

Regulated Tyrosine Phosphorylation at the Tips of Growth Cone Filopodia

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Abstract. Several types of evidence suggest that protein-tyrosine phosphorylation is important during the growth of neuronal processes, but few specific roles, or subcellular localizations suggestive of such roles, have been defined. We report here a localization of tyrosine-phosphorylated protein at the tips of growth cone filopodia. Immunocytochemistry using a mAb to phosphorylated tyrosine residues revealed intense staining of the tips of most filopodia of *Aplysia* axons growing slowly on a polylysine substrate, but of few filopodia of axons growing rapidly on a substrate coated with *Aplysia* hemolymph, which has growth-promoting material. Cytochalasin D, which causes F-actin to withdraw rapidly from the growth cone, caused the tyrosine-phosphorylated protein to withdraw rapidly from filopodia, suggesting that the protein associates or interacts with actin filaments. Phosphotyrosine has previously been found concen-

trated at adherens junctions, where bundles of actin filaments terminate, but video-enhanced contrast-differential interference contrast and confocal interference reflection microscopy demonstrated that the filopodial tips were not adherent to the substrate. Acute application of either hemolymph or inhibitors of protein-tyrosine kinases to neurons on polylysine resulted in a rapid loss of intense staining at filopodial tips concomitant with a lengthening of the filopodia (and their core bundles of actin filaments). These results demonstrate that tyrosine-phosphorylated protein can be concentrated at the barbed ends of actin filaments in a context other than an adherens junction, indicate an association between changes in phosphorylation and filament dynamics, and provide evidence for tyrosine phosphorylation as a signaling mechanism in the filopodium that can respond to environmental cues controlling growth cone dynamics.

SEVERAL pieces of evidence suggest that protein-tyrosine phosphorylation is important in the growth of neuronal processes during development, one of the key steps in establishing the circuitry of the nervous system. First, certain protein-tyrosine kinases (PTKs)¹ and protein-tyrosine phosphatases are transiently expressed by developing axons. *c-src* and *fyn*, related nonreceptor PTKs, are both expressed at much higher levels in various developing nerve tracts of the vertebrate central nervous system than in the adult, and most highly in the growth cone, the specialized ending of a growing neurite (Maness et al., 1988; Bare et al., 1993). Another nonreceptor PTK, *abl*, and three receptor protein-tyrosine phosphatases are transiently expressed on subsets of developing axons in the *Drosophila* central nervous system (Elkins et al., 1990; Hariharan et al., 1991; Tian et al., 1991). Second, protein-tyrosine phosphorylation is acutely affected by certain neurite growth-promoting molecules. The surface receptors for NGF and other neurotrophins are members of the family of *trk* PTKs (Lamballe et al., 1991; Loeb et al., 1991; Squinto et al., 1991). Binding of NGF not only causes autophosphorylation of *trk*, but

tyrosine phosphorylation of numerous other proteins as well (Maher, 1988; Schanen-King et al., 1991; Vetter et al., 1991). Soluble fragments of neural cell adhesion molecule and L1 (neurite growth-promoting molecules of the immunoglobulin superfamily), as well as antibodies to the whole proteins, decrease the tyrosine phosphorylation of membrane-bound tubulin in growth cones (Atashi et al., 1992). Third, alterations in protein-tyrosine phosphorylation have been found to affect neuritic growth. Expression of the constitutively active *v-src* gene in PC12 pheochromocytoma cells causes neurite outgrowth in the absence of the normally required NGF (Alemá et al., 1985). Pharmacological inhibition of PTKs results in increased neuritic outgrowth from cultured chick brain neurons (Bixby and Jhabvala, 1992) and PC12 cells (Miller et al., 1993). Embryonic mouse cerebellar neurons missing the *c-src* gene exhibit abnormally slow neuritic growth on an L1 substrate in culture (Ignelzi, M. A., D. R. Miller, P. Soriano, and P. F. Maness. 1992. *Soc. Neurosci. Abstr.* 18:1095a). *Drosophila* embryos missing both the *abl* PTK and fasciclin I (a neurite growth-promoting molecule of the immunoglobulin superfamily) show evidence of disrupted pathfinding by certain axons in the central nervous system (Elkins et al., 1990).

1. Abbreviations used in this paper: PTK, protein-tyrosine kinase; VEC-DIC, video-enhanced contrast-differential interference contrast.

Although these studies suggest that protein-tyrosine phosphorylation is important in neuritic growth, they do not define specific roles. In fact, some of the results are superficially contradictory: both increased and decreased PTK activity seem capable of enhancing neuritic growth (Alemá et al., 1985; Bixby and Jhabvala, 1992; Miller et al., 1993) and, whereas L1 and *c-src* have opposite effects on protein-tyrosine phosphorylation in the growth cone (Matten et al., 1990; Atashi et al., 1992), *c-src* apparently enhances neurite growth promotion by L1 (Ignelzi, M. A., D. R. Miller, P. Soriano, and P. F. Maness. 1992. *Soc. Neurosci. Abstr.* 18:1095a). There are as yet not even descriptions of any subcellular localizations of tyrosine-phosphorylated proteins suggestive of specific roles in neuritic growth.

The growth cone should be a focus of study for clues to the roles of protein-tyrosine phosphorylation in neuritic growth. It is the site at which new neuritic length is added (Goldberg and Burmeister, 1986), and interactions of the growth cone with the environment are critical in regulating both the rate and direction of growth (Bentley and Toroian-Raymond, 1986; Caudy and Bentley, 1986; Kapfhammer and Raper, 1987; Rivas et al., 1992). Though variable in form, growth cones typically display a peripheral motile region, whose thin layer of cytoplasm is filled with a dense network of actin filaments, and a more voluminous central region containing membrane-bound organelles and microtubules (Letourneau and Ressler, 1983; Forscher and Smith, 1988; Goldberg and Burmeister, 1989). Often protruding from the margin of the peripheral region are digitate filopodia filled with core bundles of actin filaments (Letourneau and Ressler, 1983; Lewis and Bridgman, 1992). Filopodia can extend tens of microns from the body of the growth cone, projecting in many directions, so they can survey large areas of the environment (Caudy and Bentley, 1986; Hammarback and Letourneau, 1986). Interactions of filopodia with environmental cues, such as other cells, can stimulate (Caudy and Bentley, 1986; Hammarback and Letourneau, 1986) or inhibit (Kapfhammer and Raper, 1987; Bandtlow et al., 1990) growth in the direction of the cue, and the loss of filopodia leads to abnormally directed axonal growth (Bentley and Toroian-Raymond, 1986). Thus, filopodia are perhaps the most important sensors of the growth cone.

We report here an interesting localization of tyrosine-phosphorylated protein in *Aplysia* growth cones in culture: the tips of filopodia. We find that the tyrosine phosphorylation there is rapidly regulated by a substrate-binding environmental cue that modifies filopodial dynamics. This and additional evidence suggest a role for tyrosine phosphorylation in mediating interactions of filopodia with environmental cues.

Materials and Methods

Culture of *Aplysia* Neurons

Juvenile *Aplysia californica* weighing 2–5 g were obtained from the Howard Hughes Medical Institute mariculture facility (Miami, FL). Neurons for culturing were obtained both by dissociation and by manual extraction of single neurons as previously described (Goldberg, 1991). Neurons were cultured in protein-free defined medium on glass coverslips that had been exposed to polylysine and then *Aplysia* hemolymph (blood), or to polylysine alone. All experiments were performed at room temperature.

