Polymerizing Microtubules Activate Site-directed F-Actin Assembly in Nerve Growth Cones

M. William Rochlin,* Michael E. Dailey, † and Paul C. Bridgman‡

INTRODUCTION

Protrusion and retraction of filopodia and lamellipodia are critical for both advance and turning of locomoting cells. Movement of the leading edge is a complex process that depends on the orchestration of mechanisms that regulate actin polymerization (Coomber, 1991; Cramer et al., 1994), cross-linking (Oster and Perelson, 1987), contraction by molecular motors (Honer et al., 1988; Mitchison and Kirschner, 1988; Smith, 1988; Rochlin et al., 1995; Lin et al., 1996, Svitkina et al., 1997; Verkhovsky et al., 1999), and cross-linking of the F-actin cytoskeleton to the substratum. To circumvent some of these complexities and learn more about protrusion and F-actin assembly in cells, several groups have focused on the ability of intracellular pathogens or other foreign agents to induce F-actin assemblies in cells. Intracellular pathogens such as Listeria, Shigella, Rickettsia, and the Vaccinia virus (Cossart, 1995; Cudmore et al., 1996; Beckerle, 1998) and extracellularly applied polycation and cell adhesion molecule (CAM) cross-linking beads (Forscher et al., 1992; Suter et al., 1998) induce F-actin assembly-dependent structures that are thought to form as a result of “hijacking” the innate cellular mechanisms involved in protrusion at the leading edge. We now report a spontaneously occurring structure that resembles the F-actin assemblies induced by those foreign agents more closely than do lamellipodia, filopodia, or ruffles. Because this structure is part of the innate motility apparatus of the growth cone, is initiated at a distance from and usually terminates within the growth cone perimeter, we term them “intrapodia.”

We have begun to investigate the role of intrapodia in growth cone motility. Although the perimeter of the growth cone has been studied extensively because it is the ultimate site at which changes in direction and advance rate can be regulated, several studies suggest that more proximal regions of the growth cone initiate...
Figure 1. Two time-lapse sequences revealing intrapodia formation and dynamics. (A–C) Intrapodium initiated in the marginal zone. (A) At the onset of intrapodia formation a protuberance is initiated at the margin between the central, organelle-rich domain and the flattened, F-actin-rich domain (arrowhead). In this case, elongation is fairly straight, perhaps because the direction of elongation is orthogonal to the nearest leading edge and therefore is not being “side-swiped” by the retrograde flow. A second intrapodium is developing near the top. Time interval between images is 5 s. (D–K) Intrapodia that developed in the peripheral domain of a growth cone from a particle released from the leading edge and carried rearward by the retrograde flow. (D) Just after release of a particle from the leading edge (arrow). During the rearward movement, but before E, the particle increases in length but then shortens to assume its original size (our unpublished results).
the series of events that lead to these changes. In particular, microtubules that terminate proximally within lamellipodia appear capable of inducing growth cone turning (Tanaka and Kirschner, 1991, 1995; Tanaka and Sabry, 1995; Suter et al., 1998), and manipulations that perturb microtubule dynamics interfere with advance (Tanaka et al., 1995; Rochlin et al., 1996) and turning (Williamson et al., 1996). One mechanism by which microtubules could exert these effects is by stabilizing sites at which the plasma membrane attaches to the substratum (Rinnerthaler et al., 1988; Bershadsky et al., 1996; Kaverina et al., 1998). Interestingly, two cues that stimulate the formation of attachment specializations first stimulate protractive F-actin assembly: CAM cross-linking beads (Suter et al., 1998) and growth factors (Hall, 1998). Microtubules are also implicated in stimulating F-actin assembly at the leading edge of non-neuronal cells (Vasiliev and Gelfand, 1976; Rinnerthaler et al., 1988; Bershadsky et al., 1991; Rosania and Swanson, 1996; Waterman-Storer et al., 1999). Given that most microtubules within the growth cone terminate in the proximal region where intrapodia are most likely to be initiated, we evaluated the relationship between microtubule polymerization and intrapodia formation. Our results suggest that microtubule polymerization triggers intrapodial initiation, and that this event precedes microtubule-based stimulation of protrusion at the leading edge.

