

# Rho Family GTPases and Neuronal Growth Cone Remodelling: Relationship between Increased Complexity Induced by Cdc42Hs, Rac1, and Acetylcholine and Collapse Induced by RhoA and Lysophosphatidic Acid

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**Rho family GTPases have been assigned important roles in the formation of actin-based morphologies in nonneuronal cells. Here we show that microinjection of Cdc42Hs and Rac1 promoted formation of filopodia and lamellipodia in N1E-115 neuroblastoma growth cones and along neurites. These actin-containing structures were also induced by injection of *Clostridium botulinum* C3 exoenzyme, which abolishes RhoA-mediated functions such as neurite retraction. The C3 response was inhibited by coinjection with the dominant negative mutant Cdc42Hs<sup>T17N</sup>, while the Cdc42Hs response could be competed by coinjection with RhoA. We also demonstrate that the neurotransmitter acetylcholine (ACh) can induce filopodia and lamellipodia in neuroblastoma growth cones via muscarinic ACh receptor activation, but only when applied in a concentration gradient. ACh-induced formation of filopodia and lamellipodia was inhibited by preinjection with the dominant negative mutants Cdc42Hs<sup>T17N</sup> and Rac1<sup>T17N</sup>, respectively. Lysophosphatidic acid (LPA)-induced neurite retraction, which is mediated by RhoA, was inhibited by ACh, while C3 exoenzyme-mediated neurite outgrowth was inhibited by injection with Cdc42Hs<sup>T17N</sup> or Rac1<sup>T17N</sup>. Together these results suggest that there is competition between the ACh- and LPA-induced morphological pathways mediated by Cdc42Hs and/or Rac1 and by RhoA, leading to either neurite development or collapse.**

The Ras-related Rho family p21 GTPases Cdc42, Rac, and Rho are involved in many aspects of cell growth and morphology (10, 20, 45). Rac activates the neutrophil oxidative burst (1), and Cdc42 is involved in bud site selection and cell polarity in the yeast *Saccharomyces cerevisiae* (13, 23). In particular, these GTPases have been shown to induce morphological changes associated with actin polymerization. In Swiss 3T3 fibroblasts, Rac1 induces membrane ruffling and lamellipodium formation (42), RhoA induces stress fiber formation (40), and Cdc42Hs induces the formation of microspikes and filopodia (26, 36), all of which are dependent on filamentous-actin organization.

The neuronal growth cone, a specialized structure at the distal end of a developing neurite, is responsible for pathfinding in the developing nervous system (4, 8, 12, 21). The growth cone guides the extending neurite towards its target by constantly protruding and retracting filopodia and lamellipodia. Filopodia appear to have a sensory role in growth cone guidance: developing grasshopper neurons deprived of their filopodia by cytochalasin treatment exhibit aberrant behavior in reaching their target organs or do not reach them at all (9, 16, 24). Lamellipodia are implicated in neurite extension and cellular movement via membrane extension (4, 46). The growth cone receives a variety of signals from molecules on the surfaces of other cells, the extracellular matrix, diffusible chemoattractants, and chemorepellents (25). These environmental cues direct the developing neurite to extend in the correct direction by reorganization of the internal actin cytoskeleton (8, 14, 29, 44).

The Rho family GTPases play central roles in signalling pathways stimulated by trophic factors. In 3T3 fibroblasts, platelet-derived growth factor-stimulated membrane ruffling requires Rac1 (42) and lysophosphatidic acid (LPA)-stimulated stress fiber formation requires RhoA (40), while bradykinin-stimulated filopodium formation requires Cdc42Hs (26). In neuronal-type cells, LPA causes neurite retraction which is mediated by Rho; conversely, injection of C3 exoenzyme toxin, which inhibits RhoA activity, leads to neurite outgrowth (22, 35, 47).

In this study we show that Rho family GTPases participated in signalling pathways influencing growth cone morphology in neuroblastoma cells. Cdc42Hs stimulated formation of filopodia and lamellipodia, and the latter was also stimulated by Rac1, while RhoA induced growth cone collapse. A search for trophic factors which could stimulate such morphological activities identified the neurotransmitter acetylcholine (ACh). The action of ACh in inducing growth cone development required the mediation of Cdc42Hs and Rac1, while neurite outgrowth stimulated by C3 exoenzyme or by serum withdrawal also required Cdc42Hs and Rac1 activity. The effects of Cdc42Hs injection on the growth cone were inhibited by coinjection with RhoA, while treatment with ACh inhibited the neurite-retracting action of LPA. Taken together, these results suggest the possibility of competition between the RhoA and Cdc42Hs and/or Rac1 pathways, which mediate changes in morphology.