Video Microscopy

Neurons were used 16–40 h after plating. Video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy and video-intensified fluorescence microscopy (of fixed growth cones) were done as previously described (Goldberg and Burmeister, 1986; Burmeister et al., 1991). Images were recorded by an optical memory disc recorder (model TQ-3031F; Panasonic Communications and Systems Company, Secaucus, NJ). Some fluorescence images were recorded by using a 35-mm camera attached directly to the microscope. Video micrographs were prepared for presentation by 35-mm photography of the video monitor.

Fluorescent Cytoskeletal Labeling and Immunocytochemistry

Cells were fixed by perfusing into the culture dish a fixative (pH 7.4) containing 0.25% glutaraldehyde, 400 mM sucrose, and 100 mM cacodylate for 7–10 min. Fixed preparations were rinsed three times with artificial seawater containing 400 mM sucrose and 0.1% Triton X-100. For staining of actin filaments, cells were then incubated at room temperature for 1 h with 0.33 μ M rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) in artificial seawater. For immunostaining of tyrosine-phosphorylated protein, cells were first incubated for 30 min in artificial seawater with 10% normal goat serum (Sigma Chemical Co., St. Louis, MO) and 0.1% Triton X-100. They were then incubated for 1 h in monoclonal antiphosphotyrosine (clone 4G10, Upstate Biotechnology, Inc., Lake Placid, NY) and used at 1:100 dilution in artificial seawater with sucrose and Triton X-100. After three rinses with artificial seawater, cells were incubated with fluorescein-conjugated goat anti-mouse antibody (Cappel; Organon Teknika Corp., Durham, NC) for 1 h. Two types of control experiments were done to assess the specificity of the staining for phosphotyrosine. In one type, preimmune mouse serum (Cappel; Organon Teknika) was used at 1:100 dilution to replace the primary antibody. In the other type, primary antibody was omitted entirely.

Statistical Analysis

Statistical evaluation of the significance of differences between means was done via Student's *t* test for single comparisons and an analysis of variance followed by Bonferroni's modified *t* test for multiple comparisons.

For the analysis of the results of micropipette puffing experiments, the filopodia of each growth cone were divided into two groups based on their proximity to the micropipette. The difference in distances from the micropipette of the nearest and farthest filopodial tips was determined. One third of this difference was added to the distance from the micropipette of the nearest tip to yield the maximum distance of a tip from the micropipette for that filopodium to be included in the "close" group. All filopodia whose tips were farther from the micropipette than this distance were included in the other group. To reduce the contributions of random fluctuations in filopodial lengths, only lengthenings of at least 2 μ m were scored positively. Filopodial responses from all the growth cones were combined for each condition (puffing of vehicle or vehicle containing genistein) and the significances of differences in the proportions of lengthening filopodia in the close group and the other group were assessed statistically by the chi-square test.

Results

Tyrosine-Phosphorylated Protein Is Concentrated at the Tips of Filopodia on a Polylysine Substrate

Neurons dissociated from the ganglia of juvenile *Aplysia* grow neurites slowly when cultured in a protein-free defined medium on a substrate coated only with polylysine (Burmeister et al., 1991). Often the short neurites are tipped by large growth cones whose peripheral region consists of numerous filopodia extending out of a wide lamellipodium (Figs. 1 and 2 A). Observations with VEC-DIC microscopy revealed considerable actin-based motility in this peripheral region, as previously described (Goldberg and Burmeister, 1986; Forscher et al., 1987; Forscher and Smith, 1988; Bur-

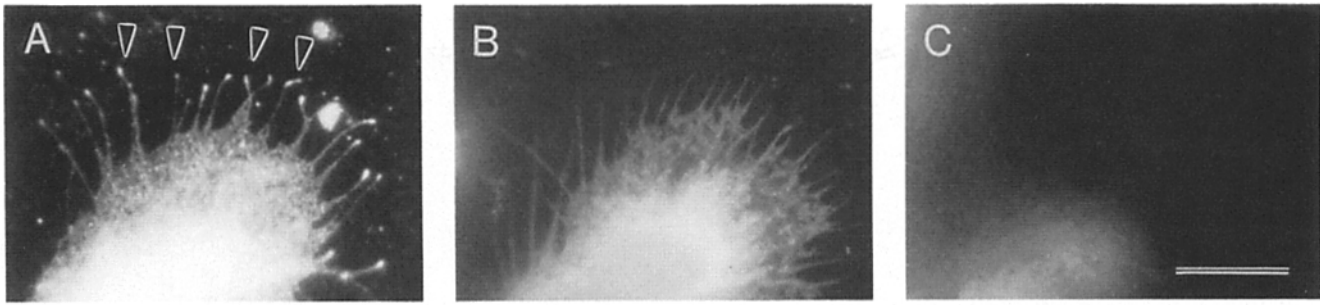


Figure 1. Distribution of phosphotyrosine in a growth cone on a polylysine substrate, as determined by immunofluorescence microscopy. (A) Almost all of the tips of the filopodia are brightly stained with an mAb to phosphotyrosine (*arrowheads* point to several filopodia). Much of the bright diffuse fluorescence in the center of the growth cone is nonspecific (some of which is autofluorescence, which is substantial when glutaraldehyde is included in the fixative), as can be seen when preimmune mouse serum replaces the antiphosphotyrosine monoclonal as the primary antibody (B) or when primary antibody is omitted (C). All of the images were photographed at the same aperture size and exposure time with a 35-mm camera attached to the microscope, and processed identically. Bar, 10 μm .

meister et al., 1991): both filopodia and the veils of membrane between them moved in and out (although net advance was slow) and there was a steady rearward flow of material in the lamellipodium.

There was a striking distribution of tyrosine-phosphorylated protein (the use of the singular is not meant to imply that there is necessarily only one species of protein) in these growth cones, as revealed by immunofluorescence using a mAb specific for phosphorylated tyrosine residues: although there was diffuse staining speckled with bright spots throughout the lamellipodium and central region of the growth cone, there was intense staining at the tips of most or all of the filopodia of almost all such growth cones (Fig. 1 A). The staining was specific in that use of preimmune pooled mouse serum to replace the primary antibody (Fig. 1 B) or omission of the primary antibody (Fig. 1 C) did not yield tip staining. The mean percentage of filopodial tips per growth cone that were brightly stained was $79 \pm 4\%$ (SEM; $n = 9$). The tips of many filopodia appeared swollen when viewed with VEC-DIC microscopy (Fig. 2 A), but we observed many filopodia without detectable tip swelling, and

these also displayed intense tip staining. An example of a growth cone with both apparently swollen and nonswollen filopodial tips, all with intense staining, is shown in Fig. 2 B. Thus, we conclude that the intense staining of the filopodial tips largely results from the presence of relatively high concentrations of phosphotyrosine at the tips, though it is enhanced in those filopodia with swollen tips by the locally increased thickness.