**MATERIALS AND METHODS**

Fetal rat superior cervical ganglion explant (SCG) cultures were prepared as described previously and grown on laminin-coated coverslips (Rochlin et al., 1995). Just before observation the coverslips was mounted in a perfusion chamber (Berg and Block, 1984). The chamber design permits simultaneous differential interference contrast (DIC) observation and perfusion. Growth cones were imaged by video-enhanced DIC microscopy. The microscope field was illuminated intermittently by a 100-W mercury lamp for purposes of time-lapse recording or for focus adjustment. At the end of some sequences, the chamber was perfused with PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, pH 6.9) (Schiwi et al., 1981) containing glutaraldehyde (0.25%; EM Science, Fort Washington, PA), saponin (0.02%; Sigma, St. Louis, MO), and rhodamine-phalloidin (80 nM; Molecular Probes, Eugene, OR; or Sigma). After a 15- to 20-min incubation, fluorescence images were collected using a slow scan, cooled charge-coupled device (Photometrics, Tucson, AZ) and stored as 512 × 512 × 16-bit digital image files. If antibody staining was to be carried out on these cultures, they were treated with 1% OsO₄ in PBS at 4°C for 5 min, washed extensively in PBS at room temperature, treated with freshly prepared 5% β-mercaptoethanol (Pierce, Rockford, IL) at room temperature for 30 min, washed extensively, and blocked for 15–30 min with 8 mg/ml BSA, 0.5% fish gelatin (Amersham, Arlington Heights, IL), and 1% normal goat serum in PBS. We stained with the primary antibodies for 1 h and the secondary antibodies for 40 min at room temperature. Primary antibodies were used at 1:1000 (mouse anti-β-actin; Sigma), 1:200 (rabbit anti-β-tubulin; Affiniti, San Diego, CA), and 1:100 (rat anti-tyrosinated tubulin; Accurate Scientific, Westbury, NY). Lower—cross-reactivity secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA) and used at 1:800 (Cy3 goat anti-rabbit), 1:400 (fluorescein goat anti-mouse), and 1:800 (Cy5 goat anti-rat).

For electron microscopy (EM), cultures were observed by DIC microscopy and fixed during intrapodia formation as described above. Actin filaments were stained with rhodamine-phalloidin immediately before and during perfusion. Cytochalasin B was used at concen-
trations ranging from 0.06 to 5 μM, and nocodazole was used at concentrations ranging from 0.33 to 6.6 μM.

Digital images were adjusted and prepared as composites using Adobe (Mountain View, CA) Photoshop and printed using a Tektronix (Wilsonville, OR) dye sublimation printer.

RESULTS

Characterization of Intrapodia

Intrapodia Formation. Rat SCG growth cones grown on laminin substrates typically are well spread, relatively thin, and highly motile. They contain two distinct cytoplasmic domains, a thickened organelle and microtubule-rich central domain and a thin actin-rich peripheral domain (Bridgman and Dailey, 1989). Intrapodia most often form at the margin between the peripheral and central domains (Figure 1, arrowheads), but are also initiated within lamellipodia. For the purposes of quantitation we defined the marginal zone as a 4-μm-thick strip separating the thin lamellipodium from the thicker central region. Fifty-seven percent of the intrapodia (n = 185) formed in the marginal zone (from recordings of four cones). Most of the remaining intrapodia formation (38%) occurred within lamellipodia. On occasion, we observed the formation of an intrapodium from a particle that appeared to have been part of the leading edge (Figure 1, D–F). Particles were observed only in very well spread cones with extremely thin lamellipodia; thus it was not possible to accurately quantitate the percentage of intrapodia that formed from particles. In growth cones observed 16–24 h after plating (and not making contact with adjacent cells), the average frequency of formation was 1.0 ± 0.4 (SD) intrapodia/min (n = 8; from 20-min recording time per cone). Intrapodia tended to form in bursts of two to four with irregular intervals between bursts (Figure 2). The average frequency of intrapodia formation increased to 4.9 ± 0.46 intrapodia/min (n = 5; 20-min recording time for each) in growth cones adhering to and growing along adjacent neurites (e.g., Figure 2), indicating that environmental factors influence the rate of intrapodia formation. Because of the variation in individual rates of intrapodia formation between cones, we also compared a set of cones before and after contact and turning toward neighboring neurites. This set of cones showed a rate of intrapodia formation of 1.9 ± 0.8/min before contact that increased to 4.0 ± 1.6/min after contact (N = 5; 5-10 min before contact and 10 min after contact). The increased formation of intrapodia in cones contacting neurites occurred in the marginal zone and new regions of lamellipodia that formed as the cone reoriented its trajectory by spreading in a direction approximately parallel with the long axis of the neurite that it contacted.

Growth Rate and Trajectory of Intrapodia. Intrapodial profiles extended at an average rate of 0.18 ± 0.01 (SEM) μm/s (n = 18). This rate is approximately the same as that reported by Forscher et al. (1992) for the retrograde flow-corrected rate of bead displacement by “inductopodia” and also the rate of Arp3/capping protein “spots” in a variety of non-neuronal cell types (Schafer et al., 1998). Intrapodia net extension rates ranged from 0.10 to 0.32 μm/s. Typically, intrapodia extension took a curvilinear path. The paths curved smoothly unless the leading tip of the intrapodium encountered another structure within the cytoplasm.
In one sequence we observed intrapodium travel in one direction (Figure 1, I and J) until its tip encountered a linear element, perhaps a microtubule. After contact, the intrapodium abruptly changed direction and advanced along the linear element (Figure 1K). These observations are consistent with the possibility that intrapodia are propelled from the rear, changes in their direction resulting from changes in resistance encountered at the front. Occasionally, intrapodia arising from the central thickened region of the growth cone would extend toward the leading edge, punching outward to create filopodia-like protrusions, or extending along previously formed filopodia (our unpublished observation). Finally, we also observed intrapodia that curved sharply and then disappeared coincident with the formation of endocytotic vacuoles (see Dailey and Bridgman, 1993, their Figure 12). These vacuoles were also observed to form without detectable intrapodial activity, but the coincidence of their occurrence with the disappearance of intrapodia suggests a relationship.