## MATERIALS AND METHODS

**Cell culture and time-lapse microscopy.** N1E-115 cells (5) were grown in Dulbecco's modified Eagle medium plus 10% fetal calf serum, with penicillin, streptomycin, and amphotericin (all from Gibco). Cells were plated at  $2 \times 10^5$  to  $3 \times 10^5$  cells/ml in serum-free Dulbecco's modified Eagle medium for approximately 20 h before microinjection. Phase-contrast time-lapse studies were carried out on a heated stage (37°C) in an atmosphere of humidified air and 10%

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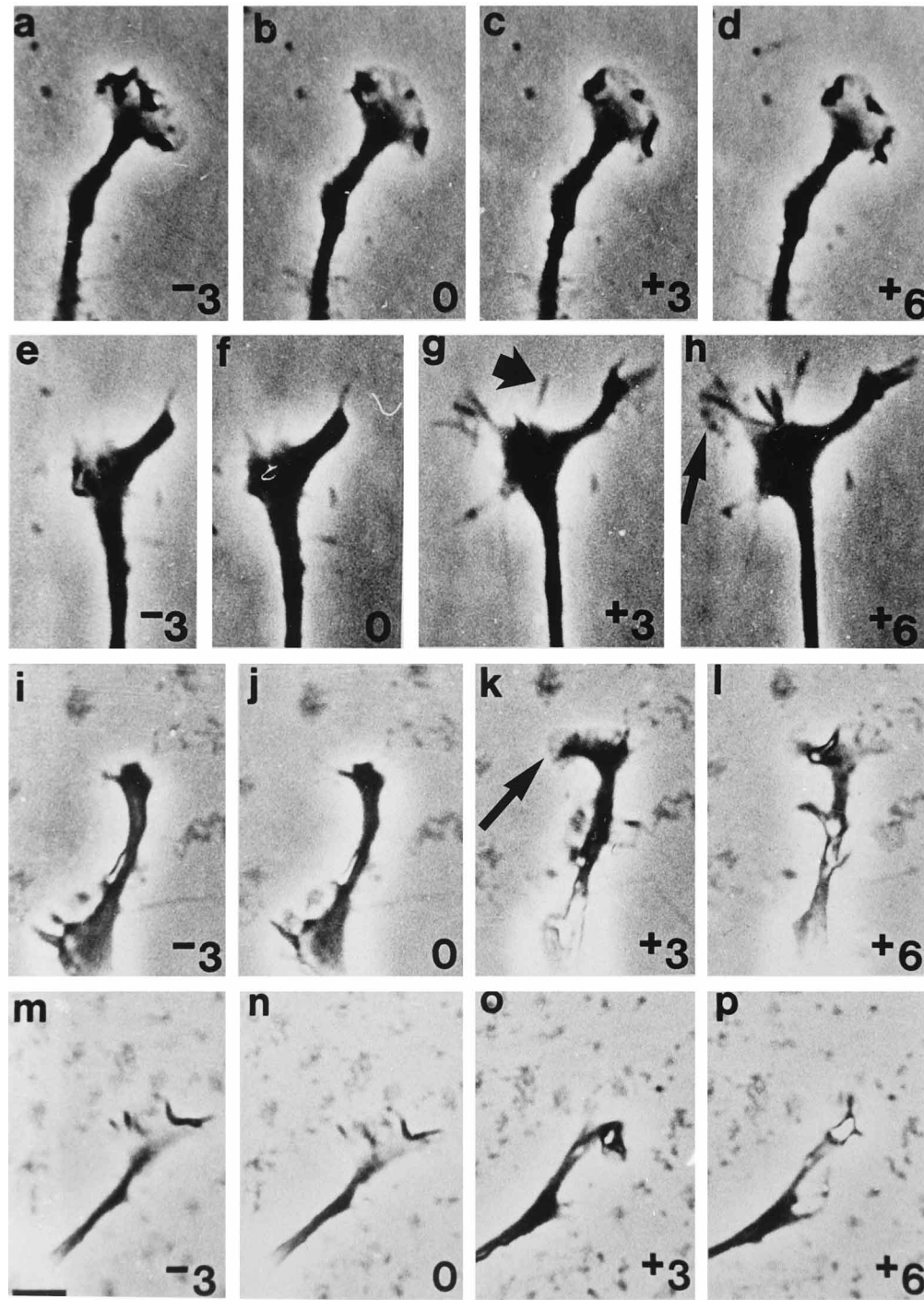


FIG. 1. Effects of Cdc42Hs, Rac1, and RhoA on growth cone morphology. Growth cones and neurites of serum-starved N1E-115 cells were monitored by phase-contrast microscopy before (a, b, e, f, i, j, m, and n) and after (c, d, g, h, k, l, o, and p) microinjection of proteins. Numbers show times (in minutes) before (–) and after (+) microinjection. (c and d) Injection with control buffer. (g and h) Injection with 1 mg of Cdc42Hs<sup>G12V</sup> per ml. The short arrow indicates a newly formed filopodium; the long arrow indicates lamellipodium veil formation. (k and l) Injection with 1 mg of Rac1<sup>G12V</sup> per ml. The arrow indicates lamellipodial formation. (o and p) Injection with 1 mg of RhoA per ml. Representative photographs are shown. Although growth cones exhibited various morphologies initially, this did not influence the rate of formation of filopodia and lamellipodia promoted by the GTPases. Bar in panel m = 15  $\mu$ m.

CO<sub>2</sub> as previously described (26), and cells were photographed with PAN F 50 film (Ilford). Time-lapse analysis was carried out by phase contrast on a Zeiss Axiovert 135 microscope over 30-min periods with a Pulnix TM-6CN video camera and Sony u-matic VO-5800PS video recorder. Images were analyzed with Bio-Rad Comos software.

**Production of Rho family proteins and mutants.** Cdc42Hs, Rac1, and RhoA GTPases and mutant proteins were expressed in *Escherichia coli*, cleaved, and purified from glutathione *S*-transferase fusion constructs (pGEX) as previously described (2, 26) and were estimated to be at least 95% pure.