Bright fluorescent cellular staining with antiphosphotyrosine antibody has been observed at normal or modified adherens junctions, both cell-substrate (focal contacts) and cell-cell (Maher et al., 1985; Tarone et al., 1985; Takata and Singer, 1988; Tsukita et al., 1991), but this was not the case here. Two types of microscopic observation showed that the filopodial tips typically did not make focal contact with the substrate (and never contacted cells during our observations). Observations of growth cones at high resolution and magnification using VEC-DIC microscopy showed that the tips were often highly mobile between transient contacts with the substrate, moving not only back and forth but also up and down above the substrate (Fig. 3). The separation of

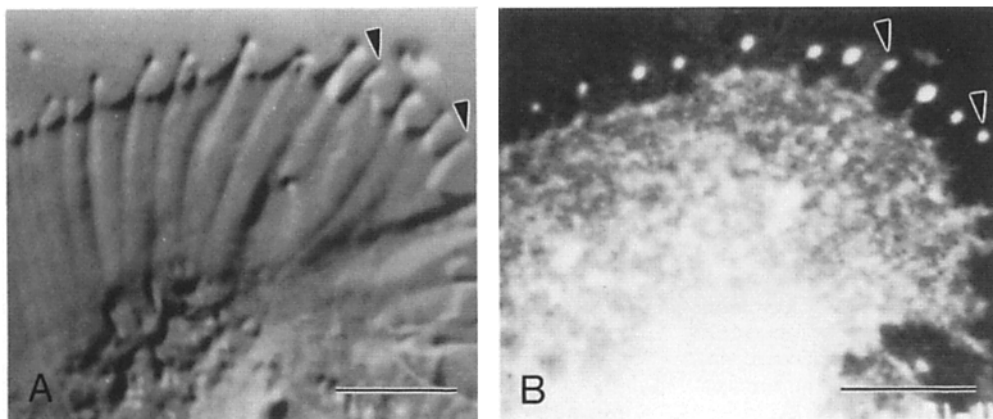


Figure 2. Video micrographs of a growth cone on a polylysine substrate. (A) VEC-DIC microscopy shows numerous short filopodia projecting from a broad lamellipodium. Many of the tips of the filopodia appear swollen. (B) Video-intensified fluorescence microscopy of the same growth cone stained with antiphosphotyrosine antibody shows nonswollen (*arrowheads*) as well as swollen tips to be brightly stained. In this and subsequent VEC-DIC/fluorescence groups, the magnification of the fluorescence image(s) is slightly greater than that of the VEC-DIC image. Bars, 5 μm .

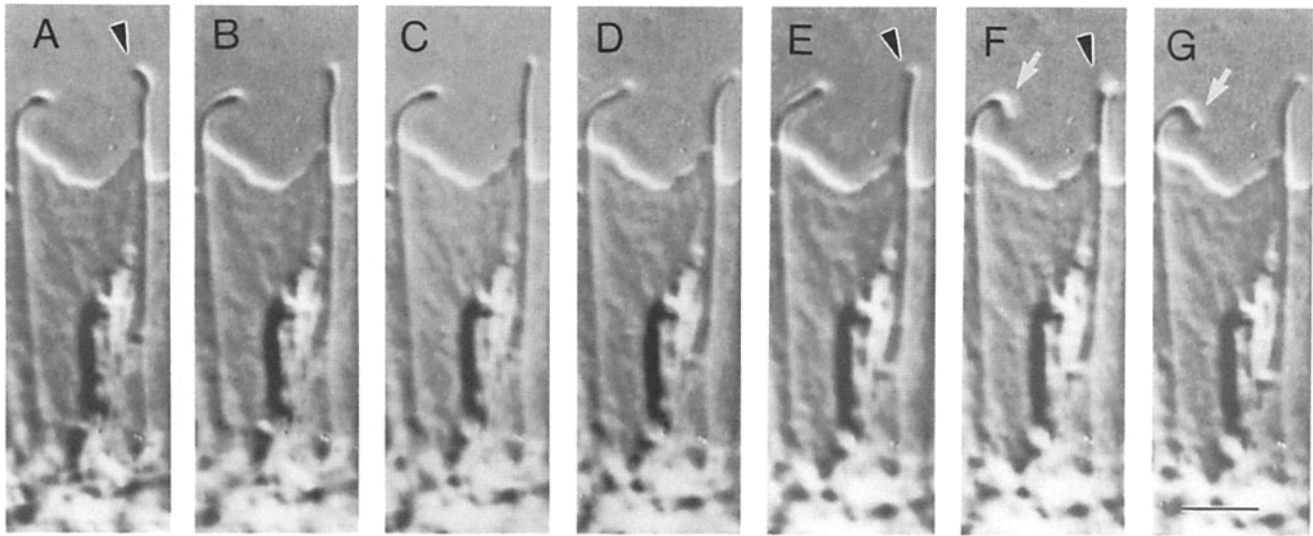


Figure 3. Rapid movement of filopodial tips is observable by VEC-DIC microscopy. The growth cone is on a polylysine substrate. One filopodium (arrowhead) is seen first swinging from left to right (A-D) and then lifting far enough off the substrate to be out of sharp focus (E and F). The left filopodium bends and lifts off the substrate (F and G). The interval between panels is 5 s. Bar, 5 μm .

the tips from the substrate was confirmed by observing living growth cones with interference reflection microscopy using a confocal scanning microscope (Fig. 4). Though there were areas of focal (black) and close (gray) contact of the peripheral region of the growth cone with the substrate, the tips of the filopodia generally exhibited a larger separation from the substrate (white). Therefore, the concentration of phosphotyrosine at the tips was not related to their adherence to the substrate.

We examined the possibility that the phosphotyrosine at the tip of the filopodium is actin associated, because a core bundle of actin filaments is the only major cytoskeletal structure in the filopodium (Letourneau and Ressler, 1983; Lewis and Bridgman, 1992), and actin and certain of its associated

proteins are substrates for tyrosine phosphorylation (Kellie et al., 1991; Howard et al., 1993). Cytochalasin has been shown to induce a rapid disappearance of the network of actin filaments from the peripheral region of these growth cones by causing the network to recede en masse into the central region (Forscher and Smith, 1988). Growth cones fixed several minutes after the application of 1 μM cytochalasin D, when the actin network had completely receded, no longer displayed bright staining with anti-phosphotyrosine antibody at the tips of filopodia (data not shown). We examined the mode of disappearance of the phosphotyrosine by fixing growth cones within 4 min of the application of cytochalasin D, when the actin network had receded only partially. In many filopodia, the phosphotyrosine accumulation was apparently caught in the process of withdrawing from the tip, sometimes having spread or fragmented (Fig. 5). These data imply an association or interaction between the phosphotyrosine at the tip and actin filaments of the filopodium.

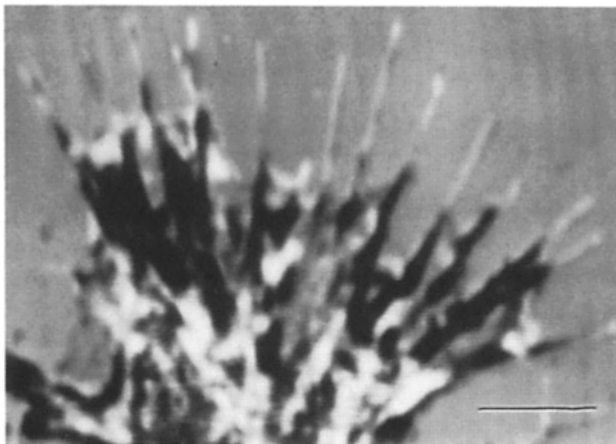


Figure 4. Confocal scanning interference reflection microscopic observation of a growth cone on a polylysine substrate. Closeness of the ventral membrane to the substrate correlates with darkness, such that the darkest areas are closest. No filopodial tips are very dark, and most are white, indicating that they have not formed focal or close contacts. Bar, 5 μm .