We also assessed the direction of intrapodal advance in relation to the direction of growth cone advance. In a rapidly advancing growth cone over a 40-min period, 80% of the intrapodia elongated forward along the axis of growth, 10% elongated perpendicular to this axis, and the remaining 10% elongated antiparallel to this axis. This suggests that intrapodia may preferentially elongate in the direction of growth cone advance.

**Comparison with Filopodia.** For comparison, we gathered rate and persistence data on filopodia. Filopodia extended slower (0.13 ± 0.04 [SEM] μm/s; n = 7) than intrapodia, and this growth was more frequently interrupted by periods of stasis (Figure 3). Also, the average lifetime of intrapodia (1.5 min; n = 33) was significantly less than that of filopodia (7.8 min; n = 9). Finally, at their peak lengths, filopodia are longer (8.0 ± 3.2 [SD] μm; n = 25), on average, than intrapodia (4.9 ± 2.1 μm; n = 20). Note that it was not possible to assess these properties for filopodia that projected upward (away from the substratum) because of the difficulty of tracking out-of-focus filopodial tips. However, we did determine the lifetime of ruffles (sheet-like and filopodial-like) for comparison. The average lifetime of these structures was 3.6 ± 1.9 (SD) min (n = 10).

**Intrapodia Contain F-Actin and F-Actin-binding Proteins.** To determine whether intrapodia contain F-actin, we stained growth cones with rhodamine-phalloidin. Upon seeing the formation of an intrapodium during live observations (Figure 4, A–C), we perfused the chamber with fixative (Figure 4D) followed by saponin and rhodamine-phalloidin (Figure 4H). Intrapodia were always associated with intense phalloidin staining, indicating that F-actin is concentrated in intrapodia compared with surrounding cytoplasm. We also labeled growth cones with anti-β-actin to ascertain whether pools of G-actin were associated with intrapodia but found no difference from the phalloidin labeling (our unpublished observation). We investigated whether actin-binding proteins that are associated with actin–plasma membrane linkage sites were present in intrapodia. The brightness of talin and α-actinin immunofluorescence staining was greater along the length of intrapodia (our unpublished observation), as was the staining for β1-integrin subunit (Figure 4, E and F).
scopic level, the leading tips were not more intensely labeled for any of these antigens than along the rest of the length of the intrapodium. Capping protein was concentrated along the length of intrapodia (Figure 4, I–K, arrowhead) as well as along actin bundles in lamellipodia that impinged on the leading edge (Figure 4K, arrows). Because capping protein binds to the barbed end of F-actin, this indicates that capped barbed ends are staggered along the length of these bundles. Comparison of the average pixel brightness within intrapodia with that of the surrounding lamellipodium indicated a greater than threefold difference (3.6 ± 0.9 [SD]; n = 5) in capping protein staining intensity.

**Electron Microscopic Observations Confirm the Presence of a High Concentration of Actin Filaments within Intrapodia.** To study intrapodial ultrastructure, growth cones were extracted and fixed during DIC observation of intrapodial activity, processed for electron microscopic observations, and reidentified (see MATERIALS AND METHODS). Stereo EM images of rotary-shadowed cytoskeleton preparations allowed the observation of intrapodial actin filament organization (Figure 5). Intrapodia contained a dense network of actin filaments that created a ridge on the dorsal surface of the growth cone. Many of the filaments within the ridge were oriented approximately parallel to the long axis of the intrapodium. Surface replicas of the dorsal membrane surface prepared by freeze etch EM also confirmed that intrapodia formed a ridge in unextracted preparations (our unpublished observation). We estimated the relative thickness of these ridges compared with that of the lamellipodium by viewing stereo pairs in a parallax measuring device. Although variable in height, the ridges were on average about twice the thickness of lamellipodia (1.8 ± 0.96 [SD] μm; n = 5). Thus, the thickness, density, and orientation of filaments within the lamel-
lipodium were focally changed by the complex network of actin filaments that make up the intrapodium. Near the leading tip of intrapodia, filaments from the lamellipodium that coursed tangential to the ridge formed by the intrapodium appeared to be pushed dorsally. It could not be determined whether these filaments were integrated into the intrapodial actin network or whether they were being pushed upward by the advancing intrapodia. The base (or trailing end) of an intrapodium was broader and flatter than the tip. F-actin at the trailing end and ventral-most portion of the ridge was integrated into the adjacent actin cytoskeleton through a complex branching network of filaments. Such a relationship has not been reported.