**Microinjection and micropipetting.** Proteins were microinjected in a solution containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.1 mM dithiothreitol, using an Eppendorf microinjector and Axiovert microscope. For micropipetting assays the glass capillary tip filled with the sample was placed at approximately 50 μm from the cell growth cone at time zero, and positive pressure of 75 hPa was applied to the pipette. Using fluorescein dye, we ascertained that retrograde diffusion of factors was minimal during the experimental time course (30 min) and that a fan-shaped gradient emerged from the tip of the micropipette. We did not see an accumulation of dye at the growth cone during the course of an experiment, presumably due to the large volume of medium in the dish (15 to 20 ml) compared with the volume of solution in the micropipette (less than 5 μl). Finally, following the method of Lohof et al. (31), we estimated the concentration of the factor at the growth cone to be approximately 1,000-fold less than that in the micropipette, on the order of low millimolar concentrations.

## RESULTS

**Effects of Cdc42Hs, Rac1, and RhoA on growth cone and neurite morphology.** To determine whether Rho family GTPases play a morphological role in neural cells, they were microinjected into the cell bodies of N1E-115 neuroblastoma cells and neurite morphology was examined by phase-contrast time-lapse microscopy. Microinjection of the GTPase-negative mutant Cdc42Hs<sup>G12V</sup> promoted the formation of filopodia within a few minutes, which was followed by formation of lamellipodia (Fig. 1e through h). Microinjection of wild-type Cdc42Hs (Fig. 2, bars 2) and another GTPase-negative mutant, Cdc42Hs<sup>Q61L</sup> (results not shown), gave results qualitatively similar to those obtained with Cdc42Hs<sup>G12V</sup>. Lamellipodium formation, but not filopodium formation, induced by Cdc42Hs was blocked by the dominant negative mutant Rac1<sup>T17N</sup> (Fig. 2, bars 3). The GTPase-negative mutant Rac1<sup>G12V</sup> promoted the formation of lamellipodia (Fig. 1i through l), as did wild-type Rac1 (Fig. 2, bars 4) and the Q61L variant (results not shown). In contrast, microinjection of RhoA caused growth cone collapse (Fig. 1m through p), as did the GTPase-negative mutant RhoA<sup>G14V</sup> (data not shown). This was occasionally followed after about 20 min by a secondary phase of lamellipodium formation (Fig. 2B, bar 5). For all three GTPase proteins (Cdc42Hs, Rac1, and RhoA) we found that the GTPase-negative mutants induced morphological responses qualitatively similar to those of the wild-type proteins, although the former appeared more potent. Injection of the "effector site" mutants (Cdc42Hs<sup>D38A</sup> and Rac1<sup>D38A</sup> [26, 51]) or dominant negative mutants (Cdc42Hs<sup>T17N</sup> and Rac1<sup>T17N</sup>) had no morphological effect (Fig. 2, bars 6, and data not shown). These results implicate Cdc42Hs and Rac1 in growth cone development. The effect of RhoA on neurites agrees with previous studies showing growth cone collapse in these and other types of cells treated with LPA, whose effects were blocked by C3 exoenzyme (22, 35, 47). Similarly, growth cone collapse induced by microinjection of RhoA or RhoA<sup>G14V</sup> was also blocked by C3 exoenzyme (data not shown), suggesting that RhoA activation is required for the collapse.

**Inhibition of RhoA results in Cdc42Hs- and Rac1-type morphologies.** Upon microinjection of C3 exoenzyme into neuroblastoma cells, we observed increased formation of both filopodia and lamellipodia (Fig. 2, bars 7 and 8, and Fig. 3), which was also observed following microinjection of the dominant negative mutant RhoA<sup>T19N</sup> (Fig. 2, bars 9). These structures closely resembled those stimulated by microinjection with

