to cause axon retraction and bending, which proceeded in a distal-to-proximal manner, although the concentration of jasp was greatest along the proximal portion of the axon (Fig. 1 F). These observations indicate that jasp acts at the growth cone to cause axon retraction. Pipette delivery of medium with vehicle had no effect on growth cones and axons.

jasp is rapidly internalized and inhibits F-actin turnover in growth cones

We determined the role of F-actin in mediating jasp-induced axon retraction. Cultures were treated first with latrunculin-A (lat-A), a drug that results in net depolymerization of F-actin by binding cytosolic G-actin, rendering it incompetent for polymerization. 5 μM lat-A caused the loss of >90% of growth cone F-actin within 5 min of treatment (unpublished data), resulting in growth cone collapse but not axon retraction. Pretreatment of cultures with lat-A fully inhibited jasp-induced axon retraction, demonstrating that the effects of jasp treatment require F-actin (Fig. 2 A).

jasp binds F-actin at the same site as phalloidin (Bubb et al., 1994). Therefore, internalization of jasp can be monitored by first treating live growth cones with jasp, and subsequently determining the reduction in staining with fluorochrome-labeled phalloidin. The relative percentage of F-actin bound by jasp can be determined by measuring the fluorescence intensity of phalloidin staining in jasp-treated growth cones relative to the intensity in control growth cones. A 10-min treatment with jasp was chosen because at this time point axons are undergoing retraction. jasp bound to 52, 85, 95, and 95% of growth cone F-actin at 5, 10, 20, and 40 nM, respectively (Fig. 2, B and C). Because growth

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cone contraction in response to jasp starts as early as 2 min after treatment, we determined the binding of jasp to F-actin after 2 min of treatment. Within 2 min of treatment, 40 nM jasp was internalized and bound F-actin to a similar degree (95%) as a 10-min treatment (unpublished data). Therefore, jasp is rapidly internalized and binds to F-actin before changes in growth cone motility and axon retraction.

To relate the effects of long-term treatment with jasp on axon extension (Fig. 1 D) to the binding of jasp to F-actin, we determined the decrease in phalloidin staining of growth cones treated with 1–2.5 nM jasp for 24 h. Axon length was decreased by 20 and 44%, which corresponds to jasp binding to 57 and 70% of available phalloidin binding sites on F-actin, at 1 and 2.5 nM, respectively. These data indicate that jasp can inhibit the long-term extension of axons by binding to 57–70% of available binding sites on F-actin in growth cones.

Depolymerization of F-actin by lat-A is dependent on the rate of filament turnover. We tested the ability of lat-A to cause the depolymerization of F-actin in growth cones treated with jasp relative to control growth cones. Staining F-actin with phalloidin is not possible in jasp-treated growth cones (see previous section). Therefore, we used an antiactin antibody to detect F-actin. Because these experiments sought to detect only F-actin and not monomeric actin, we adopted a combined fixation–extraction protocol previously used to immunocytochemically visualize microtubules in growth cones without interference from soluble tubulin (Gallo and Letourneau, 1999). This protocol results in actin antibody staining that exhibits the same staining pattern as phalloidin (Fig. 3, A and B), and is thus representative of F-actin.

jasp inhibits growth cone F-actin turnover. Growth cones were treated with 40 nM jasp or vehicle (DMSO) for 3 min before treatment with 2 μM lat-A for 2 min. In control growth cones, a 2-min lat-A treatment depolymerized 85% of F-actin. However, lat-A depolymerized only 40% of F-actin in growth cones pretreated for 3 min with 40 nM jasp (Fig. 2, F–I).

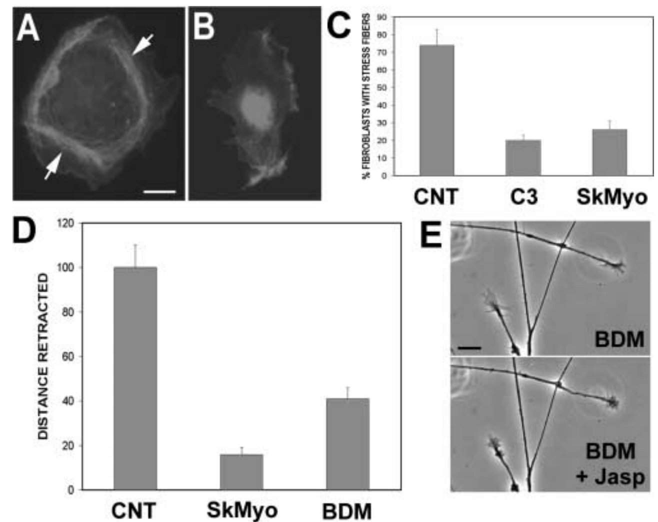

Figure 3. **Myosin activity is required for jasp-induced axon retraction.** A and B show phalloidin-stained chicken embryonic fibroblasts. (A) In control-loaded (BSA) fibroblasts, stress fibers form at the circumference by 2 h after plating the cells (arrows). However, in fibroblasts loaded with skMyosin II (SkMyo), stress fibers fail to form. (C) Trituration loading of C3 toxin or SkMyo largely decreased the percentage of fibroblasts with stress fibers 2 h after plating ($n \geq 76$ fibroblasts per group). (D) Trituration loading of DRG neurons with SkMyo, or treatment with the myosin ATPase inhibitor BDM (2 mM for 40 min), inhibits the distance axons retract after treatment with 40 nM jasp for 20 min ($n \geq 43$ axons per group). Data is presented normalized to the distance control axons retracted. (E) Example of BDM-treated growth cones before and 20 min after treatment with 40 nM jasp. Note that the growth cones do not undergo contraction. CNT, control. Bars, 10 μM.

