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RhoA-Kinase and Myosin II Are Required for the Maintenance of Growth Cone Polarity and Guidance by Nerve Growth Factor

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Abstract

Growth cones are highly polarized and dynamic structures confined to the tips of axons. The polarity of growth cones is in part maintained by suppression of protrusive activity from the distal axon shaft, a process termed axon consolidation. The mechanistic basis of axon consolidation that contributes to the maintenance of growth cone polarity is not clear. We report that inhibition of RhoA-kinase (ROCK) or myosin II resulted in unstable consolidation of the distal axon as evidenced by increased filopodial and lamellipodial extension. Furthermore, when ROCK or myosin II was inhibited lamellipodia formed at the growth cone migrated onto the axon shaft. Analysis of EYFP-actin dynamics in the distal axon revealed that ROCK negatively regulates actin polymerization and initiation of protrusive structures from spontaneously formed axonal F-actin patches, the latter being an effect attributable to ROCK-mediated regulation of myosin II. Inhibition of ROCK or myosin II blocked growth cone turning toward NGF by preventing suppression of protrusive activity away from the source of NGF, resulting in aborted turning responses. These data elucidate the mechanism of growth cone polarity, provide evidence that consolidation of the distal axon is a component of guidance, and identify ROCK as a negative regulator of F-actin polymerization underlying protrusive activity in the distal axon.

Keywords

actin; consolidation; polymerization; blebbistatin; protrusion; filopodium; lamellipodium

INTRODUCTION

Axon extension occurs through a three-step process sequentially involving protrusion, engorgement, and consolidation (reviewed in Dent and Gertler, 2003). The protrusion phase is defined as the extension of filopodia and lamellipodia from the leading edge of the growth cone. Engorgement involves the movement of microtubules and organelles into recently protruded regions of the growth cone. Finally, new axon forms behind the growth cone through consolidation. Consolidation is characterized by the suppression of protrusive activity. Furthermore, consolidation is of fundamental importance to maintaining the growth cone polarized with protrusive activity restricted to the distal tip of the axon. The mechanism of

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axon consolidation is the least understood phase of axon extension, and its relationship to guidance is unknown.

Growth cones contain actin filaments (F-actin) and myosin II (Bridgman and Elkin, 2000; Brown and Bridgman, 2003). The motor activity of myosin II requires phosphorylation of the myosin regulatory light chains. RhoA-kinase (ROCK) regulates myosin II activity by inhibiting the myosin light chain phosphatase and possibly directly phosphorylating regulatory myosin light chains (Totsukawa et al., 2000). While it has been shown that inhibition of ROCK promotes axon formation (Bito et al., 2000), the cellular mechanisms underlying the functions of ROCK have not been elucidated. The best-defined function of myosin II in growth cones is driving retrograde F-actin flow from the peripheral to the central domain (Lin et al., 1997). Interestingly, ROCK does not regulate myosin II-driven F-actin retrograde flow in growth cone lamellipodia (Zhang et al., 2003), suggesting ROCK may regulate myosin II in a different context. Thus, although ROCK activity regulates growth cones and axon extension, the functions of myosin II downstream of ROCK are not clear.

We identify ROCK and myosin II as major determinants of axon consolidation and the maintenance of growth cone polarity. In the distal axon, ROCK inhibits F-actin polymerization underlying protrusion, while promoting myosin-II dependent contractility at the growth cone neck. Inhibition of protrusive activity during consolidation of the distal axon is mediated by ROCK and myosin II, which prevent formation of filopodia and lamellipodia from pre-existing axonal F-actin patches. Investigations on the effects of inhibition of ROCK and myosin II on axon guidance by nerve growth factor (NGF) indicate that the ROCK- and myosin-II-driven mechanism of distal axon consolidation is a component of axon guidance.

METHODS

Culture

Fertilized chicken eggs were obtained from Charles River Laboratories (Wilmington, MA). Embryonic day (E) 10 dorsal root ganglion (DRG) explants and dissociated cells were prepared as previously described (Ernst et al., 2000; Gallo et al., 1997) and cultured on glass coverslips or video-dishes in defined medium (F12H; Invitrogen, Carlsbad, CA) containing 20 ng/mL NGF (R&D Systems, Minneapolis, MN). Culturing substrata were coated with 25 μ g/mL laminin (Invitrogen). Chicken embryos prior to E15 are not considered vertebrate animals by our Institutional Review Board.

DNA Transfection

DRG neurons (approximately 500,000 cells per transfection) were transfected using the Amaxa, Inc. (Cologne, Germany) Nucleofector electroporator. Transfections were performed using the G13 setting and Amaxa kit of reagents optimized for transfecting chicken neurons following manufacturer's directions. EYFP- β actin plasmids (Clontech Inc., Palo Alto, CA) were prepared by standard methods using a midi-prep kit (Quiagen, Valencia, CA). Ten micrograms of plasmid DNA were used per transfection.

Trituration Loading

Proteins were trituration loaded into dissociated neurons at a concentration of 10 mg/mL as described in Gallo et al. (2002). Loading was performed in a 30 μ L volume. Motor activity inactive myosin II and L63RhoA-GST were purchased from Cytoskeleton, Inc. (Denver, CO). The F-actin stabilizing agent jasplakinolide (Molecular Probes, Eugene, OR) causes myosin-II-dependent axon retraction that is blocked by loading cells with inactive myosin II (Gallo et al., 2002). As a positive control to confirm effective loading and myosin II inhibition in individual neurons that were imaged, cultures were treated with 2 μ M jasplakinolide for 10

min and imaged using phase contrast optics. Only neurons that retracted less than 5 μm after jasplakinolide treatment (12/15) were considered to be effectively loaded, and in turn analyzed. BSA-loaded axons retracted a mean distance of $19 \pm 4 \mu\text{m}$ following jasplakinolide treatment and only 2/15 retracted less than 5 μm after treatment.

Protein Transfection

Exogenous proteins were delivered into neurons using the Chariot protein transfection reagent (ActiveMotif Inc., Carlsbad, CA) following previously published protocols (Jurney et al., 2002; Gallo, 2003). To introduce inactive myosin II into neurons transfected with EYFP-actin for live imaging experiments we used 12 μL Chariot and 1 μg of inactive skeletal muscle myosin II.

Microscopy

Time-lapse phase imaging of cultures was performed on a Zeiss Axiovert 135M microscope (Zeiss Inc., Gottingen, Germany), equipped with a Zeiss AxioCam CCD camera. The stage was heated using an air curtain (ASI 400; Nevtek, Burnsville, VA). Time-lapse fluorescence imaging was performed using a Zeiss 200M microscope with a Zeiss three-plate insert heated-stage equipped with an Orca ER CCD camera (Hamamatsu Inc., Bridgewater, NJ). All fluorescence-imaging data acquisition and analysis were performed using Zeiss AxioVision software. Forty and 100X objectives (Zeiss) were used for phase and fluorescence microscopy, respectively. In order to standardize the sampling of growth cones in diverse treatment groups, we selected to image growth cones that, at the beginning of imaging, exhibited a polarized lamellipodial morphology, thereby decreasing the probability of biasing experimental data through uneven sample selection. We reasoned that, given all growth cones started in a similar polarized state, divergences from this initial state would be most telling of the underlying actin dynamics.

Cytochemistry

Actin filaments were visualized by the protocol described in Gallo et al. (1997) using rhodamine-phalloidin (Molecular Probes, Portland, OR). For dual labeling of F-actin and microtubules cultures were simultaneously fixed and extracted as described in Gallo and Letourneau (1999) and stained with rhodamine-phalloidin and FITC-conjugated antitubulin (Sigma). For determination of the ratio of cytoplasmic volume to myosin II regulatory light chain phosphorylation levels cells were loaded with the cell-permeable aldehyde fixable cytoplasmic volume indicator Cell-Tracker (30 min, 2.5 μM ; Molecular Probes). Cultures were fixed by replacing the medium with 8% paraformaldehyde in PBS containing 10% sucrose for 15 min and stained with a antibody specific to phosphorylated regulatory myosin light chains [1:1000 dilution in 10% goat serum + 0.1% TX-100 (GT), overnight at 4°C; see Supplemental Fig. 1 for characterization of the antibody at <http://www.interscience.wiley.com/jpages/0022-3034/suppmat>]. Following washing in PBS, cultures were stained with 1:200 goat antirabbit secondary antibody conjugated to rhodamine in GT for 45 min, washed, and mounted. Omission of primary antibody gave no staining.