Figure 5. Electron micrograph of a growth cone–containing intrapodia reveals deformation of dorsal plasma membrane, a high concentration of actin filaments, and microtubule endings. (A) Low-magnification EM image of a rotary-shadowed growth cone cytoskeleton. An intrapodium (large arrows) that was identified and tracked by video-enhanced DIC recording (our unpublished data) before fixation and extraction of the membrane elongated from a region in close proximity to the ends of microtubules (arrowheads). (B) Higher-magnification stereo pairs of the two areas indicated by the asterisks in A. The first area contains the origin of the intrapodium. A relatively broad, dense mass of actin filaments (between the arrows) forms a ridge on the dorsal surface of the cone. A microtubule (arrowheads) that appears decorated with globular material ends on one side of the ridge (the microtubule end is adjacent to the arrowhead on the right). The second area contains the leading end of the intrapodium. A bundle of actin filaments forms a narrow ridge (between arrows). At the tip of the intrapodium, numerous filament ends can be seen (arrowheads). Bars, 2.6 μm (A); 360 nm (B).
for intracellular pathogens (Cossart, 1995; Cudmore et al., 1996).

Along the length of intrapodia, free filament ends could be seen oriented with their tips upward (toward the dorsal surface). Filament ends appeared more concentrated at the tip. It was difficult to determine the length of actin filaments because of the high density and complex geometry, but the general impression was that both long and short filaments contributed to the meshwork that formed the ridge. Longer filaments oriented tangential to the long axis of intrapodia were observed along the sides of intrapodia but could not be unambiguously identified within the core of these dense structures. Katoh et al., (1999) recently reported that intrapodia in Aplysia growth cones also contain longitudinally oriented F-actin. The mixture of lengths of F-actin in intrapodia is more reminiscent of the ultrastructure of Rickettsia (Heinzen et al., 1993) and Vaccinia tails (Cudmore et al., 1996) than of Listeria tails (Tilney et al., 1992a,b; but see Zhukarev et al., 1995; Sechi et al., 1997). The polarity of some of the F-actin filaments within the intrapodium could be determined (Figure 6). Using stereo images taken at three different tilt angles (+10°, 0°, and −10°) we were able to determine the orientation of 30 filaments from three different intrapodia that were previously observed by DIC microscopy to elongate. We divided the filaments into two categories; those aligned parallel to the long axis of the intrapodium and those oriented perpendicular to the overlying membrane (parallel with the z-axis). Ninety-five percent of the filaments that were parallel to the long axis had their barbed ends oriented toward the advancing intrapodium tip. Seventy-eight percent of the filaments aligned along the z-axis were oriented with their barbed ends toward the dorsal membrane surface. We also identified an additional five intrapodia on the basis of their characteristic morphology in the same replicates. When we analyzed the orientation of filaments in these intrapodia, the results were similar: 94% of the filaments parallel to the long axis of the intrapodia were oriented with their barbed ends toward the tip, and 92% of the filaments aligned along the z-axis had their barbed ends oriented toward the dorsal membrane surface. We have not consistently observed evidence for a particle or vesicle at the tip of these structures.

**Intrapodial Particle Formation and Tip Advance Appear to Be Differentially Sensitive to Cytochalasin B.** Given the accumulation of F-actin in the tails of intrapodia, we tested whether the formation of the advancing tip was inhibited by cytochalasin B (see MATERIALS AND METHODS). Intrapodia did not form during high (≈2.5 μM) cytochalasin treatment. Latrunculin B (20 nM), a toxin derived from the Red Sea sponge, binds to G-actin (Spector et al., 1989) and had similar effects (our unpublished observation). These treatments appeared to cause a rapid thinning of lamellipodia, loss of a coherent retrograde flow, accumulations of cytoplasm into thickened islands (Figure 7), and an increase in the frequency of random particle trajectories (Evans and Bridgman, 1995). The leading edges remained thick, retracted only slightly, and never extended. We did not observe the en masse retrograde evacuation of cytoplasmic materials that was beautifully captured by Forscher and Smith (1988) in Aplysia growth cones treated with cytochalasin B.

After washout of the cytochalasin, we typically observed a flurry of intrapodial formation (Figure 7, C and D), even in growth cones in which no intrapodia had been detected during precytochalasin observation. On average, we observed 11 ± 4 intrapodia per 1.5 min before cytochalasin treatment, 24 ± 6 during the first 1.5 min after cytochalasin washout, and 15 ± 3 during the subsequent 1.5 min period (n = 5). The burst of intrapodial activity was significantly greater than the precytochalasin level (p < 0.0015) and that of the subsequent 1.5-min period (p < 0.005). This observation is consistent with cytochalasin inducing the formation of intrapodia initiators but blocking intrapodial extension. The burst of intrapodial activity after cytochalasin washout was superimposed on rapid recovery of lamellipodial thickness and an increase in protrusive activity at the leading edges.