jasp causes reorganization of the growth cone cytoskeleton without inducing F-actin polymerization

jasp inhibits F-actin turnover by preventing the recycling of actin subunits through the inhibition of depolymerization at the pointed ends of filaments. We investigated whether jasp increased the amount of F-actin present in growth cones by measuring the F-actin content of growth cones stained with actin antibodies after combined fixation and extraction. Importantly, jasp did not increase the F-actin content of growth cones at a concentration of 40 nM that reliably causes retraction of axons (10-min treatment, $P > 0.6$, compared with vehicle-treated controls [$n > 30$]; compare Fig. 2, J to K; also compare Fig. 2, F to H), Thus, jasp-induced axon retraction correlates with decreased F-actin turnover without altering the F-actin content of growth cones.

To determine the effects of inhibiting F-actin turnover on cytoskeletal organization, we stained cultures with antibodies to actin and microtubules. Growth cone F-actin underwent "clumping" after treatment with jasp (Fig. 2 K), which correlated with the contraction of the growth cone. The microtubule cytoskeleton also underwent significant reorganization during jasp-induced axon retraction, which often included development of many axonal bends (Fig. 1 B). These bends represent curvatures in the axonal microtubule array (Fig. 2 L). Lat-A treatment to depolymerize F-actin before the addition of jasp prevented the development of axonal bends (unpublished data).

Axon retraction in response to inhibition of F-actin turnover requires myosin activity

Myosins have been involved in regulating both the extension and retraction of axons (Introduction). The effects of myosins on cell motility are mediated by an interaction with F-actin, resulting in the development of contractile forces. In response to the inhibition of F-actin turnover by jasp, growth cones contracted, and axons underwent retraction. This observation suggests the hypothesis that endogenous myosin activity could be responsible for the retraction of axons in response to the inhibition of F-actin turnover. Therefore, we tested this hypothesis by (1) determining whether myosin activity was required for the retraction of axons in response to jasp, (2) investigating the role of the regulation of myosin activity through the RhoA and myosin light chain kinase (MLCK) pathways in mediating the effects of jasp, and (3) determining whether jasp treatment alters endogenous levels of myosin activity.

To inhibit actomyosin contractility in growth cones, we introduced nonfunctional skeletal muscle myosin II molecules. We used a form of skeletal muscle myosin II (skMyo-

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Table I. **Altering the activity of myosin, RhoA and MLCK does not alter growth cone F-actin content**

Treatment	Mean growth cone f-actin content in arbitrary units + SEM (n)
BSA (control)	1263 ± 163 (30)
Skmyosin II	1167 ± 142 (32)
MLCKp1	1192 ± 97 (43)
C3	1184 ± 171 (26)
CaRhoA	1114 ± 158 (56)

Dorsal root ganglion neurons were trituration loaded with protein or peptide, cultured for 3 h and then fixed and stained with phalloidin. No statistically significant differences from control trituration loaded growth cones were observed.

sin II) that is not active in in vitro actin filament sliding assays (Cytoskeleton, Inc.). We reasoned that the introduction of nonfunctional skMyosin II into cells could act as a dominant negative. To test this, we trituration-loaded skMyosin II into chicken embryonic fibroblasts and determined the percentage of cells exhibiting stress fibers. It is well established that formation and maintenance of stress fibers strictly depends on myosin II activity (Kreisberg et al., 1985). SkMyosin II decreased the percentage of fibroblasts exhibiting stress fibers to a similar extent as treatment with C3 toxin (Fig. 3, A–C), which inactivates RhoA and causes disruption of stress fibers. Thus, the results of this bioassay with fibroblasts are consistent with the idea that nonfunctional skMyosin II acts as a dominant negative myosin II in nonmuscle cells. Trituration loading of skMyosin II into DRG neurons inhibited the distance axons retracted after treatment with jasp (Fig. 3 D), indicating that myosin is required for axon retraction in response to jasp-induced inhibition of F-actin turnover.

We further tested the role of myosin in mediating jasp-induced axon retraction by treating DRG and RGC cultures with 2,3-butanedione monoxime (BDM) before treatment with jasp. BDM causes growth cone collapse at concentrations that inhibit myosin activity (Ruchhoeft and Harris, 1997). Therefore, we determined the dose of BDM that collapses 50% of growth cones and then tested the response of the remaining growth cones to jasp. BDM inhibited jasp-induced axon retraction in a manner similar to loading neurons with skMyosin II (Fig. 3 D). Inhibition of myosin activity by BDM also prevented growth cone contraction in response to jasp (Fig. 3 E), and growth cones became immotile but retained their original morphology. The addition of BDM 4 min after treatment with jasp also decreased the rate of axon retraction, indicating that myosin activity is continuously required during the process of axon retraction (unpublished data).