Tyrosine Phosphorylation Is Reduced in Filopodial Tips on a Growth-promoting Substrate

When dissociated *Aplysia* neurons are cultured in protein-free defined medium on a substrate preexposed not only to polylysine but then also to *Aplysia* hemolymph (which is then removed before addition of cells, leaving only substrate-bound material), neuritic growth is four to eight times faster than on polylysine alone and growth cones are typically smaller, with longer filopodia (Burmeister et al., 1991). On average, only $14 \pm 5\%$ ($n = 9$) of the filopodia per growth cone displayed bright staining of their tips with anti-phosphotyrosine antibody in this condition (Fig. 6 A) (mean significantly different from mean on polylysine, $P < 0.00001$). This difference in phosphorylation was not simply due to a nonspecific change in the substrate caused by covering the polylysine with protein (such as a change in surface charge). Irradiation with UV light of the growth-promoting substrate before addition of cells eliminates its ability to

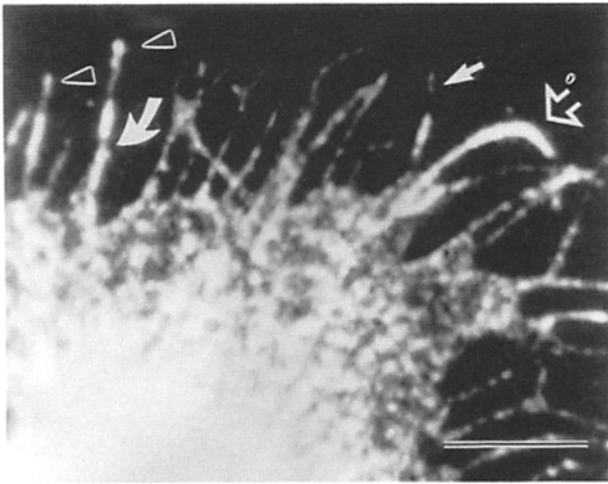


Figure 5. Tyrosine-phosphorylated protein rapidly withdraws from the tips of filopodia in response to cytochalasin D. This growth cone is in protein-free medium on a polylysine substrate. Cytochalasin D was added to the medium 4 min before fixation for immunofluorescence microscopy with antiphosphotyrosine antibody to cause the peripheral network of actin filaments to withdraw towards the central region of the growth cone. Tyrosine-phosphorylated protein seems to be withdrawing from the tips of several filopodia, spreading as it does so (*arrowheads* and *straight arrows*). In two filopodia the staining has fragmented, with some left at the tips (*arrowheads*); in one of those filopodia, there is additional fragmentation proximally (*curved arrow*). The distribution of phosphotyrosine in this growth cone before the addition of cytochalasin was presumably similar to that depicted in Fig. 2 B, because that pattern was consistent. Bar, 5 μ m.

affect the growth cone or accelerate neuritic growth (Burmeister et al., 1991). On the irradiated substrate, the pattern of staining with antiphosphotyrosine antibody was indistinguishable from the pattern on polylysine alone: most filopodial tips were brightly fluorescent (data not shown).

The disappearance of phosphotyrosine at the tips of filopodia in response to growth-promoting substrate occurs rap-

idly. This was seen by adding hemolymph to the culture medium of cells previously plated on a polylysine substrate. This has previously been shown to affect the morphology of the growth cone and accelerate neuritic growth within minutes; these effects apparently result from binding of material to the substrate (Burmeister et al., 1991). By 10–15 min after the addition of hemolymph, a mean of $16 \pm 4\%$ ($n = 9$) of the filopodia per growth cone had tips that were brightly stained with antiphosphotyrosine antibody (Fig. 6 B) (mean significantly different from mean on polylysine, $P < 0.00001$). Because the tips of most filopodia were found to be brightly stained in the absence of hemolymph (see above), it can be inferred that phosphotyrosine at the tips of many filopodia rapidly decreases when the substrate is altered acutely. These tips remained free of tight attachment to the substrate, as they could be seen with VEC-DIC microscopy to lift off the substrate (Fig. 7).

Decrease in Phosphotyrosine At Filopodial Tips Is Associated with Elongation of Filopodia

After addition of hemolymph, there was a pronounced change in behavior of the filopodia at the same time that phosphotyrosine at the tips of filopodia was disappearing. As noted above, filopodia of neurons cultured in protein-free defined medium on a polylysine substrate engaged in frequent short elongations and retractions, resulting in little net elongation. Analysis of filopodial dynamics revealed that addition of hemolymph increased the fraction of filopodia elongating during a given 10-s interval (Table I). We infer from this that the addition of hemolymph increased the fraction of time the average filopodium spent elongating. To confirm this, we followed the behavior of four filopodia on one of the growth cones for 110 s before and after the addition of hemolymph. We found that, on average, they spent 55% of the time elongating, 25% retracting, and 20% remaining stationary before the addition of hemolymph, and 75% of the time elongating, 22.5% retracting, and 2.5% remaining stationary after hemolymph. These data on individual filopodia are similar to the population data given in Table I. In addition, we found that hemolymph caused an increase in the

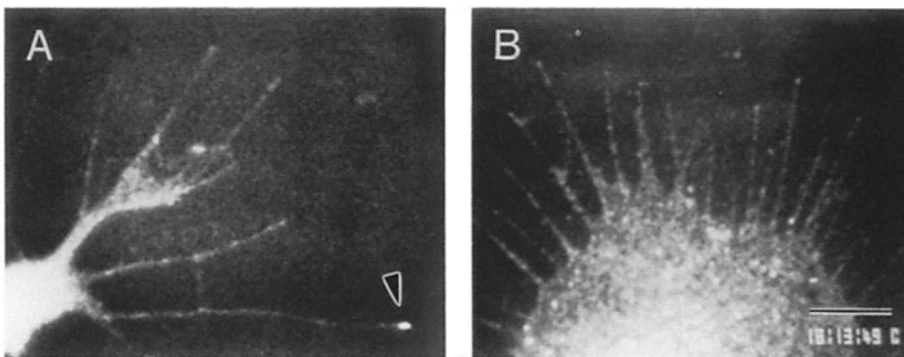


Figure 6. Distribution of phosphotyrosine in growth cones exposed to substrate-binding material from hemolymph. (A) Only one filopodium has a brightly stained tip (*arrowhead*) when the substrate has been preexposed to hemolymph as well as polylysine. This neuron had, like the neurons depicted in the previous figures, been cultured the previous day in protein-free defined medium, but exhibited much faster axonal growth because of the altered substrate. (B) Bright staining is absent from the tips of filopodia 6 min after the acute addition of hemolymph to neurons on a polylysine substrate. Compare the staining of the growth cones in these video micrographs with that of the growth cone in Fig. 2 b. Bar, 5 μ m.

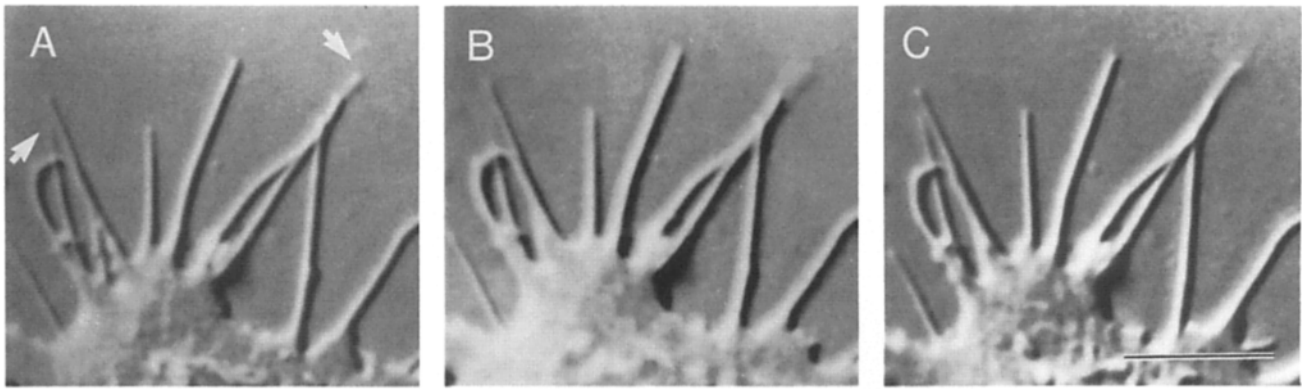


Figure 7. Lifting of filopodial tips off the substrate after filopodia have been stimulated to grow by acute addition of hemolymph. Arrows point to two filopodia whose ends go out of focus in *B* because they have lifted off the substrate. The ends return closer to the substrate in *C*. There is a 10-s interval between adjacent images. Bar, 5 μm .