How might intrapodial activity be induced by cytochalasin treatment and washout? By viewing our time-lapse images in reverse, we observed that evacuation of cytoplasm from lamellipodia was also accompanied by the intermittent retrograde transport of particles that appeared to be breaking off from the leading edge. These particles typically ended up in the cytoplasmic islands that were the site of intrapodial activity after cytochalasin washout and may therefore contribute to the burst of intrapodial activity. Also, phalloidin or anti-β-actin staining of cytochalasin-treated growth cones revealed foci of brightly staining actin in these islands and also along F-actin bundles that were resistant to the cytochalasin treatment. We suspect that these actin-rich particles are intrapodia initiators and that they seed intrapodia after cytochalasin washout.

**Influence of Microtubules on Intrapodia Formation**

**Nocodazole Alters the Formation Rate of Intrapodia.** Although actin and actin-binding proteins are thought to be the primary effectors of growth cone shape and dynamics, perturbations of microtubules have been shown to affect pseudopodial dynamics in a variety of systems (Vasiliev and Gelfand, 1976; Tanaka et al., 1995; Rosania et al., 1996). We therefore examined the influence of nocodazole on intrapodial frequency.
During live observation, we perfused the cultures with 3.3 or 6.6 μM nocodazole, two concentrations that we previously established to eliminate microtubules from the growth cone in <30 min (Rochlin et al., 1996). These treatments decreased intrapodia frequency (Figure 8A). We also observed that filopodial and lamellipodial protrusion and growth cone advance were decreased (our unpublished observation). Compared with intrapodia in untreated growth cones, intrapodia in nonadvancing growth cones in 3.3 μM
nocodazole elongated with considerably less bias toward the leading edge of the growth cone. Only 44% elongated toward the forward axis (vs. 80% in untreated cultures), and 24% elongated in the opposite direction (vs. 10% in untreated cultures). Growth cones recovered from washout of the 3.3 μM nocodazole, but the disruption caused by 6.6 μM treatments did not appear reversible during the time course of our observations.

We also tested the influence of lower doses of nocodazole on intrapodial frequency. Explant cultures were

**Figure 7.** Cytochalasin B washout causes a burst of intrapodial activity. In this case the SCG explant had been plated and maintained in a low dose of nocodazole. (A) Just after perfusion with cytochalasin. (B) One minute 33 s later. Note the more flattened appearance of the lamellipodium, the thickened leading edge, and the islands of cytoplasm that accumulate in the lamellipodium. (C and D). After perfusion of cytochalasin-free media. In this instance, intrapodia (arrowheads) emanated from one cytoplasmic island, producing a starburst appearance. Bar, 5 μm. *, Reverse shadow-cast vacuole.
maintained in 0.33 μM nocodazole, a concentration that decreases the dynamic instability of microtubule plus ends without causing depolymerization (Jordan et al., 1993). Chronic treatment with 0.33 μM nocodazole resulted in an elevated frequency of intrapodia formation (Figure 8B). Because these growth cones were larger on average than control growth cones, we determined the frequency of intrapodia formation per unit area (Figure 8C). The frequency of intrapodia per unit area was also increased in nocodazole-treated cultures, suggesting that...
the increase in frequency was not simply due to the growth cones being larger.

Surprisingly, washout of the 0.33 μM nocodazole resulted in a burst of intrapodial activity (Figures 8B and 9). In contrast, the leading edge initially retracted (coincident with the initial burst of intrapodia activity) (Figure 8D). Leading edge protrusion then resumed and increased (compared with that seen in nocodazole) after several minutes delay, as intrapodia frequency declined (Figure 8, B and D). Filopodia formation before washout (10 ± 2 per 1.5 min; n = 4) was not significantly different from the rate of formation during the 1.5 min just after washout (10 ± 4) or during the subsequent 1.5 min (8 ± 2). Finally, we also observed an increase in the rate of neurite formation at the base of the growth cone after the washout (our unpublished observation). These effects were reversible and repeatable on the same growth cone. Perfusion of medium containing the same concentration of nocodazole used for overnight growth (0.33 μM) had no effects on intrapodia frequency or leading edge protrusion.

Our previous work (Rochlin et al., 1996) (cf. Tanaka et al., 1995) established that washout of nocodazole resulted in lengthening of microtubules in the growth cone and presumably a resumption of normal dynamic instability. To determine whether the changes in intrapodial activity after alterations in nocodazole level were more likely due to changes in dynamic instability or in lengthening of microtubules, we next investigated the influence of Taxol treatment on intrapodia formation.