Axon retraction in response to jasp requires F-actin. Therefore, any experimental treatment that inhibits jasp-induced axon retraction might act by decreasing F-actin levels in growth cones. We controlled for this possibility by measuring the F-actin content of growth cones under conditions of inhibited myosin activity. SkMyosin II did not affect the F-actin content of growth cones (Table I), ruling out the alternative interpretation that interfering with myosin activity inhibits jasp-induced axon retraction by decreasing F-actin levels.

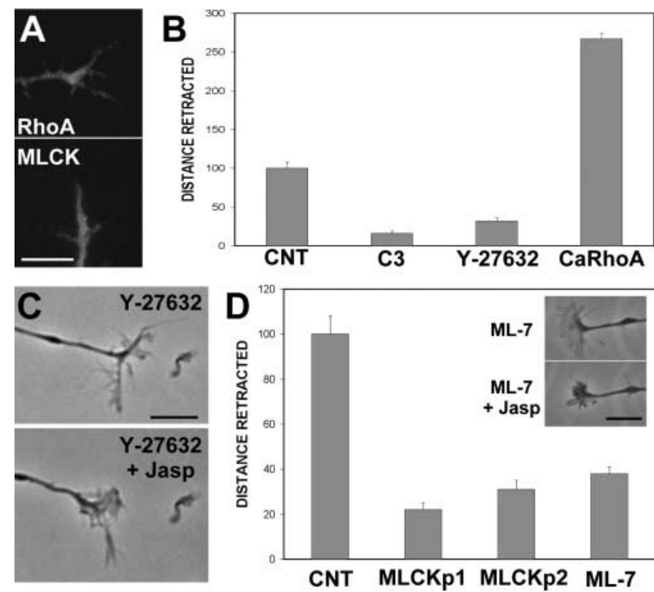

Figure 4. **RhoA and MLCK are required for jasp-induced axon retraction.** (A) Immunocytochemical localization of RhoA and MLCK in DRG growth cones. (B) Trituration-loaded C3 and the ROCK inhibitor Y-27632 (10 μM for 30 min) attenuate jasp-induced (40 nM for 20 min) axon retraction in DRG cultures. Conversely, trituration loading of constitutively active RhoA (CaRhoA) potentiates axon retraction ($n \geq 52$ axons per group). Data are presented normalized to the distance control axons retracted. (C) Example of a Y-27632–treated growth cone before and after treatment with jasp. Note that the growth cone does not undergo contraction. (D) Trituration loading of two separate MLCK-inhibitory peptides (MLCKp1 and p2) into DRG neurons inhibits jasp-induced axon retraction. The pharmacological MLCK inhibitor ML-7 (300 nM for 30 min) also inhibits the effects of jasp. However, growth cones often underwent contraction (inset; $n \geq 43$ axons per group). Data are presented normalized to the distance control axons retracted. CNT, control. Bars, 10 μM.

The activity of RhoA and MLCK regulates axon retraction in response to inhibition of F-actin turnover

Myosin II activity is positively regulated in cells by phosphorylation of the myosin regulatory light chains (mRLC) by MLCK. The RhoA pathway increases the phosphorylation state of mRLC via its effector kinase ROCK, which inactivates mRLC phosphatase (Kimura et al., 1996). MLCK, RhoA (Fig. 4 A), and ROCK (Wahl et al., 2000) are present in growth cones. Therefore, we tested the roles of RhoA, ROCK, and MLCK activity in mediating jasp-induced axon retraction.

RhoA activity was directly inhibited by trituration-loading C3 into DRG neurons before treatment with jasp. C3 inhibited the retraction of DRG axons in response to jasp (Fig. 4 B). Similarly, the ROCK inhibitor Y-27632 inhibited jasp-induced growth cone contraction and axon retraction (Fig. 4, B and C). Inhibition of RhoA signaling by C3 did not alter the F-actin content of growth cones (Table I). Collectively, these results indicate that RhoA-driven ROCK kinase activity is required for jasp-induced axon retraction.

To test whether experimentally increased levels of RhoA activity potentiate jasp-induced axon retraction, DRG neurons were loaded with a constitutively active form of RhoA (caRhoA; L63 mutation). The F-actin content of growth cones from neurons loaded with 1.5 mg/ml caRhoA was not

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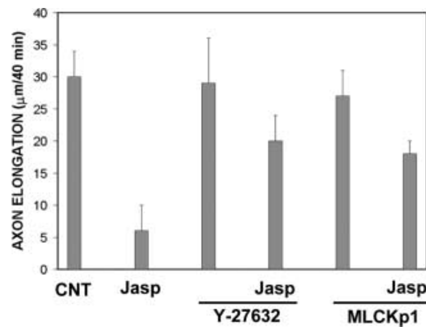

Figure 5. **RhoA and MLCK activity are required for jasp-mediated inhibition of axon extension.** A 40-min treatment with 3 nM jasp reduced axon elongation rate. The effects of 3 nM jasp on elongation rate were attenuated by inhibition of ROCK with 10 μM Y-27632 or an MLCK inhibitory peptide (MLCKp1) delivered using Chariot™ ($n \geq 36$ axons per group).