Table I. Effects of Hemolymph and PTK Inhibitors on Filopodial Dynamics

	% of filopodia			Length (μm)	Speed ($\mu\text{m}/\text{min}$)	
	Adv	Ret	Stat		Adv	Ret
			(<i>n</i>)	(<i>n</i>)	(<i>n</i>)	(<i>n</i>)
Hemolymph						
Before	50 \pm 7	27 \pm 4	23 \pm 4 (4)	2.6 \pm 0.2 (5)	1.2 \pm 0.1 (30)	1.4 \pm 0.3 (16)
After	73 \pm 3*	20 \pm 4	7 \pm 3 (4)*	5.9 \pm 0.3 (5)*	2.2 \pm 0.2 (44)§	1.4 \pm 0.2 (12)
Genistein						
Before	50 \pm 6	23 \pm 8	27 \pm 5 (4)	1.8 \pm 0.5 (5)	1.7 \pm 0.1 (30)	1.6 \pm 0.3 (14)
After	87 \pm 7*	8 \pm 5	5 \pm 3 (4)	7.5 \pm 1.3 (5)‡	2.6 \pm 0.2 (52)‡	3.1 \pm 0.6 (5)

The dynamics of filopodia before and after either hemolymph or genistein addition were analyzed. Tip positions of 15 filopodia of each of four growth cones were measured 10 s apart before and 3–9 min after the addition of hemolymph and, in another four growth cones, before and 2–5 min after the addition of genistein. For each growth cone, the percentages of filopodia that had advanced, retracted, and remained stationary during the 10 s were determined. Data shown are means \pm SEM. The speeds of individual advancements and retractions in each condition were averaged to calculate the displayed means. We also determined the average lengths of filopodia in five different growth cones before and 5–10 min after addition of hemolymph and, in another five growth cones, before and 5–10 min after addition of genistein. Data shown are means of averaged lengths. *n*, number of experiments.

* After treatment different from before treatment, $P < 0.05$.

‡ $P < 0.005$.

§ $P < 0.0001$.

speed of elongation of filopodia (Table I). As a result of these two effects, most filopodia elongated considerably (Fig. 8, *A* and *B*). Underlying this was an elongation of the core bundles of actin filaments (Fig. 8 *C*).

Inhibitors of PTKs administered to growth cones on a polylysine substrate in the absence of hemolymph also caused a decrease in phosphotyrosine at filopodial tips concomitant with filopodial lengthening. The inhibitors we

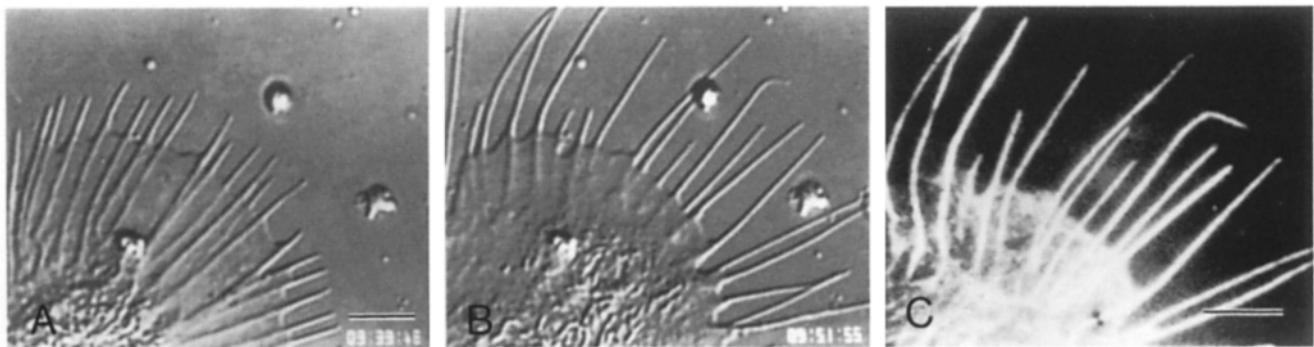


Figure 8. Elongation of filopodia and their core bundles of actin filaments when hemolymph is added acutely. VEC-DIC micrographs of a growth cone on a polylysine substrate before (*A*) and 11 min after the addition of hemolymph (*B*). Almost all of the filopodia have lengthened considerably. (*C*) A video fluorescence micrograph of this growth cone stained with phalloidin to visualize F-actin shows the core bundles to be as long as the filopodia, meaning that they must also have lengthened when growth was stimulated. Bars, 5 μm .

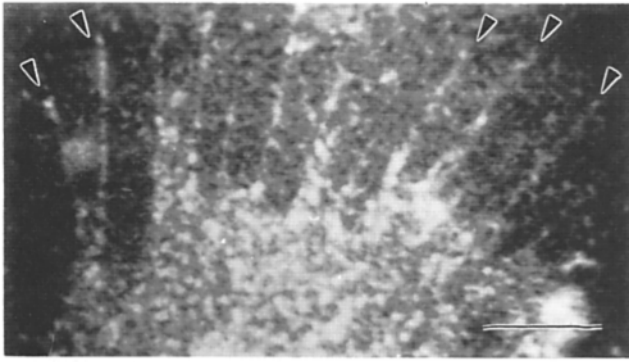


Figure 9. Suppression of protein-tyrosine phosphorylation in the growth cone by an inhibitor of PTKs. The distribution of phosphotyrosine is assessed by video fluorescence microscopy 30 min after the addition of genistein to neurons growing slowly on a polylysine substrate. Bright staining was not seen at the tips of filopodia (arrowheads) and is reduced throughout the growth cone. The image has been overexposed and contrast enhanced so that the pattern of the reduced staining could still be observed; this also brightens the staining on the substrate. The dimness of the growth cone staining is evident from its similarity to the substrate staining. Compare to Fig. 2 B. Bar, 5 μm .

used—genistein, lavendustin A, and herbimycin A—all are reported to have little effect on serine/threonine protein kinases at concentrations that cause profound inhibition of several PTKs (Akiyama and Ogawara, 1991). In addition, herbimycin A has a mechanism of inhibition different from that of genistein and lavendustin A (Fukazawa et al., 1991). As expected, there was a reduction in staining with antiphosphotyrosine antibody throughout the growth cone, including a large reduction at the tips of filopodia, within 30 min of the application of 100 μM genistein (Fig. 9). No such reduction was seen in response to 100 μM genistein (data not shown), a close structural analogue of genistein, which does not inhibit PTKs (O'Dell et al., 1991). Genistein (Fig. 10; Table I), 10 μM lavendustin A, and 5 μM herbimycin A (data not shown) each caused rapid filopodial elongation within minutes; genistein had no effect. Underlying the elongation was a lengthening of the core bundle of actin filaments of the filopodium (Fig. 10 C). Removal of genistein from the culture medium resulted in shortening of filopodia within minutes, though not completely back to their original lengths (Fig. 11). These reshortened filopodia had intense staining for phosphotyrosine at their tips (data not shown). The lengthening caused by herbimycin A also reversed when the