For presentation purposes some images were contrast enhanced using Photoshop (Adobe Systems, San Jose, CA). In all cases identical digital manipulations were performed to all images pertaining to data sets for visual comparison.

Treatment Protocol

In order to deliver reagents to cultures we removed 300 μL of culturing medium from 1 mL cultures, added the reagent to the 300 μL of medium and gently returned the medium + reagent to the culture. Blebbistatin was purchased from Toronto Research Chemicals, Inc. (North York,

Ontario, Canada), and a 200 mM stock solution was made in DMSO and stored at -20°C . Additional stocks of blebbistatin were prepared from the 200 mM stock and similarly stored. We found that in order to get a final concentration of 50–100 μM blebbistatin in solution, without precipitation, under our culturing conditions 1–2 μL of blebbistatin from 25–50 mM stocks had to be added to a minimum of 300 μL of medium and the solution mixed prior to adding it to cultures. Cytochalasin D was obtained from Sigma. Latrunculin-A was obtained from Molecular Probes. All drugs were added to cultures at a final concentration of vehicle (DMSO) of 1–2:1000. Growth cone collapse was assayed as previously described (Ernst et al., 2000). Y-27632 and HA-1152 were purchased from Calbiochem (Carlsbad, CA).

Definition of Growth Cone Neck/Distal Axon for Quantitative Analysis

The growth cone neck was defined as the region of the axon shaft that increased in mean diameter by $>30\%$ behind the leading main growth cone lamellipodium. In the majority of cases this threshold was determined by the initiation of a fan shaped lamellipodium. However, if a growth cone did not exhibit a fan shaped lamellipodium, then the growth cone neck was defined as starting 10 μm behind the distal most extent of the phase dark axon shaft. This definition was adopted because the mean proximodistal length of P-domains exhibiting lamellipodia is approximately 10 μm . Given these definitions, we considered the distal 10 μm of axon shaft as the growth cone neck. Based on these definitions, we selected growth cones that exhibited polarized morphologies and a clear P-domain at the start of the acquisition sequences in an attempt to normalize the starting point of the analysis under conditions of ROCK or myosin II inhibition.

Actin Bundle Density Determination

In order to determine the density of F-actin bundles in growth cones we followed the protocol of Bridgman et al. (2001). However, DRG neuron growth cones exhibit larger F-actin meshworks in lamellipodia than the superior cervical ganglion neurons studied by Bridgman et al. (2001). This precluded imaging in lamellipodial regions. Therefore we focused our analysis on the lamellipodial F-actin mesh-work-free region in the central domain of growth cones. In order to prevent a decrease in lamellipodial meshwork in experimental groups as a confounding factor, which may reveal bundles present in both experimental and control growth cones but more prominently under conditions of decreased lamellipodial F-actin, we focused our analysis on the central domain of growth cones extending a maximum of 5 μm from the growth cone neck.

Determination of the Growth Cone Polarity Ratio during Turning toward NGF-Beads

The area covered by lamellipodia and filopodia in the side of the growth cone toward and away from the contact with NGF-beads was measured every minute before and after contact with the beads. A line demarcating the border between the two sides of the growth cone was drawn through the middle of the growth cone by extending the axis of the axon shaft. The polarity ratio was then determined by dividing the area measurement in the region of the growth cone opposite the contact by the area closest to the contact. Thus, polarity ratio values less than one reflect growth cone polarization toward the bead and values greater than one reflect polarization away from the contact.

Quantification of Relative Mean Fluorescence Intensity Changes during Actin Patch Formation

In order to determine the dynamics of individual F-actin patches we measured the mean intensity in an area of interest defined by the maximum size of the patch. The mean intensity within the area of interest for each interframe interval was then determined and graphed. A 15% increase over baseline intensity was defined as the initiation of the rise phase of the patch

fluorescence profile because control experiments monitoring fluorescence in regions of the axon that did not give rise to patches demonstrated a maximum of 12–13% deviation in intensity from one frame to the next [Fig. 6(C), top panel]. Similarly, patches were defined as having terminated when the mean fluorescence returned to $\pm 15\%$ of prepatch base line for a minimum of three consecutive interframe intervals. See Figure 6(B) for a schematic of the parameters measured for individual patches.

NGF-Coated Bead Experiments

Generation and use of NGF-coated beads and NGF-coated bead experiments were carried out as previously described (Gallo et al., 1997).

Western Analysis of G/F Actin

Dissociated DRG cultures were treated with 4 μM jasplakinolide or DMSO for 10 min, lysed, and processed according to the protocol provided by the manufacturer for the G/F actin determination kit (Cytoskeleton Inc., Denver, CO), followed by Western blotting using an anti- β -actin antibody (Sigma) and an AP-conjugated secondary antibody. The experiment was repeated three times with similar results.

Data Analysis

Unless otherwise indicated means between groups were compared using the Welch t test that does not assume equal variances.

RESULTS

Inhibition of Myosin II Results in the Presence of Lamellipodia and Filopodia along the Distal Axon

Blebbistatin is a cell-permeable specific inhibitor of nonmuscle myosin IIA and IIB ATPase activity (Straight et al., 2003). In our experiments we used 50 μM blebbistatin because this dose effectively disrupts fibroblast myosin II-dependent stress fibers and maximally inhibits embryonic DRG axon extension rate by 40–45% (not shown), as expected for myosin II inhibition (Tullio et al., 2001). The most prominent feature of blebbistatin treatment on the morphology of DRG axons and growth cones was the presence of lamellipodial, or filopodial, protrusions on the distal axon shaft [Fig. 1(A)]. In 42% of axons ($n = 122$) treated with blebbistatin we observed lamellipodia or filopodia extending from the distal 20 μm of the axon shaft lateral to the microtubule array, compared to 9% of control axons ($n = 138$). This observation led us to focus on the role of myosin II in the distal axon, the site of consolidation, and investigate the underlying protrusive dynamics.

Inhibition of Myosin II or RhoA-Kinase Increases Protrusive Activity from the Distal Axon Shaft

Similar to inhibition of myosin II, inhibition of ROCK (10 μM Y-27632, 1 h) resulted in 45% of axons ($n = 107$) exhibiting either lamellipodial or filopodia extending from the distal axon shaft in fixed and stained preparations. In order to investigate the effects of inhibiting ROCK and myosin II on protrusive activity along the distal axon, we imaged axons using phase contrast optics and determined the frequency of filopodial and lamellipodial initiation. ROCK was inhibited using two separate pharmacological reagents, Y-27632 and HA-1552. Myosin II was inhibited using blebbistatin or by introduction of motor domain inactive myosin II into neurons through trituration loading (Gallo et al., 2002). For pharmacological experiments we used explant cultures. For trituration loading of myosin II we used dissociated neurons.

Inhibition of ROCK using Y-27632, or myosin II using blebbistatin, increased the frequency of filopodial and lamellipodial initiation from the distal axon [Fig. 1(B,C); see Methods for details on the definition of the distal axon for quantitative analysis]. Significantly, increases in protrusive activity were only observed in the distal 20 μm of axons, the site of active consolidation [Fig. 1(C)]. To confirm the results obtained using Y-27632 we used an additional ROCK inhibitor (HA-1152, 10 μM ; Ikenoya et al., 2002). Similar to Y-27632 treatment, HA-1152-treated neurons exhibited increased levels of filopodial and lamellipodial initiation from the distal 10 μm of the axon shaft [Fig. 1(D)].

In order to confirm the results obtained using blebbistatin, we trituration loaded an inactive form of skeletal muscle myosin II that acts as a dominant negative into dissociated neurons (dnMyoII, as described in Gallo et al., 2002; see Methods). Neurons were imaged 3–5 h after plating when axons were between 100–200 μm in length. Control neurons were loaded with BSA. Consistent with published results and the effects of blebbistatin (Tullio et al., 2001; Turney and Bridgman, 2005), loading with dnMyoII decreased axon extension rate by 44% relative to BSA-loaded neurons ($p < 0.005$, $n = 20$ and 19 for myosin II and BSA, respectively). Dissociated neurons loaded with dnMyoII exhibited significantly higher levels of filopodial and lamellipodial initiation in the distal 10 μm of the axon relative to BSA-loaded controls [Fig. 1(D)]. Although the absolute numbers of axonal filopodia and lamellipodia initiated in dnMyoII-loaded neurons were similar to that exhibited by blebbistatin-treated neurons, control BSA-loaded neurons exhibited higher levels of baseline protrusion than those in blebbistatin experiments performed on explants cultured for 24 h [compare values in Fig. 1(C) and (D)]. The difference in baseline activity may reflect the use of dissociated neurons for myosin II loading versus explants for blebbistatin treatment, the difference in the time in culture after which data were acquired (3–5 h in dissociated cell relative to 24 h in explant experiments), or the result of trituration loading. We therefore compared baseline filopodial initiation frequencies in dissociated neurons and dissociated neurons trituration loaded with BSA. We did not observe differences in the frequency of axonal filopodial initiation in the distal 10 μm of axons between these two groups (dissociation alone, $0.52 \pm 0.07 \text{ min}^{-1}$, $n = 10$; dissociation plus trituration loaded with BSA, $0.55 \pm 0.08 \text{ min}^{-1}$, $n = 11$; $p > 0.45$, Welch t test, one-tailed). Thus, the difference in baseline activity between the two data sets is not attributable to trituration loading or the introduction of BSA, and likely reflects a difference between dissociated cells sampled a few hours postplating and axons growing from explants sampled at 24 h *in vitro*. Regardless, introduction of inactive myosin II copied the effects of blebbistatin and increased protrusive activity from the distal axon above control levels.