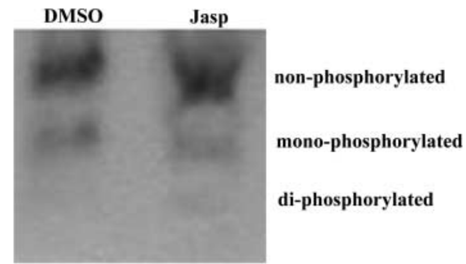

Figure 6. **jasp does not alter levels of myosin light chain phosphorylation.** Shown is a Western blot representative of myosin light chains separated using urea-glycerol PAGE. This technique separates the light chains by size and charge, thereby revealing the extent of non, mono-, and di-phosphorylated chains. Note that treatment with 40 nM jasp for 10 min did not change the profile of light chain phosphorylation. The experiment was repeated three times and produced consistent results. The slight di-phosphorylated band was associated with both control and jasp-treated samples and did not correlate with treatment across all experiments.

different from controls (Table I). Loading DRG neurons with CaRhoA caused a 167% potentiation of the distance the axon retracted in response to jasp (Fig. 4 B). This result demonstrates that increased levels of RhoA activity potentiate axon retraction that is induced by inhibition of F-actin turnover.

MLCK kinase activity was inhibited using two different inhibitory peptides and ML-7, a pharmacological inhibitor. DRG neurons were trituration-loaded with MLCK-inhibitory peptides. The first peptide (MLCKp1; Kemp et al., 1987; Kennelly et al., 1987) inhibits MLCK activity by acting as a kinase pseudosubstrate. The second peptide (MLCKp2; Kemp et al., 1987; Akasu et al., 1993) blocks the required Ca^{2+}-calmodulin–mediated activation of MLCK. Both peptides inhibited jasp-induced axon retraction to the same degree (Fig. 4 D). ML-7 also inhibited jasp-induced axon retraction (Fig. 4 D). However, when MLCK was inhibited, growth cones still underwent varying degrees of contraction (Fig. 4 D, inset). Inhibition of MLCK activity with MLCKp1 did not affect the F-actin content of growth cones (Table I).

Blocking ROCK and MLCK activity counters the inhibitory effects of jasp on the rate of axon extension

We tested whether concentrations of jasp below those that cause axon retraction in the acute treatment assay inhibited axon extension over a longer time period. We found that 3 nM jasp reduced axon extension rate by 80% relative to the control rate during a 40-min period (Fig. 5). Using the phalloidin binding assay we determined that, after a 40-min treatment, 3 nM jasp bound 47% of available F-actin sites in growth cones. Thus, attenuating F-actin turnover can reduce the mean rate of axon extension without causing axon retraction.

To determine if blocking ROCK activity could promote axonal extension in the presence of jasp, cultures were treated with 10 μM Y-27632 for 30 min before exposure to 3 nM jasp. Under conditions of blocked ROCK activity, axon extension was significantly less inhibited by 3 nM jasp and continued at 67% of the control rate (Fig. 5). To inhibit MLCK, MLCKp1 was introduced into established DRG axons using Chariot™ (see Materials and methods). Blocking MLCK activity allowed axons to extend at 66% of the control rate in the presence of 3 nM jasp (Fig. 5). These data indicate that the rate of axon extension is dependent on an interaction between F-actin turnover and myosin-driven contractility.

jasp treatment does not alter endogenous myosin activity

The phosphorylation state of mRLC is the major determinant of myosin II activity (Bresnick, 1999). The hypothesis that endogenous myosin activity is responsible for axon retraction in response to jasp-mediated inhibition of F-actin turnover demands that jasp treatment does not alter myosin activity. To test whether the levels of myosin II activity are changed when axons retract after treatment with jasp, we monitored the phosphorylation state of mRLC. Urea-glycerol PAGE separates the RLC by their charge, as well as size, revealing the presence of phosphorylated forms as bands of greater mobility (Fig. 6). Dissociated DRG neuron cultures were treated with 40 nM jasp for 10 min, and the mRLC were separated by urea-glycerol PAGE followed by Western blotting with an antimyosin light chain antibody. jasp treatment did not increase the level of mRLC phosphorylation (Fig. 6). These data indicate that the effects of jasp on axons and growth cones are not due to an increase in the levels of myosin activity.