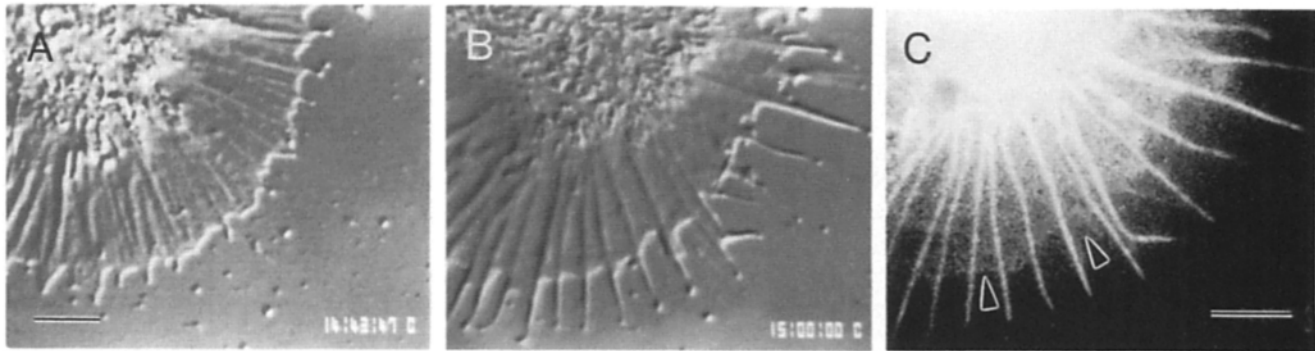


Figure 10. Elongation of filopodia and their core bundles of actin filaments in response to an inhibitor of PTKs. VEC-DIC micrographs of a growth cone on a polylysine substrate before (A) and 15 min after (B) the addition of genistein. All of the filopodia have lengthened considerably. (C) A video fluorescence micrograph of this growth cone stained with phalloidin to visualize F-actin shows the core bundles to be as long as the filopodia, meaning that they must also have lengthened in response to genistein. Bars, 5 μm .

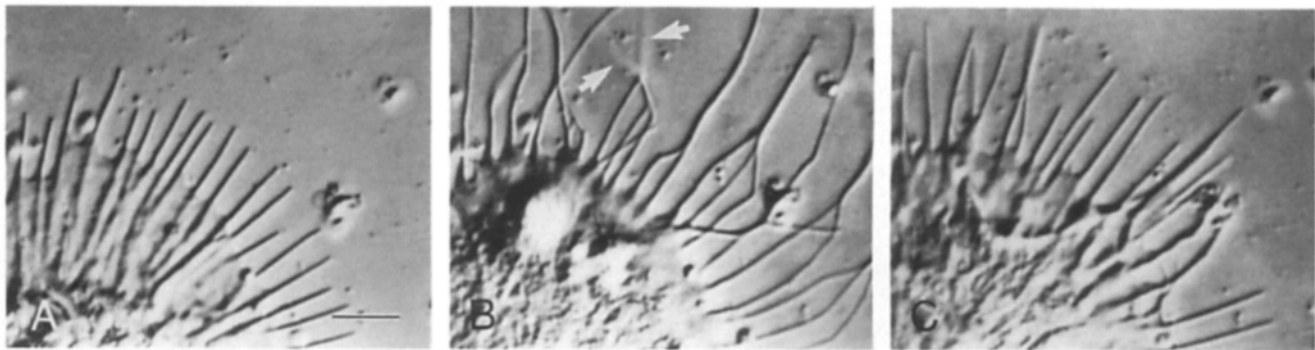


Figure 11. Reversal of filopodial elongation caused by a PTK inhibitor. VEC-DIC images 1 min before (A) and 11 min after (B) perfusion with genistein. Arrows point to filopodia waving above the substrate after rapid extension. (C) 5 min after removal of genistein, almost all of the filopodia have shortened considerably. Bar, 5 μm .

drug was washed out; we did not try to reverse the effects of lavendustin A. Analysis of filopodial dynamics before and several minutes after addition of genistein showed a change in dynamics similar to that seen with acute application of hemolymph: increases were seen in the fraction of filopodia elongating and the velocity during bouts of elongation (Table I). Lavendustin A and, to lesser extents, genistein and herbimycin A also caused some new filopodia to grow from the margins of the lamellipodium. Accompanying the filopodial lengthening caused by the PTK inhibitors was a forward movement of membrane-bound organelles within the body of the growth cone, which is also seen when hemolymph is applied acutely (Burmeister et al., 1991).

Inhibition of PTKs caused a reduction in tyrosine phosphorylation throughout the growth cone, not only at the tips of filopodia (Fig. 9). Is drug action on the filopodium alone sufficient to cause filopodial lengthening? We attempted to address this question by using a micropipette to apply inhibitor focally. Brief pulses of pressure were used to emit small puffs of vehicle (0.5% DMSO, 0.5% Fast Green [Sigma]) or vehicle containing 100 μ M genistein (the concentration used for bath application) from a micropipette several microns away from filopodia. Puffing of vehicle resulted in no significant difference in the probability of lengthening (at least 2 μ m) for filopodia relatively close to the micropipette compared with the rest of the filopodia (3 of 41 close filopodia lengthened, 4 of 58 other filopodia lengthened, seven growth cones; see Materials and Methods for details of the analysis). Even when the micropipette was positioned over the growth cone and large puffs of vehicle emitted to bathe all of the filopodia (as well as the lamellipodium), the average filopodial lengthening (0.03 ± 0.03 μ m/min, four growth cones) was not significantly different from that observed in those growth cones before the approach of the micropipette (0.09 ± 0.03). Emission of puffs of genistein from micropipettes several microns from the filopodia resulted, in contrast, in a significantly higher probability of lengthening for filopodia close to the micropipette compared with the rest of the filopodia (12 of 24 close filopodia lengthened, 5 of 48 other filopodia lengthened, six growth cones, $P < 0.001$). Fig. 12 shows a particularly striking experiment in which initially only the filopodium closest to the micropipette elongated when puffing of genistein began, followed by lengthening of its nearest neighbor; both shortened when puffing stopped. Although these results show that genistein can focally elicit filopodial lengthening, we cannot be certain that effective doses of the drug were not reaching the lamellipodium and thus we cannot be certain that reduction of protein-tyrosine phosphorylation in the filopodium is sufficient to cause its lengthening.

Discussion

We have shown here that tyrosine-phosphorylated protein can be concentrated at the tips of growth cone filopodia of *Aplysia* neurons in culture. Though numerous pieces of recent evidence point to the importance of protein-tyrosine phosphorylation in axon growth (see Introduction), there is little available information defining specific roles in the subcellular and molecular machinery of growth. Nor have specific localizations suggestive of such roles been reported. This is the first report of a localized distribution of tyrosine-

phosphorylated protein within the business end of the growing axon, the growth cone. The localization of tyrosine-phosphorylated protein at the tips of filopodia suggests that tyrosine phosphorylation could play a role in transducing environmental cues into changes in growth cone behavior. In support of this suggestion, we also report here that the phosphorylation is regulated by an environmental cue that changes filopodial dynamics.