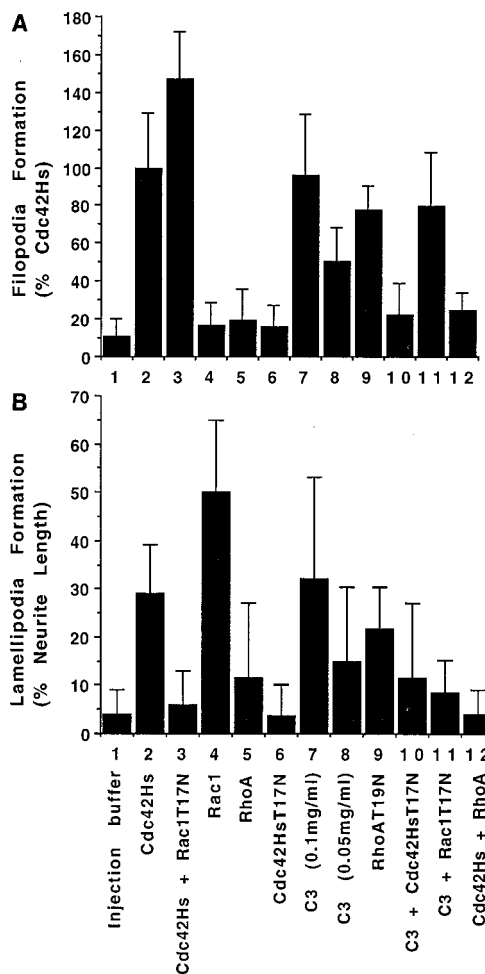


FIG. 2. Quantification of effects of Rho family GTPases on formation of growth cone filopodia and lamellipodia. The formation of filopodia (A) and lamellipodia (B) was analyzed by time-lapse microscopy. All GTPase proteins and mutants were injected at 1 mg/ml, and C3 exoenzyme was injected at 0.1 mg/ml unless otherwise indicated. (A) The y axis indicates the formation of new filopodia relative to that induced by Cdc42Hs injection (100%). (B) The y axis shows the extent of lamellipodial formation, determined by measuring the proportion of neurite length (distance from the tip of the growth cone to the soma) covered by newly induced lamellipodia, with 50% representing the maximum for each side of the neurite. Experiments were carried out over 30-min periods on a minimum of eight cells and repeated two to five times. Standard deviations are shown as error bars.

Cdc42Hs and Rac1, suggesting that inactivation of RhoA (by C3 exoenzyme) may result in the activation of the Cdc42Hs and Rac1 pathways. Coinjection of C3 exoenzyme with the dominant negative mutant Cdc42Hs<sup>T17N</sup> resulted in an inhibition of growth cone development (Fig. 2, bars 10), indicating that the morphological effects stimulated by inactivation of RhoA are indeed dependent upon Cdc42Hs activity. In contrast, coinjection of C3 exoenzyme with the dominant negative mutant Rac1<sup>T17N</sup> blocked lamellipodial activity but not induction of filopodia (Fig. 2, bars 11). Together these results suggest that inactivation of RhoA leads to activation of Cdc42Hs (and filopodium formation), which in turn leads to activation of Rac1 (and lamellipodium formation). We next coinjected Cdc42Hs with increasing amounts of RhoA. RhoA inhibited Cdc42Hs-induced increased growth cone complexity so that coinjection of approximately equimolar concentrations of