F-actin turnover is decreased during RhoA-dependent axon retraction in response to ephrin-A2

Negative guidance cues induce axon retraction. However, little is know about the role of F-actin dynamics in guidance cue–mediated axon retraction. We tested the hypothesis that axon retraction in response to negative guidance cues could utilize a similar mechanism based on actomyosin contractility and the inhibition of F-actin turnover. Ephrin-A2 is a guidance cue for temporal retinal axons that in vivo has been shown to mediate the retraction of inappropriately targeted axons (O'Leary and Wilkinson, 1999). Thus, ephrin-A2 is a valid model system for studying the cellular basis of axon retraction in response to biologically relevant guidance cues. We determined whether F-actin turnover is altered by eph-

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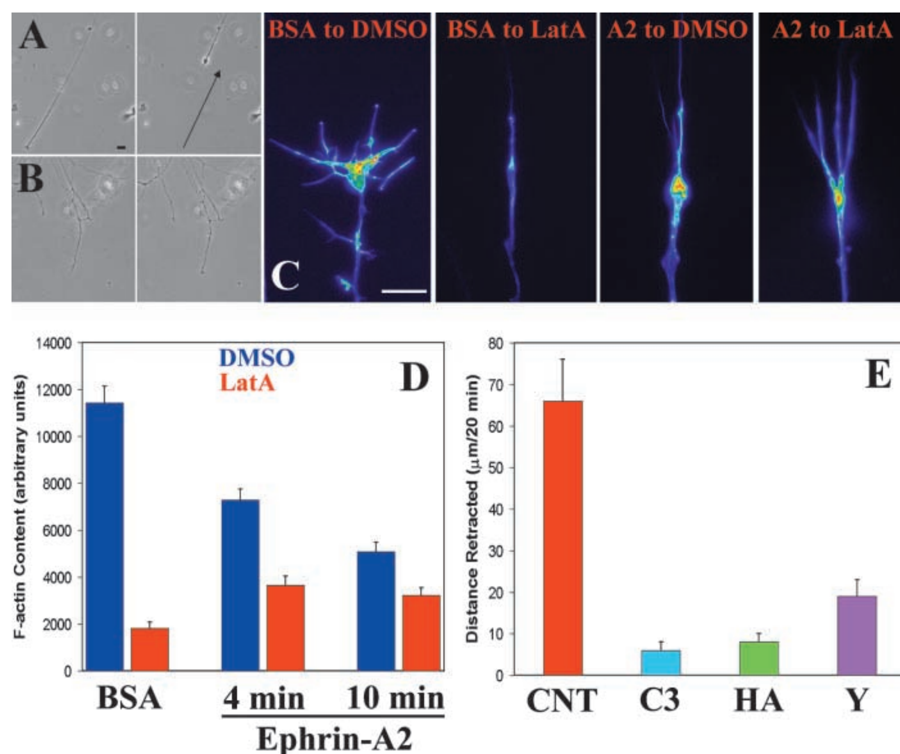

Figure 7. **Ephrin-A2 induces axon retraction that requires RhoA activity and correlates with the presence of stable F-actin.** (A) Example of temporal retinal axon retraction in response to 1 μg/ml ephrin-A2 (arrow shows distance the axon retracted during a 20-min period; ~75 μm). Bar (A) 10 μM. (B) Example of axons treated first with C3, and then with ephrin-A2. Note that none of the axons undergo retraction. (C) Phalloidin staining of temporal retinal axons treated for 10 min with 1 μg/ml BSA or ephrin-A2, and then for 2 min with DMSO or 2 μM lat-A (LatA). Note that lat-A caused significant depolymerization of F-actin in BSA-treated axons, but only partial depolymerization in ephrin-A2–treated axons. Bar (C), 5 μM. (D) Quantification of the F-actin content in the distal axons of cultures treated with BSA or ephrin-A2 for 4 or 10 min before treatment with DMSO or lat-A for 2 min ($n > 41$ in each group). (E) Inhibition of RhoA (C3) or ROCK (Y-27632 and HA-1077) blocks ephrin-A2–induced axon retraction ($n > 35$ in each group). CNT, control.

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rin-A2, and whether the activity of RhoA is required for ephrin-A2–induced axon retraction.

In vitro, treatment of temporal retinal explants with ephrin-A2 caused axon retraction within 5–6 min (Fig. 7 A). F-actin turnover was studied after 4 and 10 min of treatment with ephrin-A2. These time points reflect periods just before and during axon retraction, respectively. Treatment of control cultures with lat-A resulted in the depolymerization of 85% of F-actin (Fig. 7, C and D). In contrast, lat-A depolymerized only 50% of F-actin in axons treated with ephrin-A2 for 4 min (Fig. 7 D). Similarly, after a 10-min treatment with ephrin-A2, lat-A depolymerized only 35% of F-actin (Fig. 7, C and D). These observations demonstrate that ephrin-A2 reduces F-actin turnover.

In conjunction with the inhibition of F-actin turnover, ephrin-A2 also caused a decrease in F-actin content. Relative to controls, ephrin-A2 decreased F-actin levels by 36 and 56% at 4 and 10 min, respectively (Fig. 7 D). After lat-A treatment, ephrin-A2–treated axons exhibited 100 and 76% greater F-actin content, at 4 and 10 min, respectively, relative to control axons treated only with lat-A ($P < 0.001$ for both comparisons). Thus, relative to F-actin in control axons, the F-actin present in ephrin-A2–treated axons is significantly less sensitive to lat -A–induced depolymerization and does not represent a lat-A–insensitive population of F-actin normally present in control growth cones. These data demonstrate that ephrin-A2–induced axon retraction correlates with depolymerization of F-actin and inhibition of F-actin turnover.