Significance of Filopodial Tips

The tip of the filopodium is an intriguing place to find a large amount of tyrosine-phosphorylated protein. The growth cone is the most important part of the axon in determining the rate and direction of growth, and the filopodium is probably the most important part of the growth cone in detecting the environmental cues that guide the axon. Filopodia increase markedly in number when growth cones of developing axons reach points *in vivo* where a choice must be made among potential pathways (Tosney and Landmesser, 1985; Bovolenta and Mason, 1987). The importance of filopodia in pathfinding has been documented most thoroughly in the grasshopper. Pioneer axons (the first in an area) growing through the developing leg towards the central nervous system are guided by interactions of filopodia with specific guidepost cells (Bentley and Caudy, 1983; Caudy and Bentley, 1986; O'Connor et al., 1990). In some places, contact of a single filopodium with the guidepost cell reorients the axon (Caudy and Bentley, 1986; O'Connor et al., 1990). When the formation of filopodia is suppressed by the use of cytochalasin, the pioneer axon grows in a disoriented fashion (Bentley and Toroian-Raymond, 1986). The efficacy of single filopodia in steering has been seen *in vitro* as well, where it was shown that the neurite of a chick sensory neuron would grow across a band of inhospitable substrate when a filopodium first extended over that band to contact a favorable substrate (Hammarback and Letourneau, 1986). Axonal pathfinding is mediated by negative as well as positive interactions of the growth cone with environmental cues (Patterson, 1988), and here, also, contact of a filopodium with the cue can produce the effect: freezing and then collapse of the growth cone occurs (Bandtlow et al., 1990).

The tip of the filopodium may be particularly important. First, it is often the distal part of the filopodium that makes contact with the environmental cue, and this contact can be sufficient to reorient growth towards a positive cue (Hammarback and Letourneau, 1986; O'Connor et al., 1990) or stop growth in response to a negative cue (Bandtlow et al., 1990). Second, the tip may be specialized to interact with the environment. The filopodia of chick retinal neurons in culture attach to the substrate and other cells preferentially at their tips, and this adhesion seems to be mediated by fine fibrils that project from the tip (Tsui et al., 1985). The filopodium of the grasshopper pioneer neuron sometimes invaginates deeply into a guidepost cell, so that a considerable length of the filopodium is making contact, yet only directly opposite the tip do coated vesicles form in the guidepost cell (Bastiani and Goodman, 1984).

Lastly, actin subunits are probably preferentially added at the distal tip of the filopodial core bundle of filaments. This would be expected from the orientation of the filaments. Most probably have their "barbed" ends at the tip (Lewis and Bridgman, 1992); this is usually the preferred end for addi-

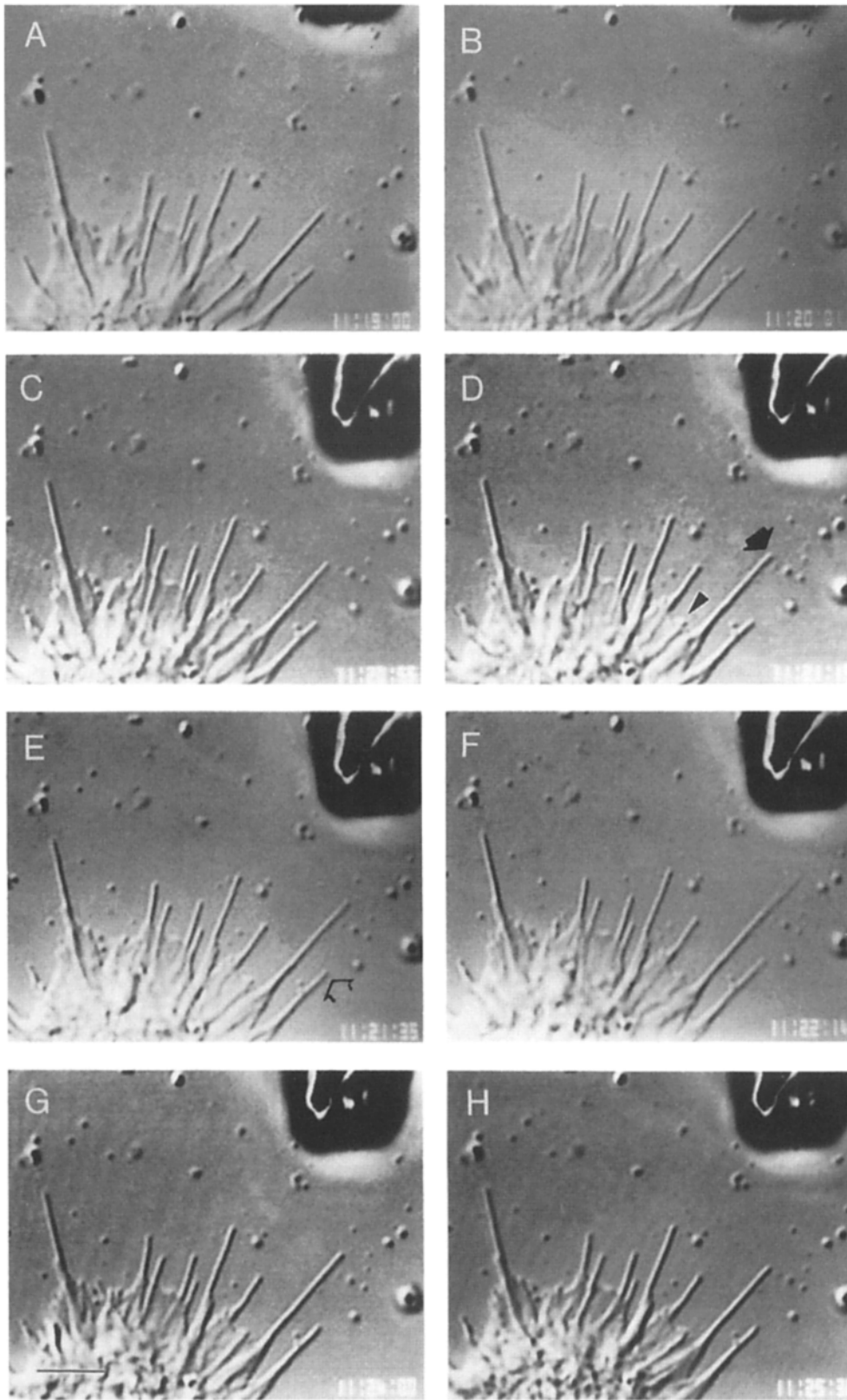


Figure 12. Local effect of an inhibitor of PTKs on filopodia. (A) A microelectrode filled with genistein (top right corner) is positioned several microns away from a growth cone on a polylysine substrate, without positive pressure being applied. (B) 1 min later, with positive pressure still off, there has been very little movement of filopodia. This growth cone was selected because its filopodia displayed relatively little spontaneous length changes, thus simplifying the interpretation of the experiment. Movement of filopodia can be assessed by comparison with numerous stationary particles on the substrate. (C) 54 s later, the microelectrode has just been moved closer to the growth cone and brief (4-ms) pulses of positive pressure are being applied repetitively (2 Hz) to the microelectrode to emit puffs of genistein (starting a few seconds before the image was taken). The puffs are visualized by the inclusion of Fast Green with the genistein, but cannot be seen in these single frames. A rapid bath perfusion of drug-free medium rapidly sweeps the emitted drug to the right. There has been essentially no movement of filopodia in the interval between this and the preceding image. (D) The filopodium closest to the puffs (*filled arrow*) has begun to elongate in the 20-s interval between this and the preceding image. The arrowhead denotes a filopodial nub next to the growing filopodium, which does not elongate during drug application. (E) The filopodium has grown more during the next 20 s as the puffing continues. In addition, a shorter filopodium next to it (*open arrow*) has started to grow. (F) Both filopodia have continued to grow as puffing has continued during the next 29 s. No other filopodia on the growth cone have grown appreciably during

the drug application. (G) Positive pressure was turned off 1 min before this image was taken. Both filopodia that were growing during drug application have started to shorten. (H) The longer filopodium continues to shorten during the 90-s interval between this and the previous image. Both filopodia that had grown have returned nearly to their original lengths. Other filopodia of the growth cone have not changed length substantially. Bar, 5 μm .

tion of subunits (Pollard and Cooper, 1986). Confirmation of the preferential addition of subunits to the tip has come from two types of experiment. Monomeric actin microinjected into mouse sensory neurons and PC12 cells in culture first appears incorporated into the filopodial core bundles at the distal tips (Okabe and Hirokawa, 1991). Also, after cytochalasin has caused the withdrawal of the peripheral actin network of the *Aplysia* growth cone, F-actin first appears at the distal margin of the growth cone upon drug washout (Forscher and Smith, 1988). Preferential addition of actin subunits at the distal tip implicates this as a key site of control of the length of the filament bundle and thus, presumably, of the filopodium.