Inhibition of ROCK or Myosin II Disrupts Growth Cone Polarity by Allowing Disto-Proximal (DP) Migration of Growth Cone Lamellipodia onto the Axon Shaft

Growth cones are highly polarized structures. Lamellipodia and filopodia formed at the growth cones are retained at the tip of the axon and do not normally migrate onto the consolidated axon shaft. Inhibition of ROCK or myosin II resulted in the DP migration of lamellipodia from the growth cone onto the axon shaft [Fig. 2(A)], in addition to increased *de novo* protrusive activity as described in Figure 1. DP migration was not routinely observed in controls that retained polarized growth cones with lamellipodial activity restricted to the tip of the axon. Inhibition of myosin II or ROCK increased the percentage of axons that exhibited DP migration by 6–10-fold (Table 1). Inhibition of ROCK and myosin II disrupts the maintenance of growth cone polarity by allowing lamellipodia formed at the growth cone to migrate onto the axon shaft. We interpret DP migration as reflective of a failure to maintain the growth cone in a polarized state.

In order to gain insights into the mechanism that normally prevents DP migration, thereby maintaining growth cone polarity, we analyzed time-lapse sequences of growth cones

expressing EYFP-beta actin. In control growth cones, we observed that some lamellipodia migrated toward the neck of the growth cone [Fig. 2(B)]. However, these lamellipodia did not continue migrating onto the axon shaft. When lamellipodia reached the neck of the growth cone protrusive activity was shut down in concert with the formation of actin bundles in association with the lamellipodia [e.g., Fig. 2(B), 144–192 s; also see next section, Fig. 3(A,B)]. The lamellipodium was subsequently contracted into the neck of the growth cone. These observations on lamellipodia are consistent with the report that filopodia can also exhibit displacement toward the neck of the growth cone after which they are consolidated into the axon (Bray and Chapman, 1985). In contrast, when cultures were treated with the ROCK inhibitor Y-27632 lamellipodia failed to stop at the growth cone neck and continued exhibiting protrusive activity as the lamellipodium migrated onto the axon. Figure 2(C) shows an example of the failure of lamellipodia undergoing DP migration to stop at the growth cone neck in Y-27632-treated cultures. In this example the whole growth cone lamellipodium migrated approximately 15 μm back onto the axon, leaving the former site of the growth cone devoid of protrusive activity.

We next analyzed the EYFP-actin time-lapse sequences in order to determine the frequency at which lamellipodia underwent migration toward the base of the growth cone, and characterized the behavior of the lamellipodium when it reached the growth cone neck and stopped extending further. Fifty-seven percent of control growth cones exhibited one to three attempts at DP migration, as shown in Figure 2(B) ($n = 14$, 6 min sampling period). In 66% of attempts at DP migration we observed actin bundles developing in association with the migrating lamellipodium as it reached the growth cone neck [Fig. 2(B), 144–192 s; Fig. 3(A)]. In cases where DP migration was not associated with *de novo* bundle formation, migrating lamellipodia halted when the edge of the lamellipodium encountered pre-existing filopodial F-actin bundles at the growth cone neck. In 33% of cases in which bundles formed *de novo*, the bundles appeared perpendicular to the lamellipodium undergoing DP migration [Fig. 3(A)]. In the remaining cases of *de novo* bundle formation, bundles formed parallel to the edge of the lamellipodium [Fig. 2(B), 54–84 s; Fig. 3(B)]. Lamellipodia subsequently retracted into the growth cone neck. Thus, the DP migration of growth cone lamellipodia terminated in concert with the formation of F-actin bundles or arriving at an existing filopodial F-actin bundle.

Sixty-four percent of Y-27632-treated growth cones exhibited attempts at DP migration, similar to controls ($n = 14$, 6 min sampling period). However, only one out of nine DP migrations stopped at the growth cone neck. In Y-27632-treated axons, we did not observe bundle formation or stopping at existing filopodial bundles, as noted in controls, and DP migration from the growth cone onto the distal axon continued for 5–15 μm along the distal axon before the lamellipodium was absorbed into the axon shaft. In the one instance of DP migration that was terminated at the growth cone neck we observed bundle formation similar to that noted in controls. Collectively, these observations indicate that growth cone polarity is maintained by a ROCK-myosin-II-based mechanism that prevents the DP migration of growth cone lamellipodia beyond the growth cone neck by blocking lamellipodial protrusion in concert with the formation of F-actin bundles and contraction into the neck. The lack of bundle formation in lamellipodia attempting DP migration in Y-27632-treated axons suggests that ROCK activity is required for the formation of actin bundles at the growth cone neck.

ROCK Activity Drives F-Actin Bundle Formation in Growth Cones

As described above, live imaging of EYFP-actin at the growth cone neck revealed that DP-lamellipodial migration terminated in conjunction with the formation of F-actin bundles [Fig. 3(A,B)], and that when ROCK was inhibited DP lamellipodial migration was enhanced and F-actin bundle formation was diminished. We therefore investigated whether activation of ROCK, downstream of RhoA, is sufficient to promote F-actin bundles in growth cones.

Neurons were transfected with constitutively active RhoA protein (L63RhoA) using Chariot as a delivery reagent, fixed, and stained to reveal F-actin. Neurons were transfected with 0.5 μ g of L63RhoA for 2 h. Greater concentrations of L63RhoA resulted in growth cone collapse that prevented analysis of F-actin organization. L63RhoA-transfected axons and growth cones contained increased numbers of F-actin bundles in the central domain [Fig. 3(C); see Methods]. We quantified the number of F-actin bundles as described in Bridgman et al. (2001) and determined that L63RhoA-transfected growth cones contained 180% greater density of F-actin bundles than control growth cones. The increase in growth cone F-actin bundle density in response to L63RhoA was abolished by treatment with the ROCK inhibitor Y-27632 [Fig. 3(D)]. Inhibition of myosin II using blebbistatin did not alter the density of L63RhoA induced F-actin bundles in growth cones [Fig. 3(D)], indicating that myosin II is not a downstream effector of ROCK in F-actin bundle formation. These data confirm that ROCK activity drives the formation of F-actin bundles in growth cones.

ROCK Drives Myosin II Activity in the Distal Axon

Inhibition of myosin II and ROCK resulted in increased protrusive activity by the distal axon and allowed lamellipodial DP migration. We have previously shown that RhoA is active in growth cones and distal axons (Gehler et al., 2004a), and myosin is localized to growth cones and axons (Rochlin et al., 1995; Wahl et al., 2000; Zhang et al., 2003). However, although myosin II has been shown to be under regulation by ROCK in neurons (Wahl et al., 2000), previous determinations relied on Western blotting and do not provide information regarding the state of myosin II activation at the subcellular level. Therefore, we sought to determine whether ROCK drives myosin II activity in the distal axon, the site of axon consolidation.

Myosin II activity requires the phosphorylation of myosin II regulatory light chains at serine 19 (rMLC). Serine 19 is phosphorylated by myosin light chain kinase and dephosphorylated by the myosin II phosphatase. The myosin II phosphatase is in turn under negative regulation by ROCK, and ROCK can also directly phosphorylate serine 19 (Totsukawa et al., 2000; Ueda et al., 2002). We used an antibody raised against serine 19 phosphorylated chicken rMLC to detect activated myosin II in axons [Fig. 4(A); see Supplemental Fig. 1 for validation of the antibody specificity]. Importantly, phospho-rMLC staining was present in the distal 20 μ m of axons, the site of consolidation affected by inhibition of ROCK and myosin II [Fig. 4(A,B)]. Phosphorylated rMLC were found throughout axons and quantitative analysis of phospho-rMLC staining levels ratioed to a volumetric marker revealed that the activity of myosin II is relatively uniform in distal axons [Fig. 4(C)].