Next, we investigated whether ephrin-A2–induced, like jasp-induced, axon retraction involves regulation of actomyosin contractility through RhoA. Similar to jasp, treatment of cultures with lat-A before the addition of ephrin-A2 almost completely blocked axon retraction in response to ephrin-A2 (mean distance retracted of 1.9 μm/20 min; $n = 36$). Ephrin-A2–induced axon retraction was blocked by 80–90% when RhoA or ROCK were inhibited (Fig. 7, B and E). Collectively, these data demonstrate that axon retraction in response to ephrin-A2 correlates with decreased rates of F-actin turnover and requires F-actin and RhoA activity, indicating that biologically relevant signaling molecules can cause axon retraction through a mechanism similar to that operating in jasp-induced axon retraction.

Discussion

In this work, we investigated the combined roles of F-actin turnover and actomyosin contractility in the process of axon extension and retraction. We demonstrate that when actin filament turnover is inhibited, endogenous myosin-driven forces result in axon retraction. These observations reveal for the first time that axon extension and retraction depend on an interaction between the degree of actin filament turnover and endogenous levels of myosin-based contractility. Additionally, we report the new observation that F-actin turnover is inhibited during axon retraction in response to a biologically relevant guidance cue, ephrin-A2.

jasp is rapidly internalized in growth cones and inhibits growth cone F-actin turnover. Cramer (1999) used jasp to demonstrate that polymerization of F-actin at the leading edge requires G-actin released by filament turnover. Our data are consistent with a similar requirement for F-actin turnover in providing the actin monomers required for growth cone–protrusive activity. First, treatment with jasp alone caused growth cone contraction and cessation of protrusive activity. Second, when myosin activity was inhibited, growth cones did not contract in response to jasp, but remained flattened. However, lamellipodial and filopodial protrusion was inhibited. These observations indicate that

myosin activity is required for growth cone contraction in response to the inhibition of F-actin turnover by jasp, and that in the absence of myosin-based contractility, growth cones become quiescent due to the lack of available G-actin for polymerization at the leading edge. These observations are consistent with the model proposed by Lin et al. (1996) for the regulation of growth cone leading edge protrusion by actin polymerization and retrograde flow.

The response of the growth cone to treatment with jasp, which we refer to as contraction, differs from growth cone collapse in response to pharmacological depolymerization of F-actin (Letourneau et al., 1989) or guidance cues that cause collapse (Fan et al., 1993; Ernst et al., 2000; Wahl et al., 2000). Unlike during growth cone collapse, in response to jasp, filopodia often remained attached to the substratum while the growth cone lamellipodium contracted (Fig. 1 A). Also, growth cones became phase dark and exhibited clumping of F-actin without depolymerization (Fig. 2 K). The demonstration that myosin and RhoA activity are required for growth cone contraction also differentiates contraction from collapse because inhibition of RhoA activity only partially blocks collapse in response to repellant guidance cues (e.g., ephrin-A5; Wahl et al., 2000). Thus, we interpret the response of growth cones to jasp as a myosin-driven contraction of the lamellipodium.

The effects of jasp on growth cones are similar to the responses of nonneuronal cells to jasp. Treatment with jasp causes accumulation of F-actin in the central domain as the growth cone undergoes contraction. In nonneuronal cells, jasp causes the formation of F-actin aggregates (Lee et al., 1998; Bubb et al., 2000). The formation of aggregates occurs as F-actin filaments accumulate in the cytoplasm and is attenuated in *Dictyostelium* mutants lacking myosin II (Lee et al., 1998). Similarly, when RhoA or myosins are inhibited, growth cones do not undergo contraction. Thus, the observations on growth cones provide evidence for the conservation of the myosin-based mechanism involved in the responses of both nonneuronal and neuronal cells to inhibition of F-actin turnover by jasp.

The observation that inhibiting F-actin turnover results in myosin-dependent growth cone contraction and axon retraction, without altering myosin activity, demonstrate a novel mechanism in the regulation of axon extension. In the presence of "normal" rates of F-actin turnover, myosin activity drives retrograde flow, whereas actin filament polymerization drives leading-edge protrusion. When filament turnover is inhibited, F-actin contracts centripetally through a myosin-based mechanism. Thus, in growth cones, fast F-actin turnover and endogenous myosin-based contractility combine to allow growth cone motility and axon extension to continue. When F-actin turnover is inhibited, endogenous myosin-driven forces cause axon retraction. Bradke and Dotti (1999) have suggested that local F-actin turnover in growth cones specifies axonal versus dendritic extension, and Bito et al. (2000) have shown that endogenous RhoA activity limits axon formation. Hence, our observations may have relevance to the process of axonogenesis as well as the extension of preestablished axons.