Signaling Mechanisms in the Filopodium

It is not understood how the interaction of the filopodium with most environmental cues produces a change in growth cone behavior. It has been suggested that tight adhesion and the subsequent production of tension by filopodia causes trailing materials to move forward (Bray and Chapman, 1985; Heidemann et al., 1990) and that asymmetric pulling causes turning (Bray, 1982). Reorientation of axonal growth away from multiple adherent filopodia towards the direction of a single filopodium has suggested the involvement of biochemical signaling events in addition to, or instead of, such mechanical events (Caudy and Bentley, 1986). The paralysis and collapse of growth cones upon contact of a filopodium with a negative cue (Bandtlow et al., 1990) is also suggestive of biochemical or ionic signaling events. The only such signaling event so far demonstrated to operate in filopodia in response to an environmental cue is an increase in $[Ca^{2+}]_i$ in response to certain neurotransmitters (Davenport et al., 1993). Our results here establish tyrosine phosphorylation as another potential signaling mechanism in the filopodium. It should be emphasized that tyrosine phosphorylation could participate in a strictly mechanical transduction of an environmental cue by the filopodium; for example, it could regulate the linking of a membrane receptor occupied by a substrate-bound ligand to the bundle of actin filaments in the filopodium.

We have shown that, in the *Aplysia* growth cone in culture, the tyrosine phosphorylation in the filopodium is responsive to an environmental cue, substrate-binding material of *Aplysia* hemolymph, that alters the dynamics of the filopodium. Growth cones rapidly respond to this material by reducing the amount of phosphotyrosine in the tips of the filopodia. The substrate-binding material in *Aplysia* hemolymph and ganglion-conditioned medium is uncharacterized, other than as a protein >100 kD (Goldberg et al., 1991). Because there may be multiple UV-sensitive proteins of hemolymph that bind to the polylysine substrate, it is possible that the change in filopodial dynamics and the reduction in phosphotyrosine at the tips of filopodia are not caused by the same protein. We think this unlikely because reduction of phosphotyrosine by application of inhibitors of PTKs caused a similar change in dynamics. There is recent precedent for rapid tyrosine dephosphorylation in the growth cone being caused by substrate-binding, neurite growth-promoting proteins. Soluble fragments of neural cell adhesion molecule and L1 cause tyrosine dephosphorylation of tubulin in plasma membranes of growth cone particles obtained by fractionation of embryonic rat brain (Atashi et al., 1992).

The functional significance, if any, of this is unknown, and tubulin is not the sole target for tyrosine dephosphorylation.

Role of Tyrosine Phosphorylation in the Filopodium

It seems likeliest that tyrosine-phosphorylated protein at the tips of the *Aplysia* filopodia has a role associated with the actin filaments of the core bundle. These filaments comprise the major intracellular feature of the filopodium (Letourneau and Ressler, 1983; Lewis and Bridgman, 1992), and the rapid rearward movement of the phosphorylated protein when the actin network is induced to withdraw by application of cytochalasin (Fig. 5) implies an interaction of the protein with the network. It also suggests that the tyrosine-phosphorylated protein is inside the filopodium rather than in the plasma membrane, since the 2A1 membrane antigen has been shown to remain concentrated at the edge of embryonic mouse growth cones after treatment with cytochalasin (Sheetz et al., 1990). We suspect that the tyrosine-phosphorylated protein is a component of the subplasmalemmal Triton-insoluble globular material in which the distal ends of filopodial actin filaments appear to be embedded in electron micrographs of growth cones (Lewis and Bridgman, 1992).

What role in regulating the actin filaments of the filopodium might be played by protein-tyrosine phosphorylation? Three possible roles of tyrosine phosphorylation are suggested to us by its location in the filopodium. One possibility is that tyrosine phosphorylation regulates the linkage, via accessory proteins, of the actin filaments to membrane receptors mediating adhesion to the substrate or a cell. This is suggested by the fact that large concentrations of phosphotyrosine have previously been found only at the adherens junction (Maher et al., 1985; Tarone et al., 1985; Takata and Singer, 1988; Tsukita et al., 1991), which resembles the tip of the filopodium in being the point of termination of a bundle of actin filaments (Burrige et al., 1988). Such a role is not in evidence in our experiments, because our microscopic data demonstrate the heavily phosphorylated tips to be nonadherent (Figs. 3 and 4); nor do they become adherent upon dephosphorylation (Fig. 7). A second possibility is that tyrosine phosphorylation regulates the bundling of actin filaments. There is evidence that bundles of actin filaments homologous to those of the growth cone filopodium form in motile fibroblasts by an initial coalescence of the ends of filaments at the membrane (Izzard, 1988). Talin, which is a constituent of the focal contact (and is a substrate for tyrosine phosphorylation [Kellie et al., 1991]), is concentrated at the ends of these bundles even in the absence of contact with the substrate (DePasquale and Izzard, 1991). It was therefore suggested that talin functions in coalescing the filament ends (DePasquale and Izzard, 1991). We have no information relevant to the possible involvement of tyrosine phosphorylation at the tips of filopodia in coalescence of the ends of the actin filaments.

A third possible role for tip phosphorylation is in regulating the length of actin filaments in the bundle. As discussed above, actin is preferentially incorporated into filaments of the filopodium at the tip, so this should be a key site for regulation of filament dynamics. We showed that reduction of phosphotyrosine at the tip coincided with lengthening of the filopodium (and its core bundle of actin filaments) when the growth cone responded acutely to alteration

of the substrate. We also showed that application of inhibitors of PTKs caused a reduction of tip phosphorylation accompanied by lengthening of the filopodium. The fact that filopodia shortened rapidly on removal of the inhibitors argues that tyrosine dephosphorylation shifts the intrinsic balance between lengthening and shortening of the filament bundle towards lengthening, rather than removing an external impediment to lengthening such as attachment to the substrate, for shortening would not be expected to occur if the latter were true. Thus, these data indicate an association between reduction in tip phosphorylation and filopodial lengthening and raise the possibility that the dephosphorylation is involved in causing the lengthening. However, we cannot rule out the possibility that filopodial lengthening is secondarily caused by primary effects elsewhere in the growth cone of hemolymph and PTK inhibitors. Identification of the phosphorylated protein (or proteins) at the tips of filopodia and presentation of immobilized dephosphorylating agents to individual filopodia could clarify this issue.

Conclusion

By projecting out from the obscuring scrim of phosphotyrosine staining in the body of the growth cone, having a well-defined consistent internal cytoskeletal structure, and being an observable entity before and after treatment with PTK inhibitors or growth-promoting material, the filopodium is an informative location to detect a focal concentration of phosphotyrosine. From a cell biological perspective, this work shows that tyrosine-phosphorylated protein can be concentrated at the barbed ends of actin filaments in a context other than an adherens junction, and indicates an association between changes in phosphorylation and changes in filament dynamics. From a neurobiological perspective, it provides evidence for tyrosine phosphorylation as a signaling mechanism in the filopodium that can respond to environmental cues controlling growth cone dynamics and therefore suggests one way in which tyrosine phosphorylation may be involved in axonal pathfinding during development and regeneration.

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