**Taxol Alters Intrapodia Formation Rate.** Taxol, like nocodazole, decreases the rate of rapid polymerization events and catastrophe among populations of microtubules (Jordan et al., 1993). In contrast to nocodazole, Taxol promotes assembly of microtubules. In explant cultures grown overnight in low doses (7 or 12 nM) of Taxol, neurite outgrowth length decreased to 80% (n = 5) and 50% (n = 6), respectively, of the control rate of growth. As was the case for nocodazole-treated cultures, neurites were thicker, suggesting that the presence of Taxol did not decrease the rate of axoplasm production. In contrast to cultures grown in nocodazole, extremely large growth cones were not observed in the presence of Taxol. Intrapodial formation rate also appeared elevated in these cultures compared with controls (our unpublished observation, but compare Figure 8E before washout with the control rates stated above in Intrapodia Formation). Washout of Taxol did not elicit an elevation in intrapodia frequency. However, perfusing Taxol-containing media through the chamber resulted in a burst of activity similar to that observed after washout of nocodazole (Figure 8E). Because washout of nocodazole and perfusion of Taxol would be expected to have opposite effects on microtubule dynamic instability, but both treatments would be expected to increase microtubule polymerization, our data suggest that microtubule polymerization, rather than a change in dynamic instability per se, is capable of increasing intrapodial frequency. We next examined whether microtubule endings were located near sites of intrapodia initiation.

**Microtubule Endings Are Found near Some Sites of Intrapodia Initiation.** To establish whether microtubule endings are present at sites of intrapodia initiation, we fixed growth cones during live observation of intrapodia formation. We attempted to fix growth cones...
Actin Polymerization Is Necessary for Intrapodial Elongation

Our light and electron microscopic analysis demonstrated that intrapodia contained more F-actin than surrounding regions of the lamellipodia. Of the filaments that we could analyze, 90% of those that were aligned parallel to the direction of elongation at the time of fixation had their barbed ends oriented toward the tip, consistent with a role for actin polymerization in pushing the tip forward. Cytochalasin and latrunculin B, two drugs that interfere with F-actin polymerization, blocked elongation of intrapodia. Cytochalasin has two effects on actin: at low concentrations it caps F-actin, and at higher doses it binds to monomers (Cooper, 1987). Given the difficulty of determining the intracellular concentration of cytochalasin and the effect of the cellular environment on its binding properties, we cannot extrapolate from the extracellular cytochalasin concentration which of the actin-binding properties is most responsible for the effects we describe (Cooper, 1987). Latrunculin B, a cell-permeant drug that does not bind to F-actin but facilitates depolymerization by binding G-actin (Spector et al., 1989), had effects similar to those of cytochalasin B (our unpublished observations). This is consistent with the possibility that the impact of cytochalasin B results from its G-actin sequestering capability. These data demonstrate that actin polymerization is essential for intrapodia formation.

Most Intrapodial Leading Tips Are Likely to Be Associated with the Plasma Membrane

Intrapodia tips and tails are adjacent to and elevate the dorsal plasma membrane of lamellipodia. Thus, the plasma membrane at the front edge of the tip is approximately perpendicular to the direction of actin polymerization that is propelling the tip forward. No discrete structure (e.g., a vesicle) has been observed at the leading tips of intrapodia in growth cones (but see below). Furthermore, the association of intrapodia with elevated β1-integrin staining is consistent with the possibility that intrapodia are linked to a membrane-associated protein. We cannot at this time rule out the possibility that the elevation in β1-integrin staining and other F-actin-associated proteins (talin, α-actinin, and capping protein) is due to the increased fluorescence path length caused by