In this paper, we did not directly address the issue of which myosin type drives jasp-induced axon retraction. However, myosin II is the most likely candidate, given its established role in the contractility of nonneuronal cells and its presence in growth cones (Letourneau, 1981; Bridgman and Dailey, 1989; Letourneau and Shattuck, 1989). In growth cones, myosin IIB localizes to areas of the lamellipodium undergoing retraction (Rochlin et al., 1995), providing additional evidence that myosin II may be responsible for the retraction/contraction of the growth cone. Furthermore, MLCK and RhoA have well established roles in regulating myosin II activity (Bresnick, 1999), and are required for jasp-induced axon retraction.

We found that F-actin turnover in the distal axons of temporal RGCs is decreased by treatment with ephrin-A2. Ephrin-A2 is a good model for axon guidance cues that cause axon retraction, because of its in vivo role as a cue that promotes the retraction of inappropriately targeted retinal axons (Orioli and Klein, 1997; O'Leary and Wilkinson, 1999). The relevance of axon retraction to neurodevelopment was directly demonstrated by in vivo live imaging of axon pathfinding error correction in the projection of zebrafish retinal axons (Hutson and Chien, 2002). It is at present unclear whether all aspects of action retraction in vitro reflect retraction in vivo (e.g., axonal buckling). However, the mechanism by which ephrin-A2 causes axon retraction shares similarities with that underlying jasp-induced axon retraction. In particular, ephrin-A2–induced axon retraction requires RhoA activity. The related ligand ephrin-A5 increases RhoA activity in retinal neurons (Wahl et al., 2000). Although in our investigation we did not measure RhoA activity, it is likely that ephrin-A2 also causes increased RhoA activity. It will be of interest to investigate the role of GTPases in regulating F-actin turnover during ephrin-A2–mediated axon retraction.

The experiments with ephrin-A2 indicate that axon retraction in response to guidance cues utilizes a similar mechanism as jasp-induced axon retraction. F-actin is required for ephrin-A2–induced axon retraction. Contrary to this requirement, ephrin-A2 caused the depolymerization of ~50% of growth cone F-actin. However, F-actin depolymerization coincided with decreased F-actin turnover. Thus, guidance cues could induce axon retraction by activating myosin activity through RhoA-ROCK while at the same time generating a favorable substrate for actomyosin contractility by inhibiting F-actin turnover. The molecular basis for the coordinated depolymerization and stabilization of F-actin in response to negative guidance cues will have to be investigated further. However, the inactivation of cofilin by guidance cues could result in the stabilization of F-actin (Aizawa et al., 2001), whereas additional pathways could cause F-actin depolymerization through filament severing or perhaps rapidly sequestering actin monomers making them unavailable for recycling, or both.

Collectively, the results of the present study suggest a model for the regulation of axon extension by an interaction between myosin-based contractility and F-actin turnover (Fig. 8 A). Endogenous actomyosin-based force generation in axons can be a negative regulator of axon extension. RhoA and MLCK contribute to the negative regulation of axon extension by myosin. Treatment of neurons with the RhoA-inactivating enzyme C3 potentiates axon extension (Bito et al., 2000). Similarly, blocking MLCK activity with a pseu-

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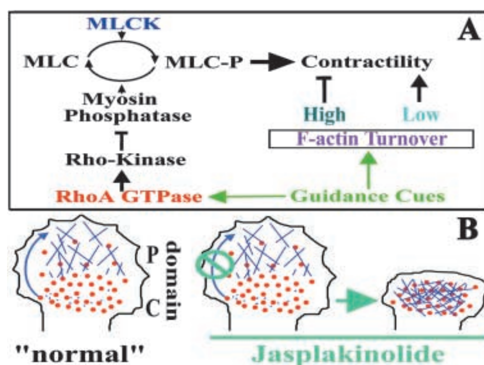

Figure 8. **Working model for the regulation of axon extension by F-actin turnover and myosin-driven contractility.** (A) Regulation of axon extension by the combination of F-actin turnover and actomyosin contractility. (B) Diagram of relationship between F-actin (blue) and myosin II (red) in the peripheral (P) and central (C) domains of growth cones. Under "normal" conditions, F-actin is depolymerized at the interface between the P- and C-domains and the monomers are turned over (arrow). In the presence of jasp, F-actin is not depolymerized and filament turnover is blocked. F-actin undergoes retrograde transport and accumulates in the myosin II–enriched C-domain resulting in increased actomyosin contractility.

dosubstrate peptide increases axon length by 27% (unpublished data). While axons are extending, MLCK activity drives the phosphorylation of mRLC and activates myosin. Endogenous levels of RhoA activity also contribute to the regulation of mRLC phosphorylation by acting through the RhoA effector kinase ROCK, which is also capable of directly phosphorylating mRLC in addition to inhibiting the myosin phosphatase (Bresnick, 1999). The finding that attenuation of F-actin turnover inhibits axon extension and results in axon retraction in a manner dependent on RhoA and MLCK activity demonstrates that the negative regulation of axon extension by RhoA and MLCK is countered by the rapid turnover of F-actin. In the absence of F-actin turnover, endogenous myosin activity generates sufficient contractile force to produce axon retraction. The mechanism by which the myosin activity is either permissive or inhibitory for axon extension depending on F-actin turnover is unclear. However, under normal conditions, myosin II is enriched in the central domain of growth cones (Bridgman and Dailey, 1989) whereas F-actin is localized mostly to the peripheral domain (Fig. 8 B). In the absence of F-actin depolymerization, F-actin is expected to undergo retrograde flow and accumulate in the central domain. Consistent with this expectation, we observed F-actin clumping toward the central domain in response to jasp. Thus, the accumulation of F-actin in the myosin II–rich central domain may increase the ability of myosin II to interact with F-actin and generate contractile forces.