We observed increased protrusive activity following inhibition of ROCK or myosin II. This suggests that myosin II is downstream of ROCK in the mechanism that suppresses protrusive activity from the distal axon. Therefore, we next determined whether ROCK inhibition resulted in decreased levels of phospho-rMLC staining in the distal 20 μ m of axons. Cultures were first treated with Y-27632 or DMSO, then fixed and stained for phospho-rMLC. The phospho-rMLC staining was ratioed to volume. Y-27632 treatment decreased rMLC-p in the distal 20 μ m of axons by 83% relative to DMSO-treated controls [Fig. 4(B,D)]. Y-27632 did not affect the intensity of pan-MLC staining (data not shown). These data demonstrate that myosin II is under regulation by ROCK in the distal axon and support the observations that inhibition of ROCK or myosin II results in unstable consolidation of the distal axon.

Spontaneously Formed F-Actin Patches Are Precursors to Protrusive Activity in the Distal Axon

The observation that inhibition of ROCK or myosin II results in increased protrusive activity along the distal axon provided interesting phenomenological data. However, these data do not provide insights into how ROCK and myosin II mechanistically regulate the axonal actin

cytoskeleton resulting in suppression of protrusive activity contributing to the consolidation of the distal axon. Therefore, in order to elucidate the cytoskeletal basis of protrusive activity along the distal axon we examined the dynamics of EYFP-actin. We identified spontaneously formed and transient patches of EYFP-actin fluorescence in distal axons [Fig. 5(A)]. These patches are reflective of F-actin and not an accumulation of monomeric actin. First, patches colocalized with phalloidin staining, a reagent that only binds F-actin [Fig. 5(B)]. Second patches did not form in the presence of latrunculin-A (4 μ M) or cytochalasin-D (15 μ g/mL), cell-permeable inhibitors of F-actin polymerization (not shown). Third, jasplakinolide is a cell-permeable inducer of F-actin polymerization (Bubb et al., 1994). Treatment with a high dose of jasplakinolide (4 μ M) increased the intensity of existing patches at the expense of the diffuse EYFP-actin signal in axons [Fig. 5(C)], the latter presumably reflective of soluble actin. Indeed, 4 μ M jasplakinolide resulted in a significant shift of actin from the monomeric soluble pool onto existing filaments as determined by cellular fractionation of soluble and polymeric forms of actin followed by Western blotting [Fig. 5(D)]. Finally, F-actin patches were detected along nontransfected axons stained with phalloidin with the same frequency as EYFP-actin patches [Fig. 5(E)]. We found 1.05 ± 0.19 puncta/10 μ m of axon in EGFP-transfected axons ($n = 23$) and 1.13 ± 0.15 puncta/10 μ m of axon in nontransfected axons in the same cultures ($n = 37$; $p > 0.8$, t test). Thus, transient patches of F-actin form spontaneously within distal axons.

Spontaneously formed EYFP-actin patches served as precursors for axonal filopodia and lamellipodia formation [Fig. 6(A)]. The distal 20 μ m of EYFP-actin-expressing axons spontaneously generated F-actin patches at a frequency of 4.9 ± 0.5 patches per minute ($n = 15$ axons). Six percent ($n = 471$) of axonal F-actin patches gave rise to a filopodium or lamellipodium. Moreover, 83% ($n = 28$) of spontaneously formed axonal filopodia or lamellipodia were preceded by detectable F-actin patch formation 6–25 s before emergence of the filopodium. Ninety-seven percent of patches that gave rise to protrusive activity resulted in the formation of a filopodium. Lamellipodial formation accounted for only 3% of protrusive activity. Thus, consistent with the report of Lau et al. (1999), pre-existing axonal F-actin patches serve as precursors to protrusive activity.

ROCK Regulates Axonal F-Actin Patch Elaboration and Subsequent Protrusive Activity

Inhibition of ROCK or myosin II increased protrusive activity from the distal axon. In order to investigate further the underlying cytoskeletal dynamics, we took advantage of the identification of spontaneously formed axonal F-actin patches as precursors to protrusive activity in the distal axon. We tested the hypothesis that ROCK and myosin II control aspects of patch elaboration and/or the frequency with which patches give rise to protrusive activity. We refer to the increase in size and fluorescence intensity of axonal F-actin patches as elaboration. Based on the observation that inhibition of ROCK or myosin II increased filopodial and lamellipodial formation from the distal axon a number of models can be envisioned. For example, inhibition of ROCK/myosin II could increase protrusive activity by increasing the frequency of patch formation independent of the frequency of transition from patch to a protrusive structure. Alternatively, inhibition of ROCK/myosin II could increase the transition from patch to protrusive activity without altering the frequency of patch formation. Furthermore, ROCK/myosin II could alter both the frequency of patch formation and the transition into protrusive activity.

We first determined whether inhibition of ROCK (Y-27632) or Chariot peptide-mediated transfection with dominant negative myosin II altered the frequency of patch formation or the frequency at which patches transitioned into protrusive activity. We used dnMyoII to inhibit endogenous myosin II because blebbistatin is not a suitable reagent for live imaging of EYFP-actin due to phototoxicity (Kolega, 2004). dnMyoII was loaded into axons using Chariot as a

cell-permeable transfection reagent (see Supplemental Fig. 2 for verification of transfection efficiency; Gallo, 2003). Neither ROCK nor myosin II inhibition altered the frequency of patch formation [Fig. 6(B), leftmost bars labeled *patch frequency*]. However, both ROCK and myosin II inhibition increased the frequency of transition from patch formation into a protrusive event [Fig. 6(B), bars labeled *transition frequency*]. Thus, these data indicate that myosin II is downstream of ROCK in regulating the frequency with which spontaneously formed F-actin patches give rise to protrusive structures in the distal axon.

In order to further determine whether ROCK and myosin II are also involved in the elaboration of patches we performed a detailed quantitative analysis of patches. The top panel of Figure 6 (B) shows the general scheme of the measurements we performed on patches (see Methods for additional details). Briefly, we measured the mean intensity of EYFP-actin fluorescence as a function of time in regions of the axons that gave rise to patches. From the time sequence of the changes in intensity of individual patches we next extracted a number of measurements. We determined the lifespan of patches, reflective of the duration of fluorescence increase above baseline. We also obtained the maximum intensity value for each patch, reflecting the highest point in the plot of EYFP-intensity versus time [Int. Max; Fig. 6(B)]. We interpret Int. Max as reflective of the maximal total amount of actin filaments present during each patch's lifespan. In addition we determined the time it took for each patch to attain maximal intensity (t_{max}). In order to investigate whether the overall form of the patch intensity profile was altered we compared the ratio of the duration of the rise to Int. Max to the duration of the falling phase of patch intensity ($T_{\text{rise}}/T_{\text{fall}}$). This metric would report if, after experimental treatments, the overall profile of patch intensity is altered in terms of time spent undergoing net actin polymerization (T_{rise}) versus net actin depolymerization (T_{fall}). Finally, we measured the rate of fluorescence intensity increase during the rise phase of each patch's profile (Int. Max/ T_{rise}). Changes in this metric could result from increased net polymerization or depolymerization, respectively.

Inhibition of ROCK resulted in F-actin patches that persisted for longer time periods and attained greater maximal intensities. Inhibition of ROCK increased patch life span by 40%, Int. Max by 25%, and t_{max} by 140% [Fig. 6(B)]. No effect on $T_{\text{rise}}/T_{\text{fall}}$ or Int. Max/ T_{rise} was detected. Interestingly, although inhibition of myosin II mimicked the effects of ROCK inhibition on the transition frequency into protrusive activity, it did not alter any additional measurements [Fig. 6(B)]. Examples of the effects of ROCK inhibition on the temporal profile of patch intensity are shown in Figure 6(C), and are summarized in diagrammatic form in Figure 6(D). These data indicate that endogenous ROCK activity in the distal axon inhibits the extent of F-actin polymerization in patches that act as precursors to protrusive activity. Thus, ROCK modulates patch elaboration by negatively regulating the total extent of F-actin polymerization that occurs without altering the overall temporal profile of patch elaboration, as indicated by no effect of ROCK inhibition on $T_{\text{rise}}/T_{\text{fall}}$. In a heuristic sense, endogenous ROCK activity puts "the breaks" on net F-actin patch elaboration, but does not control the initial formation of patches in the distal axon. Thus, ROCK contributes to stable consolidation of the distal axon by suppressing the elaboration of F-actin patches that serve as precursors to protrusive activity. In contrast, although inhibition of myosin II copied the effects of ROCK inhibition on the transition frequency from patch formation into protrusive activity, unlike ROCK inhibition it did not alter any of the measured parameters of F-actin patch elaboration [Fig. 6(B)]. Thus, as evidenced by the finding that inhibition of myosin II only copied the effects of ROCK inhibition on the transition from F-actin patch into protrusive activity, myosin II downstream of ROCK is specifically involved in the inhibition of protrusive activity from spontaneously formed patches.