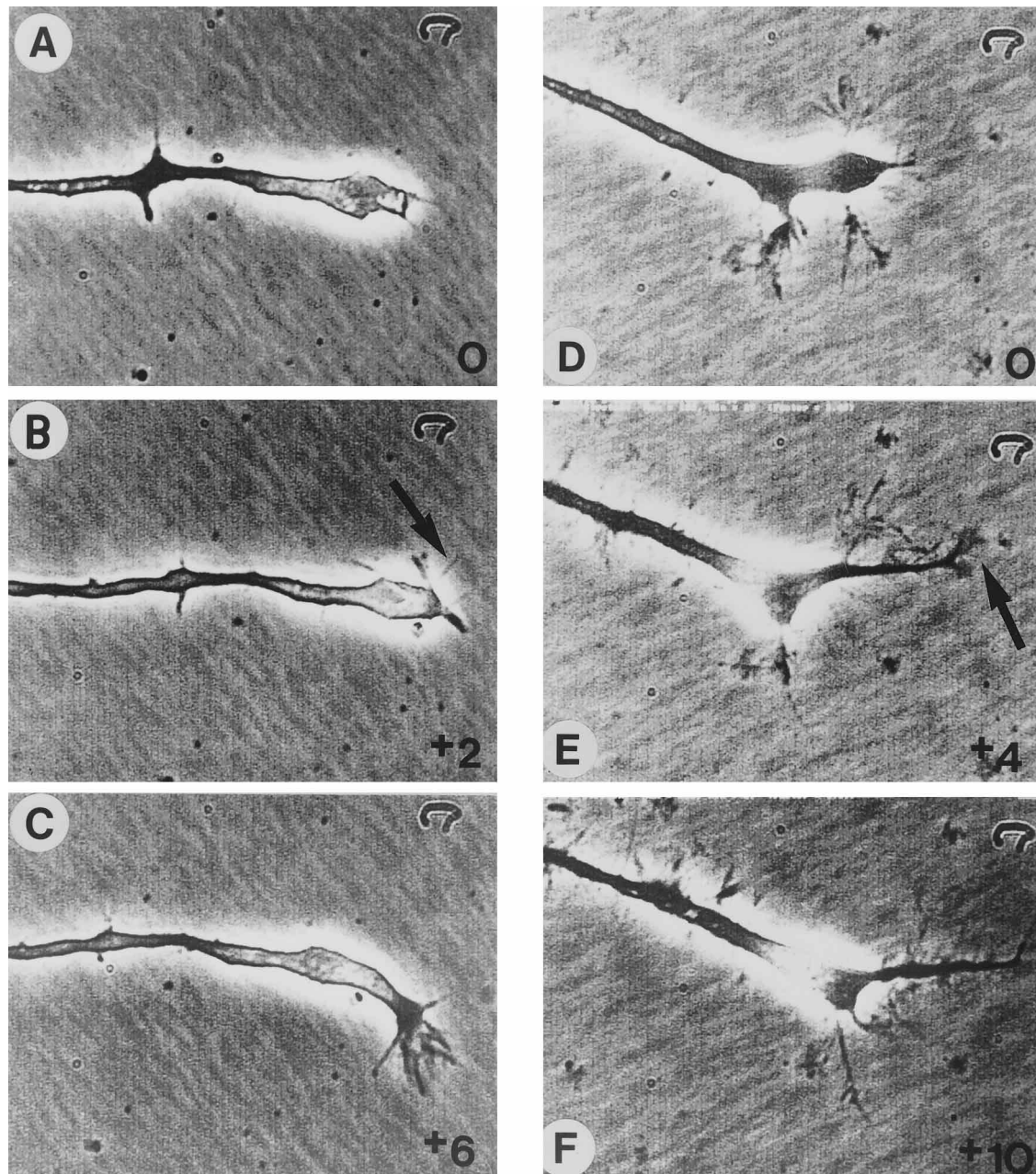


FIG. 3. C3 exoenzyme induces increased growth cone complexity. Growth cones and neurites of serum-starved N1E-115 cells were monitored by phase-contrast microscopy. Two examples (A through C and D through F) before (A and D) and after (B, C, E, and F) microinjection of 100  $\mu$ g of C3 exoenzyme per ml are shown. Times (in minutes) following injection are indicated in the bottom right-hand corners. The arrow in panel B indicates a filopodium. The arrow in panel E indicates lamellipodial veil formation on an extending neurite.

these GTPases resulted in negligible effects on growth cone and neurite morphology (Fig. 2, bars 2 and 12).

**Neurite outgrowth requires Cdc42Hs and Rac1 activity.** Filopodia and lamellipodia are important structural components of developing neurites (4, 8, 12, 16, 21, 24). During the initial phase of neurite outgrowth induced by serum starvation, rounded cells produced many filopodia and some lamellipodia around their circumferences (data not shown). The overall activity was reduced over several hours to a few discrete regions. Structures in these regions consolidated, and short neurites were formed. These immature neurites continued to produce filopodia and lamellipodia along their lengths and on

their growth cones and gradually extended in this manner. Formation of filopodia and lamellipodia can be promoted by microinjection of C3 exoenzyme (Fig. 2 and 3) or the dominant negative mutant RhoA<sup>T19N</sup> (Fig. 2, bars 9) or by serum starvation (data not shown). Over extended periods all treatments lead to neurite outgrowth in neuroblastoma cells (reference 22 and data not shown) and C3 exoenzyme also induces neurite outgrowth in PC12 cells (35), suggesting that the morphological processes of filopodium and lamellipodium formation and neurite extension are linked. We induced neurite outgrowth by both serum withdrawal and microinjection with C3 exoenzyme (Fig. 4 and 5). The proportion of cells with neurites of 30  $\mu$ m