Materials and methods

Reagents

jasp was purchased from Molecular Probes, Inc. and dissolved in DMSO. Exozyme C3, constitutively active (L63) RhoA, and skMyosin II were purchased from Cytoskeleton, Inc. and dissolved in cell-loading buffer. ML-7 was purchased from BIOMOL Research Laboratories, Inc. and dissolved in DMSO. Y-27632 and HA-1077 were obtained from BIOMOL Research Laboratories, Inc. and prepared in water. MLCKp1 and MLCKp2 were obtained from BIOMOL Research Laboratories, Inc. and Calbiochem, respectively; both were resuspended in cell loading buffer. Brain-derived neurotrophic factor and neurotrophin-3 were gifts of Dr. J. Cantello (Regeneron Pharmaceuticals, Inc., Tarrytown, NY). NGF was purchased from R&D Systems. All other reagents were purchased from Sigma-Aldrich.

Cell culture

Embryonic chick DRG (E9) and retinal explants (E7) were cultured as described previously (Ernst et al., 2000) in defined Ham's F12 medium (GIBCO BRL) containing additives and 20 ng/ml brain-derived neurotrophic factor for DRG, and grown on laminin (Invitrogen) or polylysine-coated (Sigma-Aldrich) glass coverslips (both at 25 μg/ml). For live videomicroscopy, glass coverslips were affixed using aquarium sealant to plastic dishes with holes (22-mm diameter) drilled in the center.

Cell trituration and Chariot™-based delivery

Proteins and peptides were trituration-loaded into dissociated DRG neurons as described in Jin and Strittmatter (1997) in cell loading buffer containing 5 mg/ml of protein or peptide. All experiments with trituration-loaded cells were performed within 3–5 h after plating the cells. In our culturing system, DRG neurons exhibit axons that are between 50 and 200 μm long 3–5 h after plating.

The Chariot™ peptide (Active Motif LLC) method was used to load peptides into neurons. 1.2 μl Chariot™ peptide stock solution was mixed with 2 μg MLCK peptide (BIOMOL Research Laboratories, Inc.), or 6 μl Chariot™ peptide with 1 μg C3 for 30 min at RT as per the manufacturer's directions. BSA was used as an inert control protein. The Chariot™ peptide–protein complex was added to cultures for 3–4 h before use in experiments.

Videomicroscopy

Growth cones and axons were visualized with an inverted microscope (model IX70; Olympus) using phase-contrast optics (20–60× objectives). Time lapse sequences were obtained using a Spot camera (model 2.1.0; Diagnostic Instruments) driven by digital image acquisition software (MetaVue™ 4.6r7; Universal Imaging Corp.). The procedure for quantification of fluorescence was essentially the same as in Ernst et al. (2000). The integrated pixel intensity of the distal 10 μm of axon plus the growth cone, if one was present, was obtained by subtracting the background intensity. jasp was applied locally by placing a pipette ~20 μm above growth cones (10-μm-diam tip, filled with medium containing 40 μM jasp) and allowing jasp to diffuse onto growth cones or axons.

Immunocytochemistry

Phalloidin staining was used to visualize F-actin as described previously (Gallo and Letourneau, 1998; Molecular Probes, Inc.). For antibody staining of F-actin, cultures were simultaneously fixed and extracted using 0.2% glutaraldehyde and 0.1% TX-100 in PHEM buffer and stained as described in Gallo and Letourneau (1999). Omission of the primary antibody (1:20 in 10% normal goat serum [NGS]; Sigma-Aldrich) gave no staining. Microtubule staining was performed exactly as described in Gallo and Letourneau (1999).

For determination of RhoA and MLCK localization, cultures were fixed with 4% PFA and −20°C methanol for 15 min, respectively, and then TX-100 treated. Primary antibodies in NGS were applied for 1 h (1:400 of rabbit anti-RhoA [Santa Cruz Biotechnology, Inc.] or 1:400 of monoclonal mouse anti-MLCK [Sigma-Aldrich]). Secondary antibodies were applied for 1 h in NGS (1:400 of rhodamine-labeled goat anti–rabbit and goat anti–mouse, respectively; Cappel). Omission of primaries gave no staining.

Western analysis

Urea-glycerol PAGE was used to monitor the phosphorylation state of myosin light chains as described in Yee et al. (2001). Proteins from purified, dissociated DRG neurons (Gallo and Letourneau, 1999) from a total of 100 ganglia per sample were used. Cells were scraped off the dish in 10% TCA. Protein samples were then run on urea-glycerol PAGE followed by Western blotting with an antibody raised to a conserved sequence in myosin light chains.

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