Inhibition of Myosin II or ROCK Blocks Growth Cone Turning by Preventing Growth Cone Repolarization

Growth cone guidance by attractant signals requires the repolarization of the growth cone toward the source of the attractant. NGF guides growth cones *in vitro* and is thus a useful model system for studying the mechanism of axonal guidance (Gallo et al., 1997). In order to determine whether myosin II activity is required for NGF-mediated growth cone guidance, we compared the turning behavior of growth cones toward NGF-coated beads in the absence and presence of blebbistatin. As previously characterized, the turning behavior of growth cones following contact with NGF-beads consists of five phases (Gallo et al., 1997): (1) initial filopodial contact with the bead; (2) stabilization and retention of the filopodial contact with the bead; (3) engorgement of the contact; (4) consolidation (i.e., suppression of protrusive activity) on the side of growth cone in the direction opposite to the contact; and (5) reformation of a polarized growth cone resulting in axon turning toward the NGF-coated bead. Ninety percent of control growth cones established filopodial contacts with beads and turned towards the site of filopodial contact, as previously described [Fig. 7(A)]. In contrast, only 35% of blebbistatin-treated growth cones that made contact with NGF-beads turned towards the site of contact [Fig. 7(B)]. Significantly, blebbistatin-treated growth cones responded to contact with NGF-beads by going through the first three phases of turning in a manner not distinguishable from controls [Fig. 7(C), also see below]. However, turning towards the beads was aborted when the side of the growth cone away from the contact failed to consolidate and continued generating new lamellipodia or filopodia in the direction opposite the contact with the bead [18/20 growth cones that did not turn failed to exhibit consolidation of the side away from the contact; Fig. 7(C)]. Growth cones treated with the ROCK inhibitor Y-27632 also failed to turn toward NGF-beads due to disruption of consolidation [Fig. 7(B); 9/13 growth cones that did not turn failed to exhibit consolidation of the side away from the contact].

In order to perform a quantitative analysis of the disruption of consolidation under conditions of myosin II inhibition during turning toward NGF-beads we measured the ratio of growth cone area directed away from the contact relative to that toward the contact, which we term the polarity ratio (see Methods). Prior to contact with NGF-beads both control and blebbistatin-treated growth cones exhibited similar polarity ratios of approximately one ($p > 0.4$ for ratio comparison between controls and blebbistatin). In control growth cones turning toward NGF-beads the polarity ratio decreased as growth cones turned and consolidated the area directed away from the contact [Fig. 7(D)]. In the presence of blebbistatin growth cones responded with a decrease in polarity ratio in a manner similar to controls during the first 2 min following contact [Fig. 7(D)]. However, following this initial response to the NGF-beads the polarity ratio of blebbistatin-treated growth cones returned to precontact levels as growth cones inappropriately protruded lamellipodia and filopodia away from the contact with the bead [Fig. 7(C,D)]. Thus, growth cones in the presence of blebbistatin initially respond to contact with NGF-beads but fail to remain polarized towards the contact due to persistent protrusive activity in the direction opposite the bead.

DISCUSSION

Axon extension and guidance occur through a three-phase process sequentially involving protrusion, engorgement, and consolidation of the axon (reviewed in Dent and Gertler, 2003). Growth cone polarity is due the preferential protrusion of filopodia and lamellipodia at the tip of the axon, and polarity is maintained by the process of consolidation, which suppresses protrusive activity from the distal axon. The mechanism underlying the suppression of protrusive activity during consolidation is the least understood phase of axon extension and guidance. This study yielded three major observations regarding the mechanism of axon consolidation. First, ROCK-myosin II activity is required for suppression of protrusive activity

during consolidation of the distal axon shaft. Second, ROCK negatively regulates the elaboration of axonal F-actin patches that serve as precursors to protrusive activity while promoting formation of F-actin bundles. Finally, unstable consolidation of the distal axon prevented growth cones from fully executing a positive turning response toward NGF.

The mechanism of filopodial initiation has received significant attention in recent years. The ability to quantitatively track the elaboration of axonal F-actin patches and their transition into protrusive activity provides a novel model system for the study of filopodial initiation. Svitkina et al. (2003) proposed that filopodia form through reorganization of F-actin branched dendritic networks. In addition, Steketee et al. (2001) described a structure termed the basal ring that appears to be one of the first steps in filopodial formation. The observation that axonal filopodia form from pre-existing patches of F-actin appears consistent with the dendritic network model. However, the specific molecular components driving filopodial formation in neurons may be different from those in non-neuronal cells (Stasser et al., 2004).

The elaboration of axonal F-actin patches is negatively regulated by ROCK. Myosin II downstream of ROCK controls the transition from patch to protrusive activity but not additional effects of ROCK on patch elaboration. We suggest that cofilin/actin depolymerizing factor may be the effector downstream of ROCK that regulates patch elaboration. The elaboration of patches reflects the net polymerization and depolymerization of F-actin. Decreased ROCK activity is expected to result in increased cofilin/actin depolymerizing factor activity through decreased phosphorylation of cofilin by the ROCK-regulated LIM-kinase (Maekawa et al., 1999; Kuhn et al., 2000; Gehler et al., 2004b). The observation that patch elaboration is prolonged and patches attain greater maximal intensities when ROCK is inhibited is suggestive of a role of cofilin downstream of ROCK in regulating the turnover of F-actin in axonal patches.

Our data indicate that myosin II is the downstream effector of ROCK that negatively regulates filopodial formation from patches. Myosin II drives F-actin retrograde flow in growth cone lamellipodia and filopodia (Lin et al., 1997). As with the extension of the tip of an established filopodium, emergence of a filopodium from a patch is likely countered by the ongoing F-actin retrograde flow driven by myosin II. In the absence of myosin II activity filopodia forming from patches would be subjected to decreased F-actin retrograde flow and may thus form at a greater frequency.

The data suggest a working model for the suppression of protrusive activity contributing to the stable consolidation of the distal axon shaft and the maintenance of growth cone polarity. In the distal axon, ROCK positively regulates myosin II and negatively regulates the elaboration of patches of F-actin that serve as precursors to protrusive activity (Fig. 8). In turn myosin II negatively regulates the transition from patch to a protrusive structure. Thus, during axon consolidation protrusive activity along the distal axon is inhibited by ROCK and myosin II. In addition, ROCK and myosin II are required to prevent the inappropriate migration of growth cone lamellipodia onto the axon that otherwise results in the disruption of growth cone polarity. The termination of DP lamellipodial migration correlated with the ROCK-dependent formation of actin bundles associated with the lamellipodia, or arrival at an existing actin bundle. Myosin II may be required for the contraction of the lamellipodium and bundles into the neck of the growth cone. Interestingly, Steketee et al. (2002) report that shaft adhesions along filopodial F-actin bundles block the protrusion of lamellipodia, an observation consistent with the correlation between the presence of actin bundles and termination of lamellipodial DP migration. Schaefer et al. (2002) have described actin arcs in the central and lateral domains of *Aplysia* growth cones that appear analogous to bundles formed at the growth cone neck. Furthermore, actin arcs and the bundles described in this article share a dependence on ROCK activity (Zhang et al., 2003). In addition, Shimizu et al. (2005) also reported that ROCK is required for the formation of actomyosin bundles in non-neuronal cells. We suggest that myosin

II in growth cones and axons operates through a dynamic network contraction mechanism (Verkhovsky et al., 1999). Thus, F-actin bundles lateral to the central domain/growth cone neck would be contracted toward the base of the growth cone, contributing to myosin II-dependent consolidation.