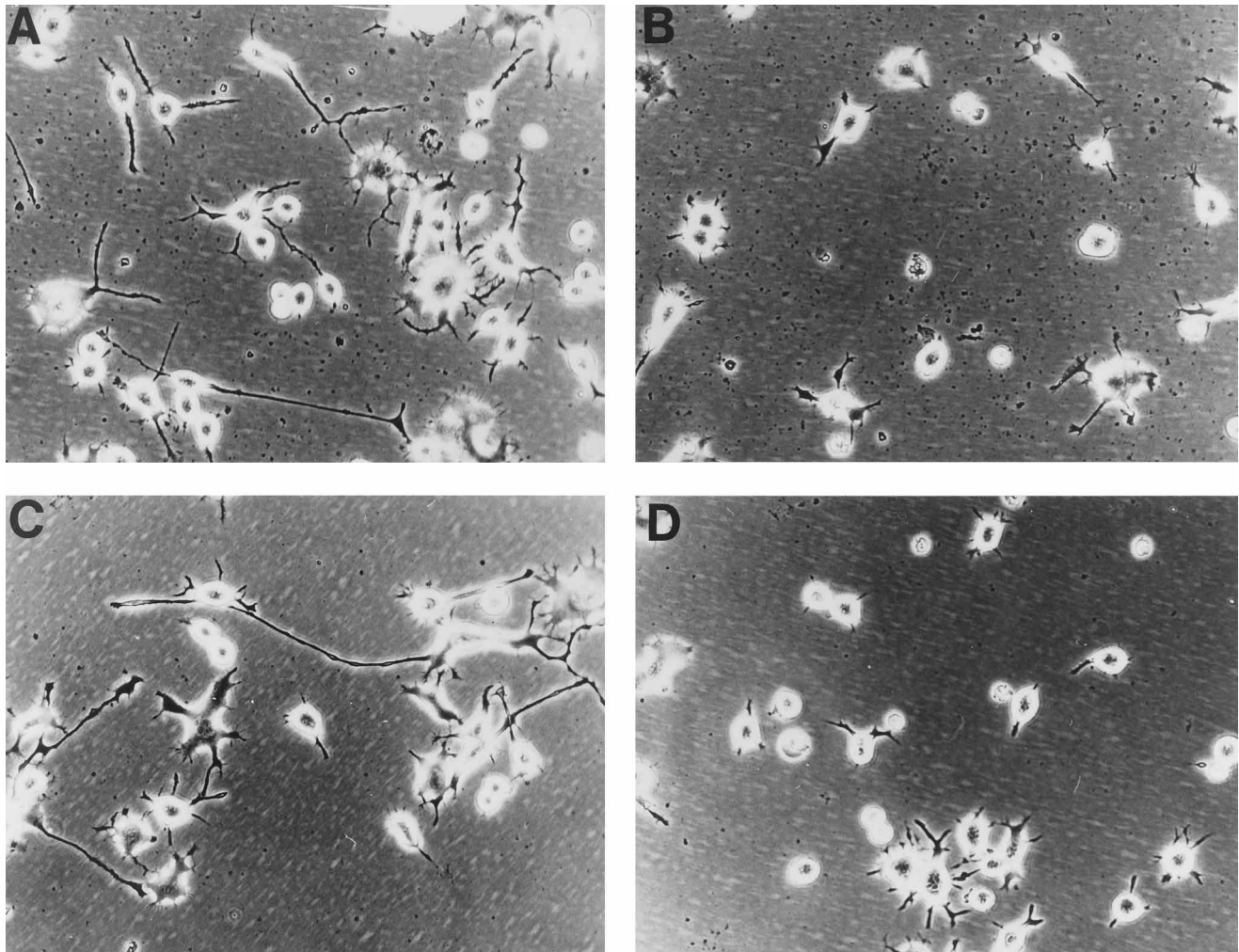


FIG. 4. Requirement for Cdc42Hs in neurite outgrowth. Neuroblastoma cells were serum starved (A) or microinjected with the dominant negative mutant Cdc42Hs<sup>T17N</sup> at 2 mg/ml prior to starvation (B). Cells were microinjected with C3 exoenzyme at 0.1 mg/ml (C) or coinjected with C3 exoenzyme plus Cdc42Hs<sup>T17N</sup> (D). All cells were examined 24 h later. Cells were microinjected on grids to allow relocation, and all cells in panels B through D were microinjected. Experiments were repeated three times, giving similar results.

or longer grown in serum was increased when cells were serum starved (Fig. 5; compare bars 1 and 5) or injected with C3 exoenzyme (Fig. 5; compare bars 1 and 2) and examined 24 h later. Microinjection with the dominant negative mutant Cdc42Hs<sup>T17N</sup> inhibited this increase in neurite outgrowth (Fig. 4 and Fig. 5, bars 3 and 7), as did coinjection with the dominant negative mutant Rac1<sup>T17N</sup> (Fig. 5, bars 4 and 8). This indicates that Cdc42Hs and Rac1 activities are required for efficient neurite outgrowth. The extent of neurite outgrowth seen either on serum starvation or on C3 exoenzyme microinjection was not increased further when both treatments were combined (data not shown). Treatment with LPA reduced the neurite outgrowth induced by serum withdrawal (Fig. 5, bar 6). This suggests that LPA is the main factor in serum responsible for preventing neurite outgrowth and that withdrawal and/or blockage of RhoA activation is an important factor in neurite outgrowth, which involves Cdc42Hs and Rac1.

**ACh induces increased growth cone complexity mediated by Cdc42Hs and Rac1.** When LPA was administered through a micropipette, polarized retraction away from the pipette, presumably caused by RhoA activation (22), was observed (results not shown). We then screened several neurotrophins and neu-

rotransmitters, some of which have trophic effects on neural cells (28, 30), for the ability to stimulate changes in growth cone morphology. Of these, we found ACh to induce formation of filopodia and lamellipodia along the neurite and on the growth cone, but only when administered locally through a micropipette (Fig. 6a through d and Fig. 7, bars 4). Other agents, which included nerve growth factor, platelet-derived growth factor, phorbol 12-myristate 13-acetate, bradykinin, neuropeptide Y, and noradrenaline, were ineffective (data not shown). Interestingly, simple addition of ACh to the medium did not elicit significant morphological changes (Fig. 7, bars 5), demonstrating that ACh needs to be present in a concentration gradient to be effective. This is in keeping with a similar requirement for ACh in order to elicit a turning response in cultured *Xenopus laevis* spinal neurons (52), whose growth cones can detect a 10% gradient of dibutyl cyclic AMP concentration across their width (31). However, neurite growth could occur either towards or away from the source of ACh under our experimental conditions. The effects of choline were investigated in the same way as those of ACh to rule out its involvement via acetylcholinesterase activity on ACh. No response was observed. Furthermore, the action of ACh was

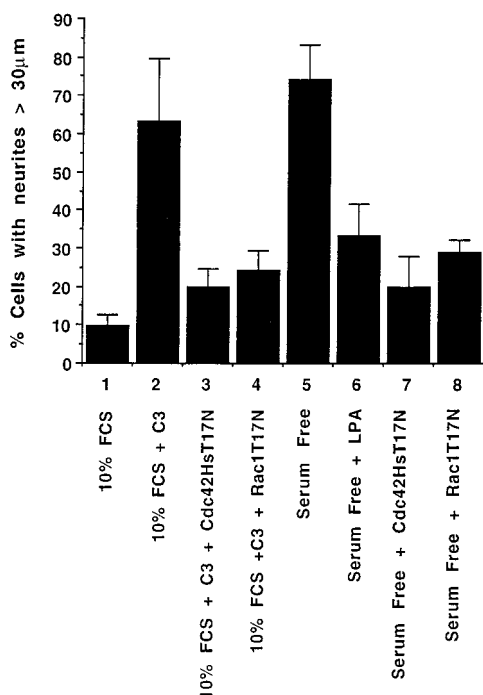


FIG. 5. Effects of Rho family GTPases on neurite outgrowth. Neuroblastoma cells were plated at a density of  $2 \times 10^5$  cells per ml. An area of cells that had been treated or microinjected was relocated after 24 h and the percentage of cells possessing neurites of 30  $\mu$ m and above was determined. Cells were grown either in the presence of 10% fetal calf serum (FCS) (bars 1 through 4) or in serum-free medium (bars 5 through 8). C3 exoenzyme was microinjected at 0.1 mg/ml and dominant negative proteins were injected at 1 mg/ml. LPA was added at 1  $\mu$ g/ml (bar 6). Experiments were repeated at least three times.