DISCUSSION

We describe elemental features of a spontaneous, intralamellipodial, F-actin-rich structure that appears similar to those induced by pseudosubstrata (polyca- tion-coated beads; Forscher et al., 1992), intracellular pathogens (Cossart, 1995; Cudmore et al., 1996), and to a lesser extent, vesicles in non-neuronal cells and cell extracts (Heuser et al., 1992; Southwick et al., 1997; Frischknecht et al., 1999) and Arp2/3 spots in fibroblasts (Schafer et al., 1998) (cf. Welch et al. 1997). Our data indicate that elongation of these spontaneous structures, termed intrapodia, depends absolutely on actin polymerization. We succeeded in altering the rate at which they occur by using drugs that target actin or microtubules. The direction of intrapodia elongation correlates with the direction of growth cone advance and intrapodial frequency is influenced by growth cone interaction with neurites. Still, the distribution of intrapodia, their transient nature, and the variability of their elongation patterns indicate that intrapodia are not primarily responsible for guid-
Figure 10. Microtubule endings are associated with intrapodial tails. Red corresponds to actin staining, green to microtubule staining. Growth cones in the presence or absence of microtubule-perturbing treatments were monitored during time-lapse DIC recordings and fixed during intrapodia formation. Arrowheads in D–F represent the initiation sites of intrapodia as determined before fixation during DIC observations. At high magnification, the microtubule staining sometimes appears discontinuous. Because similarly fixed samples prepared
the sides of intrapodia. Presumably, cation-coated beads induce a specialization in the Aplysia growth cone plasma membrane to produce inductopodia (Forscher et al., 1992), indicating that plasma membrane–based signaling is sufficient to induce intrapodia. All of the intracellular pathogens use either their own membrane surfaces (Listeria, Shigella, and Rickettsia) or cloak themselves in the host cell’s intracellular membranes (Vaccinia virus), to provide a discrete organizer at the leading tip of the actin structure. In addition, there is evidence that the necessary actin-organizing proteins used by Vaccinia (Cudmore et al., 1996) and Listeria (Friederich et al., 1995), when present in the plasma membrane of infectable cells but separated from the pathogen, give rise to surface protrusions reminiscent of the protrusions induced by the intact pathogen. Finally, as noted by a reviewer, Listeria tails that are associated with the plasma membrane are more likely to contain long axial actin filaments (Sechi et al., 1997) than those that are advancing through cytoplasm at a distance from the plasma membrane (Tilney et al., 1992a,b), consistent with the possibility that the plasma membrane stabilizes or contributes to this component of filamentous actin tails. These data are consistent with the possibility that the plasma membrane contains the actin polymerization machinery that mediates intrapodia formation and that it may also influence the organization of actin filaments in the intrapodial tails. A minority of intrapodia, those that arise from particles derived from the leading edge, are likely to be vesicle tipped. The behavior of these particles is unlike that of conventional intrapodia. In the example shown (Figure 1, D–K), the intrapodia-generating particle had elongated and shortened before the intrapodia type elongation (our unpublished observation). After the intrapodium elongation commenced, the particle appeared to split into two oppositely directed intrapodia. This would seem to suggest that opposite sides of a vesicle induce intrapodia. Note, however, that Schaefer et al. (1998) have reported splitting of Arp3/capZ spots, which are evidently plasma membrane specializations. One of the intrapodia tips we described (Figure 1K) turned and advanced along linear elements (presumably microtubules). This is also difficult to explain for a plasma membrane specialization but not for a vesicle. There are reports of vesicle-tipped “actin rockets” that course through the cytoplasm in non-neuronal cell types (Heuser and Morisaki, 1992; Southwick et al., 1997; Frischknecht et al., 1999).

**Cytochalasin B Washout Caused a Burst of Intrapodial Activity**

Although the direct action of cytochalasin and latrunculin may be to sequester G-actin (Cooper, 1987), a
The site at which most intrapodia form. It is noteworthy that cytochalasin-resistant F-actin clusters are found at microtubule endings (Kelley et al., 1996), and microtubules, like leading edges, are sites from which F-actin network assembly is initiated after washout of cytochalasin in Aplysia growth cones (Forscher and Smith, 1988). A correlation between microtubule endings and ruffle formation at the leading edge of non-neuronal cells has also been reported (Rinnerthaler et al., 1988; Rosania and Swanson, 1996; Waterman-Storer et al., 1999). The correlation between microtubule endings and intrapodia initiation sites, combined with the correspondence between microtubule polymerization and intrapodia initiation, supports involvement of microtubule endings or closely associated structures with induction of intrapodia.

Incorporating Microtubules, F-Actin, and the Plasma Membrane into a Model of Intrapodia Formation

We focus on the plasma membrane-associated intrapodia initiators because vesicle-tipped initiators are rare in growth cones and because they may simply represent ectopic leading edge fragments (endosomes) of limited importance in growth cone motility. We wish to address two questions raised by our observations: 1) how might microtubule polymerization induce intrapodia initiators in the overlying plasma membrane; and 2) why do microtubules induce intrapodia rather than filopodia?

Microtubules can influence the F-actin and plasma membrane by mechanical means, by virtue of transporting organelles (Dailey and Bridgman, 1991), or by enzymes associated with the microtubules. Polymerizing microtubule tips are able to drag endoplasmic reticulum tubules (Waterman-Storer et al., 1995; Waterman-Storer and Salmon, 1998) and may therefore be able to push actin filaments encountered at the growing tip (perhaps via dynein activity, Waterman-Storer et al., 1998). Although microtubules appear unable to resist the pressure of the constitutive retrograde flow of F-actin from the leading edge of lamellipodia (Waterman-Storer and Salmon, 1997; Suter et al., 1998), they are able to grow into such regions, suggesting either an association of these microtubules with a non-rearward-moving F-actin population or the ability to polymerize without becoming cross-linked to the F-actin involved in the rearward flow. Microtubules appear to be drawn toward plasma membrane specializations associated with ventral surface integrin-based focal adhesions (Kaverina et al., 1998) and sites of CAM cross-linking (Lin and Forscher, 1993; Suter et al., 1998) and may therefore be capable of associating with a subpopulation of F-actin that is connected to plasma membrane tethers, perhaps via molecules that bind both microtubules and F-actin.
Polymerization of microtubules associated with this subpopulation of F-actin could lead to concentration or alignment of F-actin at the tip of the microtubule and thereby a concentration of the plasma membrane-associated tethers of these filaments, which in turn might accelerate cross-linking of these tethers. Cross-linking of CAMs by coated beads precedes and is necessary for indocytodectomy formation on the dorsal plasma membrane of *Aplysia* growth cones (Suter et al., 1998) and is implicated as an initial step in the formation of actin–plasma membrane specializations at contact sites (Chrzanska-Wodnicka and Burridge, 1996; Suter et al., 1998). To date, however, there is little direct evidence supporting the possibility that microtubule polymerization moves F-actin in lamellipodia.