The maintenance of growth cone polarity is likely due to a balance of protrusion promoting activities localized to the distal axon and the presence of the protrusion inhibitory mechanism described in this article. Inhibition of ROCK-myosin II impaired consolidation of the distal axon (<20 μm from the growth cone neck), but not the proximal axon (>20 μm from the growth cone neck). This observation indicates that consolidation of the proximal axon involves additional mechanisms other than ROCK-myosin II activity. We suggest the lack of increases in protrusive activity from the proximal axon under conditions of ROCK-myosin II inactivation is due to decreased positive regulation of protrusive activity relative to the distal axon and growth cone. For example, microtubule dynamics have been shown to positively regulate protrusion and actin polymerization through a Rac1-mediated mechanism (Gallo, 1998; Rochlin et al., 1999; Grabham et al., 2003; Rodriguez et al., 2003) and growth cones contain more dynamic microtubule tips than axons (Stepanova et al., 2003). Alternatively, growth cones may contain specific cytoskeletal regulatory proteins not prevalent or active in the proximal axon (e.g., GAP-43; Strittmatter et al., 1992). Future work will address the issue of the mechanism of consolidation of the proximal axon. The consolidation of the proximal axon is of relevance to the mechanism of collateral branch formation, which is initiated by the protrusion of axonal filopodia (Gallo and Letourneau, 1999). Our results indicate that formation of axon collateral branches from the proximal axons requires additional signaling intermediates than the ROCK-myosin II axis in sensory neurons. For example, NGF-mediated collateral branching likely involves the activation of multiple pathways, including PI3-kinase (Gallo and Letourneau, 1998).

Myosin II activity is required for guidance toward a source of NGF. Turney and Bridgman (2005) reported that inhibition of myosin II blocks guidance by substratum-bound laminin. However, whether myosin II activity is required for guidance in response to growth factors is unknown. We now demonstrate that myosin II is also required for axon guidance by NGF. Our observations identify the suppression of protrusive activity during axon consolidation as a component of axon guidance. Failure to suppress protrusive activity from the side of the growth cone away from the contact with NGF correlates with the failure of growth cones to turn toward a source of NGF. Growth cone turning toward NGF-coated beads is achieved by repolarization of the growth cone toward the source of NGF (Gallo et al., 1997; Gallo and Letourneau, 2000; Gallo and Letourneau, 2004). Growth cones with inhibited ROCK-myosin II did not turn due to the failure to suppress protrusive activity from the site opposite contact with NGF, although they exhibited an initial turning response toward NGF similar to controls. Thus, growth cones failed to maintain polarity specifically toward the contact with NGF and aborted turning. Previous work has established that neurotrophins induce transient decreases in RhoA-ROCK activity and rMLC phosphorylation (Yamashita et al., 1999; Fujita et al., 2001; Gehler et al., 2004a). Thus, during guidance NGF may elicit localized decreases in myosin II activity, generating an imbalance in contractility across the growth cone, resulting in turning toward the side of least contractility and consolidation of the side exhibiting the greatest level of myosin activity (i.e., the direction opposite to contact with NGF). The observation that ROCK inhibits F-actin patch elaboration suggests that local decreases in ROCK activity induced by NGF could also promote F-actin polymerization.

In conclusion, these data identify ROCK and myosin II as important elements of the mechanism that drives the consolidation of the axon shaft behind the advancing growth cone, and thereby maintains growth cone polarity driven by the preferential protrusion of filopodia and lamellipodia at the tip of the axon. We propose that growth cone polarity is not just the result

of protrusive activity specific to the tip of the axon, but rather that it is due to a local balance of forces between protrusion promoting factors and protrusion inhibitory factors such as ROCK and myosin II.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Bito H, Furuyashiki T, Ishihara H, Shibasaki Y, Ohashi K, Mizuno K, Maekawa M, et al. A critical role for a Rho-associated kinase, p160ROCK, in determining axon outgrowth in mammalian CNS neurons. *Neuron* 2000;26:431–441. [PubMed: 10839361]
- Bray D, Chapman K. Analysis of microspike movements on the neuronal growth cone. *J Neurosci* 1985;5:3204–3213. [PubMed: 4078625]
- Bridgman PC, Dave S, Asnes CF, Tullio AN, Adelstein RS. Myosin IIB is required for growth cone motility. *J Neurosci* 2001;21:6159–6169. [PubMed: 11487639]
- Bridgman PC, Elkin LL. Axonal myosins. *J Neurocytol* 2000;29:831–841. [PubMed: 11466474]
- Brown J, Bridgman PC. Role of myosin II in axon outgrowth. *J Histochem Cytochem* 2003;51:421–428. [PubMed: 12642620]
- Bubb MR, Senderowicz AM, Sausville EA, Duncan KL, Korn ED. Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J Biol Chem* 1994;269:14869–14871. [PubMed: 8195116]
- Dent EW, Gertler FB. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* 2003;40:209–227. [PubMed: 14556705]
- Ernst AF, Gallo G, Letourneau PC, McLoon SC. Stabilization of growing retinal axons by the combined signaling of nitric oxide and brain-derived neurotrophic factor. *J Neurosci* 2000;20:1458–1469. [PubMed: 10662836]
- Fujita A, Hattori Y, Takeuchi T, Kamata Y, Hata F. NGF induces neurite outgrowth via a decrease in phosphorylation of myosin light chain in PC12 cells. *Neuro-Report* 2001;12:3599–3602.
- Gallo G. Involvement of microtubules in the regulation of neuronal growth cone morphologic remodeling. *J Neurobiol* 1998;35:121–140. [PubMed: 9581969]
- Gallo G. Making proteins into drugs: Assisted delivery of proteins and peptides into living neurons. *Methods Cell Biol* 2003;71:326–339.
- Gallo G, Lefcort FB, Letourneau PC. The trkA receptor mediates growth cone turning toward a localized source of nerve growth factor. *J Neurosci* 1997;17:5445–5454. [PubMed: 9204927]
- Gallo G, Letourneau PC. Localized sources of neurotrophins initiate axon collateral sprouting. *J Neurosci* 1998;18:5403–5414. [PubMed: 9651222]
- Gallo G, Letourneau PC. Different contributions of microtubule dynamics and transport to the growth of axons and collateral sprouts. *J Neurosci* 1999;19:3860–3873. [PubMed: 10234018]
- Gallo G, Letourneau PC. Neurotrophins and the dynamic regulation of the neuronal cytoskeleton. *J Neurobiol* 2000;44:159–173. [PubMed: 10934319]
- Gallo G, Letourneau PC. Regulation of growth cone actin filaments by guidance cues. *J Neurobiol* 2004;58:92–102. [PubMed: 14598373]
- Gallo G, Yee HF Jr, Letourneau PC. Actin turnover is required to prevent axon retraction driven by endogenous actomyosin contractility. *J Cell Biol* 2002;158:1219–1228. [PubMed: 12356866]
- Gehler S, Gallo G, Veien E, Letourneau PC. p75 neurotrophin receptor signaling regulates growth cone filopodial dynamics through modulating RhoA activity. *J Neurosci* 2004a;24:4363–4372. [PubMed: 15128850]