potentiated by coaddition of the acetylcholinesterase inhibitor eserine (5  $\mu$ g/ml) (data not shown), confirming ACh as the active agent causing the morphological changes. The response to ACh was dose dependent (Fig. 7C) and was abolished by preaddition of atropine, a muscarinic ACh receptor (mAChR) inhibitor (Fig. 7, bars 6), but not by *d*-tubocurarine, a nicotinic ACh receptor inhibitor (Fig. 7, bars 7). Pertussis toxin also inhibited the ACh response (Fig. 7, bars 8), which is consistent with the mAChR being G protein linked (18, 48) in this pathway. Finally, the morphological response to ACh was significantly decreased after 1.5 to 2 h of treatment, and following washout of the medium, the response could not be elicited upon readdition of the ACh source (data not shown). Although the reason for this is not known, one explanation could be desensitization of the mAChRs (50).

To determine whether Rho family GTPases were involved in ACh-induced morphological changes, we microinjected dominant inhibitory mutant proteins prior to ACh treatment. Microinjection with Cdc42Hs<sup>T17N</sup> blocked ACh-induced formation of filopodia but not of lamellipodia (Fig. 6e and f and Fig. 7, bars 9); conversely, Rac1<sup>T17N</sup> blocked ACh-induced formation of lamellipodia but not of filopodia (Fig. 6g and h and Fig. 7, bars 10). These results show a requirement for both Cdc42Hs and Rac1 in ACh-induced morphological responses.

**LPA-induced growth cone collapse is inhibited by ACh.** In view of the possible competing effects of microinjected RhoA and Cdc42Hs on growth cone collapse and development, we examined whether LPA and ACh had a similar antagonistic relationship. We found that ACh was indeed able to block LPA-induced neurite retraction (Fig. 8). This blocking effect

was limited to cells close to and in front of the fan-shaped gradient of ACh administration, with LPA-induced neurite retraction still being observed in more distal cells (Fig. 8), and was abolished when the LPA concentration was increased 10-fold (results not shown). The blocking effect occurred irrespective of the direction of the ACh source relative to the neurite or growth cone, provided it was proximal.

## DISCUSSION

Of the Ras superfamily GTPases, Ras and Rab2 have been shown to stimulate neurite outgrowth of PC12 cells and primary neurons (6, 7, 11, 37). Expression of mutated Rac and Cdc42 in *Drosophila melanogaster* disrupts axon and dendrite formation, respectively (33), while expression of Rac<sup>G12V</sup> in mice reduces Purkinje cell axon terminal formation (32). In this study we have shown that growth cone and neurite morphology can be altered by direct microinjection of Rho family GTPases. In neuroblastoma neurites, as with fibroblasts, Cdc42Hs promoted formation of filopodia and lamellipodia (26, 36) whereas Rac1 promoted formation of lamellipodia only (42). RhoA, which promotes the formation of stress fibers in fibroblasts (40), causes growth cone collapse and neurite retraction in neuroblastoma cells. Interestingly, blockage of Rho activity by microinjection of C3 exoenzyme has an effect very similar to that seen upon Cdc42Hs injection, with new filopodia and lamellipodia being formed within minutes of microinjection. Both structures can be blocked by coinjection of the dominant negative mutant Cdc42Hs<sup>T17N</sup>, while the dominant negative mutant Rac1<sup>T17N</sup> blocked lamellipodial activity only. This suggests that inactivation of RhoA can lead to sequential activation of Cdc42Hs and Rac1. C3 exoenzyme can also promote neurite outgrowth (22), which involves the formation of filopodia and lamellipodia, and subsequent consolidation of neurite structures (19, 46, 49). Cdc42Hs and Rac1 are both implicated in neurite outgrowth, as microinjection of dominant negative mutants reduces neurite extension caused by either C3 microinjection or serum starvation. Serum starvation may promote neurite outgrowth as a result of the absence of LPA (normally present in serum) and thus consequent loss of RhoA activation within the cell.

In fibroblasts, cellular contraction initiated by Rho-mediated phosphorylation of myosin light chains and by consequent assembly of myosin filaments precedes formation of stress fibers (15). It has been proposed that myosin-dependent alignment of actin filaments (stress fibers) then occurs, leading to peripheral aggregation of integrins connected to the filaments by actin-binding proteins. Tyrosine phosphorylation of these integrin-containing focal-adhesion complexes is required, since inhibitors of tyrosine kinases, such as genistein, will block RhoA-mediated formation of focal adhesions and stress fibers (41). It is not clear whether stress fibers occur in neuroblastoma cells. However, with respect to RhoA action, parallels between neurite retraction in neuronal-type cells and contraction in fibroblasts can be drawn, since both processes require RhoA and tyrosine phosphorylation (15, 22, 43, 47). Interestingly, nerve growth factor-primed PC12 cells treated with genistein display increased neurite outgrowth (34). This finding is consistent with the proposal that inhibition of RhoA (and thus its downstream effects) leads to neurite outgrowth, presumably through Cdc42Hs-mediated actions.