Microtubules are associated with a variety of enzyme activities, a subset of which are implicated in the regulation of the actin cytoskeleton (Kolodney and Elson, 1995; Best et al., 1996; Nagata et al., 1998). Small Ras-related GTPases cdc42, Rac, and Rho evidently control filopodial protrusion, ruffle formation and leading edge protrusion, and focal adhesion assembly in a variety of cell types (for review, see Hall, 1998), making them candidates for molecules through which microtubule polymerization may act. Several lines of evidence support a role for Rac1 in intrapodia initiation. Rac1 is implicated in ruffling and in pinocytosis (Ridley et al., 1992), consistent with roles in both intrapodial F-actin assembly and the occasional formation of reverse phase organelles. Rac1 is also involved in the curvilinear, F-actin polymerization-based motility of spots of capZ/Arp3 in non-neuronal cells (Schafer et al., 1998) and may be present in the *Listeria* F-actin tails (David et al., 1998). In addition, it has been found to localize with microtubules in non-neuronal cells, and colchicine-induced microtubule depolymerization blocked ruffling caused by serum treatment, platelet-derived growth factor, and phorbol 12-myristate 13-acetate (Best et al., 1996), each of which normally stimulates protrusive events via a pathway involving Rac1 (Ridley et al., 1992; Nobes and Hall, 1995). Dominant negative Rac1 blocks the ruffling that normally accompanies microtubule repolymerization after nocodazole washout in fibroblasts (Waterman-Storer et al., 1999). Recent observations implicate guanine nucleotide exchange factors, which bind to small GTPases and stimulate their activity, in the linkage of Rac to the polymerizing ends of microtubules (Ren et al., 1998; Glavin et al., 1999). One possibility suggested by these data is that microtubule polymerization delivers Rac1 to the plasma membrane and initiates protrusive-type actin assembly at a discrete site. If the site is not immobilized (e.g., via ECM attachment), it is propelled by the actin polymerization away from the microtubule, giving rise to an intrapodium.

Filopodia and ruffle-like sheets are capable of forming on the dorsal surface of our SCG growth cones (our unpublished observations); so why do intrapodia also form? We speculate that the tip specializations, the plasma membrane–linked protein complexes that induce F-actin assembly, are similar in intrapodia, filopodia, and ruffles, but levels of active F-actin-plasma membrane cross-linking proteins determine whether protrusion parallel to the direction of F-actin assembly (i.e., filopodia or ruffles) occurs at sites at which this F-actin assembly is stimulated. In the presence of high levels of cross-linking activity, recently assembled F-actin becomes linked to the plasma membrane. If the plasma membrane is “sticky,” it is pulled forward by the tip, and if it is “slippery” (as in the case of intrapodia), the tip glides through the plasma membrane without pulling the plasma membrane along with it. The stickiness would be determined by the levels of the cross-linking proteins or their levels of activation (e.g., phosphorylation; Weed et al., 1998).

We have not determined the role of intrapodia in growth cone motility in vitro or in vivo. Nonetheless, the reproducible occurrence of intrapodia after wash-out of actin–monomer binding drugs or microtubule polymerization-stimulating drugs may provide an assay for determining the role of candidate signaling molecules in the early stages of formation of plasma membrane specializations involved in more typical forms of protrusion or the generation of adhesion specializations. Our finding that microtubule polymerization triggers intrapodia formation before (perhaps at the expense of) leading edge protrusion should encourage workers to incorporate this early, proximal event in models of microtubule-based motility alterations. Whether intrapodia occur in vivo is not known, but the frequency with which they occur in vitro suggests that the signaling events that underlie their initiation in vitro are active and significant in vivo.

ACKNOWLEDGMENTS

We thank Dr. John Cooper and Dr. Dorothy Schafer for comments on the manuscript and the gift of the anti-capping protein antibody. This work was supported by National Institutes of Health grant NS26510 (to P.C.B.).

REFERENCES