- Gehler S, Shaw AE, Sarmiere PD, Bamburg JR, Letourneau PC. Brain-derived neurotrophic factor regulation of retinal growth cone filopodial dynamics is mediated through actin depolymerizing factor/cofilin. *J Neurosci* 2004b;24:10741–10749. [PubMed: 15564592]
- Grabham PW, Reznik B, Goldberg DJ. Microtubule and Rac 1-dependent F-actin in growth cones. *J Cell Sci* 2003;116:3739–3748. [PubMed: 12890754]
- Ikenoya M, Hidaka H, Hosoya T, Suzuki M, Yamamoto N, Sasaki Y. Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. *J Neurochem* 2002;81:9–16. [PubMed: 12067241]
- Jurney WM, Gallo G, Letourneau PC, McLoon SC. Rac1 mediates endocytosis during ephrin-A2 and semaphorin 3A induced growth cone collapse. *J Neurosci* 2002;22:6019–6028. [PubMed: 12122063]
- Kolega J. Phototoxicity and photoinactivation of blebbistatin in UV and visible light. *Biochem Biophys Res Comm* 2004;320:1020–1025. [PubMed: 15240150]
- Kuhn TB, Meberg PJ, Brown MD, Bernstein BW, Minamide LS, Jensen JR, Okada K, et al. Regulating actin dynamics in neuronal growth cones by ADF/ cofilin and rho family GTPases. *J Neurobiol* 2000;44:126–144. [PubMed: 10934317]
- Lau PM, Zucker RS, Bentley D. Induction of filopodia by direct local elevation of intracellular calcium ion concentration. *J Cell Biol* 1999;145:1265–1275. [PubMed: 10366598]
- Lin CH, Espreafico EM, Mooseker MS, Forscher P. Myosin drives retrograde F-actin flow in neuronal growth cones. *Biol Bull* 1997;192:183–185. [PubMed: 9057289]
- Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, et al. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 1999;285:895–898. [PubMed: 10436159]
- Rochlin MW, Dailey ME, Bridgman PC. Polymerizing microtubules activate site-directed F-actin assembly in nerve growth cones. *Mol Biol Cell* 1999;10:2309–2327. [PubMed: 10397767]
- Rochlin MW, Itoh K, Adelstein RS, Bridgman PC. Localization of myosin II A and B isoforms in cultured neurons. *J Cell Sci* 1995;108:3661–3670. [PubMed: 8719872]
- Rodriguez OC, Schaefer AW, Mandato CA, Forscher P, Bement WM, Waterman-Storer CM. Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat Cell Biol* 2003;5:599–609. [PubMed: 12833063]
- Schaefer AW, Kabir N, Forscher P. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J Cell Biol* 2002;158:139–152. [PubMed: 12105186]
- Shimizu Y, Thumkeo D, Keel J, Ishizaki T, Oshima H, Oshima M, Noda Y, et al. ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. *J Cell Biol* 2005;168:941–953. [PubMed: 15753128]
- Stekette M, Balazovich K, Tosney KW. Filopodial initiation and a novel filament-organizing center, the focal ring. *Mol Biol Cell* 2001;12:2378–2395. [PubMed: 11514623]
- Stekette M, Balazovich K, Tosney KW. Three functionally distinct adhesions in filopodia: shaft adhesions control lamellar extension. *J Neurosci* 2002;22:8071–8083. [PubMed: 12223561]
- Stepanova T, Slemmer J, Hoogenraad CC, Lansbergen G, Dortland B, De Zeeuw CI, Grosveld F, van Cappellen G, Akhmanova A, Galjart N. Visualization of micro-tubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). *J Neurosci* 2003;23:2655–2664. [PubMed: 12684451]
- Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, Sellers JR, Mitchison TJ. Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. *Science* 2003;299:1743–1747. [PubMed: 12637748]
- Strasser GA, Rahim NA, VanderWaal KE, Gertler FB, Lanier LM. Arp2/3 is a negative regulator of growth cone translocation. *Neuron* 2004;8:81–94. [PubMed: 15233919]
- Strittmatter SM, Vartanian T, Fishman MC. GAP-43 as a plasticity protein in neuronal form and repair. *J Neurobiol* 1992;23:507–520. [PubMed: 1431834]
- Svitkina TM, Bulanova EA, Chaga OY, Vignjevic DM, Kojima S, Vasiliev JM, Borisy GG. Mechanism of filopodia initiation by reorganization of a dendritic network. *J Cell Biol* 2003;160:409–421. [PubMed: 12566431]

- Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J Cell Biol* 2000;150:797–806. [PubMed: 10953004]
- Tullio AN, Bridgman PC, Tresser NJ, Chan CC, Conti MA, Adelstein RS, Hara Y. Structural abnormalities develop in the brain after ablation of the gene encoding nonmuscle myosin II-B heavy chain. *J Comp Neurol* 2001;433:62–74. [PubMed: 11283949]
- Turney GS, Bridgman PC. Laminin stimulates and guides axonal outgrowth via growth cone myosin II activity. *Nat Neurosci* 2005;8:717–719. [PubMed: 15880105]
- Ueda K, Murata-Hori M, Tatsuka M, Hosoya H. Rho-kinase contributes to diphosphorylation of myosin II regulatory light chain in nonmuscle cells. *Oncogene* 2002;21:5852–5860. [PubMed: 12185584]
- Verkhovsky AB, Svitkina TM, Borisy GG. Network contraction model for cell translocation and retrograde flow. *Biochem Soc Symp* 1999;65:207–222. [PubMed: 10320940]
- Wahl S, Barth H, Ciossek T, Aktories K, Mueller BK. Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J Cell Biol* 2000;149:263–270. [PubMed: 10769020]
- Yamashita T, Tucker KL, Barde YA. Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* 1999;24:585–593. [PubMed: 10595511]
- 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]

Figure 1.

Inhibition of ROCK or myosin II resulted in unstable consolidation of the distal axon. (A) Examples of distal axons double stained for tubulin (green) and F-actin (red). Note that axons treated with 50 μ M blebbistatin for 1 h exhibit lamellipodia (arrowheads). (B) Time-lapse sequences of control and blebbistatin-treated axons (numbers denote min). Note that the blebbistatin-treated axon exhibits filopodial and lamellipodial protrusion from the distal axon. At min 1 multiple small lamellae form along the distal axon followed by initiation of additional lamellipodia and filopodia. (C) Quantification of the frequency of lamellipodial or filopodial initiation along the axon as a function of distance from the growth cone neck in controls and blebbistatin- or Y-27632-(10 μ M) treated axons. Both treatments increase protrusive activity in the distal, but not proximal axon, relative to controls (DMSO treated, $p < 0.001$). $n = 15-20$ axons per group. (D) Quantification of protrusive activity in the distal 10 μ m of axon in dissociated neurons triturated loaded with dominant negative myosin II (dnMyoII), or BSA as a control, and axons growing from explants treated with the ROCK inhibitor HA-1152 (10 μ M) or DMSO. All bars = 10 μ m.

Figure 2.

ROCK and myosin II are required for maintenance of growth cone polarity. (A) Example of disto-proximal lamellipodial migration from a growth cone treated with HA-1152. White arrowheads denote lamellipodia formed from the axon *de novo*. The black arrowheads track the disto-proximal migration of growth cone lamellipodia onto the axon shaft. At 40 s the growth cone reverses polarity, exhibiting lamellipodial extension toward the axon. By 60 s lamellipodia at the growth cone have begun to re-extend in the “appropriate” direction, away from the axon tip, and growth cone polarity is re-established. The lamellipodium initiated at 40 s continues to migrate disto-proximally up to 150 s. As the lamellipodium migrates it breaks up into smaller lamellae, after which it is retracted into the axon shaft. (B) Example of two

unsuccessful attempts at disto-proximal lamellipodial migration in a control EYFP-actin-expressing growth cone. Note two instances of the emergence of a lamellipodium at 54 and 132 s (arrowhead) and movement toward the neck (54–84 and 132–204 s, direction of movement shown by arrows at 60 and 144 s). Upon arrival at the growth cone neck (defined by arrows at 54 s) both lamellae stop protruding and undergo contraction into the neck (72–84 and 162–204 s). In the first example (36–84 s) the termination of lamellipodial migration correlates with the presence of a bundled arclike structure at the base of the lamellipodium (36–60 s, denoted at 60 s by set of three arrows with concave heads), toward which the lamellipodium retracts (60–84 s, arrow at 84 s denotes retraction of lamellipodium into the arc-like bundle). Similarly, in the second example, parallel actin bundles form as the lamellipodium reaches the growth cone neck (144–192 s). Note the contraction of the edges of the lamellipodium, now defined by actin bundles, between 162–192 s (arrows at 192 s). Arrow at 240 s shows the distance the growth cone neck advanced during the sequence. All images were identically digitally enhanced in order to reveal the relevant details of actin organization at the growth cone neck. Due to the significantly greater actin signal present in the peripheral domain, and some portions of the central domain, these manipulations result in saturation of the signal in these regions. (C) Y-27632-treated growth cone exhibiting DP lamellipodial migration. At 48 s the growth cone neck appears to consolidate (arrows). However, as shown by the remainder of the sequence, the growth cone lamellipodia begin (arrowhead at 60 s) and continue to migrate along the axon shaft (direction of migration shown by arrow at 90 s). In this case the whole of the protrusive activity originally present at the axon tip has displaced approximately 15 μm (arrow at 270 s) behind the tip (denoted by arrowheads at 132–174 s). All bars = 10 μm , bar in (B) is also valid for (C).

Figure 3.

ROCK activity increases F-actin bundle density in growth cones. (A) and (B) show time-lapse sequence of F-actin bundle formation during consolidation (numbers in panels reflect seconds elapsed). Panels focus on the growth cone neck. The green arrowhead in (A) and (B) denotes the direction of the leading edge of the axon. (A) Formation of filopodia-like bundles in the lamellipodium (red arrowhead) starting at 18 s. The bundles grow in length between 18–30 s both away from the lamellar edge and toward the base of the growth cone (arrows at 30 s, compare to 18 s). Starting at 42 s the bundles undergo “zippering” (arrowheads) and are coalesced. The coalesced bundles are subsequently retracted into the base of the growth cone (48–66 s). (B) Formation of a bundle parallel to the lamellipodium (red arrowhead). The white

dots at 0 s denote a diffuse region of fluorescence at the base of the lamellipodium. The diffuse region diminishes in size as a function of time resulting in the formation of a bundlelike linear structure aligned parallel to the axis of the axon by 36 s (blue arrowheads denote the detectable ends of the bundle). The lamellipodium subsequently retracts toward the newly formed bundle (red arrowhead at 60 s). (C) Examples of phalloidin-stained growth cones and distal axons transfected with BSA (control) or constitutively active L63RhoA. Images were inverted because black on white increases visual contrast. For demonstration purposes, an F-actin bundle is denoted by blue arrowheads in a BSA-transfected control growth cone. Note the increase in F-actin bundles in the central domain of L63RhoA- transfected growth cones relative to BSA-transfected controls. In order to clearly reveal the F-actin bundles in the central domain images were contrast enhanced (equal enhancement for all panels). This resulted in saturation of the signal in the F-actin rich peripheral domain, which contains much greater amounts of F-actin than the central domain. (D) RhoA activation of ROCK increases F-actin bundle density in growth cones. Quantification of F-actin bundle density in growth cones (bundles/ $10\ \mu\text{m}^2$) was performed as described in Bridgman et al. (2001). L63RhoA increased bundle density in a ROCK-dependent manner. Mean + SEM is shown (n = number of growth cones). L63RhoA density was compared to BSA, and L63RhoA+Y-27632 ($10\ \mu\text{M}$) density was compared to L63RhoA. Blebbistatin did not alter the increase in bundle density elicited by L63RhoA.

Figure 4.

ROCK drives myosin II activity in the distal axon. (A) Immunostaining for phosphorylated rMLC (rMLC-p) in axons extending from a DRG explant (to the right of picture). Bar = 50 μ m. (B) Growth cones double stained for rMLC-p (red) and F-actin (green, phalloidin). Top panels show a control growth cone. Arrowheads in the F-actin panel denote the growth cone neck. Bottom panels show two Y-27632-treated growth cones. Note that the staining intensity of rMLCp is decreased in Y-27632-treated axons relative to the control growth cone shown above. See text for quantitative analysis of rMLC-p staining intensity. Bar = 10 μ m. (C) Quantification of the ratio of rMLC-p staining to the volumetric marker CellTracker in the growth cone (GC) and axon. No statistically significant difference was found across these

domains (Welch *t* test, $n = 30$ axons), although there is an apparent trend toward lower rMLC-p with distance from the growth cone. (D) Quantification of rMLC-p staining in the distal axon in control DMSO-treated axons and Y-27632-treated axons. Numbers in bars reflect sample sizes.

Figure 5.

Distal axons contain spontaneously formed and transient patches of F-actin. (A) Example of EYFP-actin patch formation in the distal axon. A patch becomes evident at 6 s (arrow) and persists beyond the time shown in this sequence. An additional patch forms at 12 s (arrow) and disappears by 18 s. (B) Example of an EYFP-actin expressing axon fixed and labeled with rhodamine phalloidin. EYFP-actin patches colocalize with phalloidin staining. (C) Treatment with 4 μ M jasplakinolide causes increases in fluorescence in regions previously exhibiting relatively high EYFP-actin signal (arrowheads) at the expense of the relatively homogeneous EYFP-actin signal in the axon (arrows), reporting cytosolic actin. The panel labeled *Post* reflects the distribution of EYFP-actin 10 min after treatment with jasplakinolide. (D) Cytosolic and cytoskeletal fractionation experiment with or without jasplakinolide treatment followed by Western blotting for actin. A 10 min treatment with 4 μ M jasplakinolide causes the incorporation of the majority of available actin into filament form. (E) Example of phalloidin-stained axon exhibiting F-actin patches independent of EYFP-actin transfection.

Figure 6.

Axonal F-actin patches are precursors to protrusive activity. (A) Pseudocolored sequence of an EYFP-actin-expressing distal axon. Two patches begin to form at 0 s (blue and red arrows). The patches elaborate over the next 9 s. At 9 s a new patch is formed (red arrowhead). Between 6 and 9 s one of the two patches that formed at 0 s gives rise to a filopodium (yellow arrow). Similarly, at 18 s the patch that formed at 9 s also gives rise to a filopodium (yellow arrow). (B) Quantification of EYFP-actin patch elaboration. The top half of the panel shows a schematic diagram of the parameters we measured to quantitatively describe patch elaboration (a pseudocolored example of patch elaboration is shown below the diagram). All the data are expressed relative to the control values shown below each set of bars. Fifteen axons per group were used to obtain the data. The first two patches to form during each sequence were used for collecting data on the elaboration of patches. All patches in time-lapse sequences were counted in determining patch initiation and transition frequency, and lifespan. (C) Examples of

individual color-coded patch intensity temporal profiles graphed as a function of time. In regions of the axon that did not give rise to patches the intensity was stable over time (Baseline). Control patches rapidly attained maximal intensity and returned to baseline. Inhibition of ROCK (Y-27632) increased patch lifespan and maximum intensity, as described quantitatively in (B). (D) A schematic of the generalized effects of ROCK inhibition on the temporal profile of patch intensity.

Figure 7.

Inhibition of ROCK and myosin II prevents growth cone turning toward nerve growth factor by inhibiting consolidation of the distal axon. (A) Sequence of events during NGF-bead contact-mediated growth cone turning. Numbers in panels reflect minutes elapsed since the first frame in the sequence (bead diameter = 10 μ m). Following filopodial contact with the NGF-coated bead the axon turns toward the bead and undergoes consolidation (arrows at 14 min). Following turning the axon continues to extend in the new direction dictated by the turn. (B) Inhibition of ROCK (Y-27632) or myosin II (blebbistatin, bleb) blocks growth cones turning toward NGF beads. The sample size (growth cones observed) is denoted by the numbers in the bars. (C) In the presence of Y-27632 or blebbistatin (shown) growth cones made

filopodial contact with NGF-beads and initiated turning (minutes 24 and 42). Although the behavior of growth cones under these conditions was similar to controls up to this point [compared to min 14 in (A)], the distal axon failed to consolidate and filopodia and lamellipodia continued to extend in directions opposite to the contact with the NGF-bead [min 32 and 45 show clear instances of deconsolidation, see panel (D) for a quantitative analysis]. As a result, growth cones were able to redirect protrusive activity away from the site of contact with NGF-beads and turning was not completed. The inset of the region denoted by the box at 57 min shows that filopodial contacts with the bead were retained (arrowheads) although turning was aborted. (D) Quantitative analysis of growth cone polarity during turning in the presence of blebbistatin using the polarity ratio (see Methods). Precontact ratios did not differ between control and blebbistatin-treated growth cones ($p > 0.4$). Ratios measured at individual time points following contact were compared to the mean precontact ratio within treatment group. Both control and blebbistatin-treated growth cones exhibited decreased polarity ratios during the first 1–2 min following contact (denoted by a bracket), reflecting the initiation of a turning response toward the bead. As expected, control growth cones maintained decreased polarity ratios until the turn was terminated through consolidation of the growth cone area far from the contact. However, blebbistatin-treated growth cones did not maintain polarity toward the bead and by 3 min following contact the polarity ratio returned to precontact levels. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

Figure 8.

A model for the negative regulation of axonal F-actin patches during axon consolidation. An elaborating patch is shown along with the text. Patch initiation is independent of ROCK and myosin II (step i). During elaboration of the patch ROCK activity inhibits the lifespan and intensity of patches (steps ii–iv). Myosin II downstream of ROCK in turn negatively regulates the probability that the patch will give rise to protrusive activity (step iii). Thus, ROCK and myosin II activity inhibit protrusive activity during consolidation by acting on F-actin patches, which are precursors to protrusive activity.

Table 1
Effects of ROCK and Myosin II Inhibition on DP Lamellar Migration

Treatment	% Axons Exhibiting DP Migration (n)
Pharmacological	
DMSO	14% (14)
Y-27632	93% (14)
HA-1152	93% (14)
Blebbistatin	95% (22)
Trituration loading	
BSA	7% (19)
dnMyoII	75% (20)

Data were obtained from 20 min imaging periods with 10 interframe intervals. Pharmacological inhibition data were obtained from explant cultures, and trituration loading data were obtained from dissociated neuron cultures.