We have identified a signal transduction pathway in which the neurotransmitter ACh causes an increase in growth cone complexity through mAChR activation and the action of Cdc42Hs and Rac1 in inducing filopodium and lamellipodium formation, respectively (Fig. 9). Unlike the bradykinin pathway

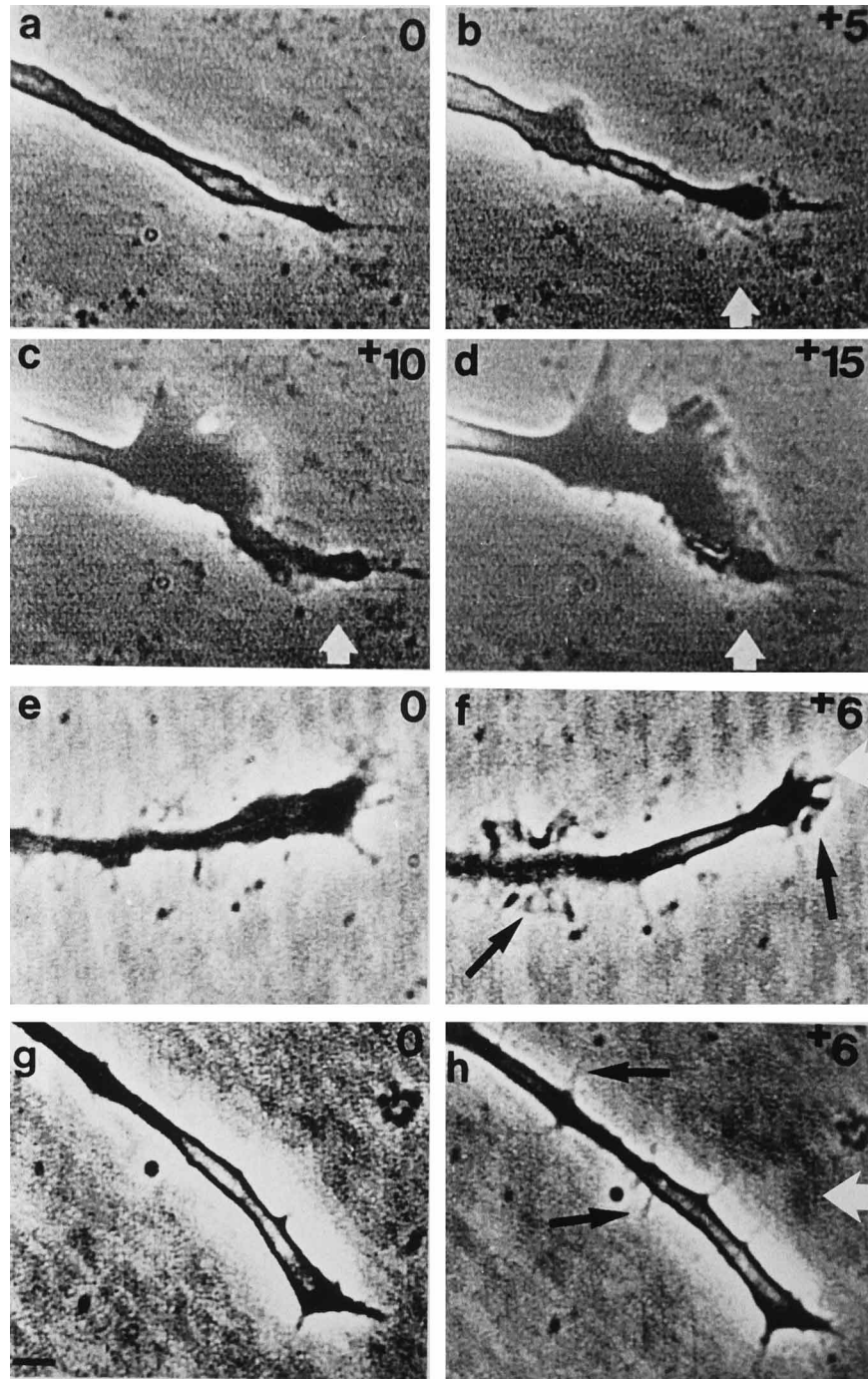


FIG. 6. Requirement for Cdc42Hs and Rac1 in ACh-induced growth cone development. (a to d) ACh-induced neuroblastoma growth cone development. ACh (1-g/ml micropipette concentration) was applied 50  $\mu$ m from the growth cone at time zero (a) and monitored by time-lapse video microscopy, shown every 5 min for a total of 15 min as indicated in panels b through d. White arrows indicate the direction of the ACh gradient. (e and f) Microinjection with 1 mg of the dominant negative mutant Cdc42Hs<sup>T17N</sup> per ml 5 min prior to application of ACh at time zero (e) and after 6 min (f). Note lamellipodium formation, indicated by black arrows. (g and h) Microinjection with 1 mg of the dominant negative mutant Rac1<sup>T17N</sup> per ml 5 min prior to application of ACh at time zero (g) and after 6 min (h). Note filopodium formation, indicated by black arrows. Photographs of representative cells are shown. Bar in panel g = 5  $\mu$ m.

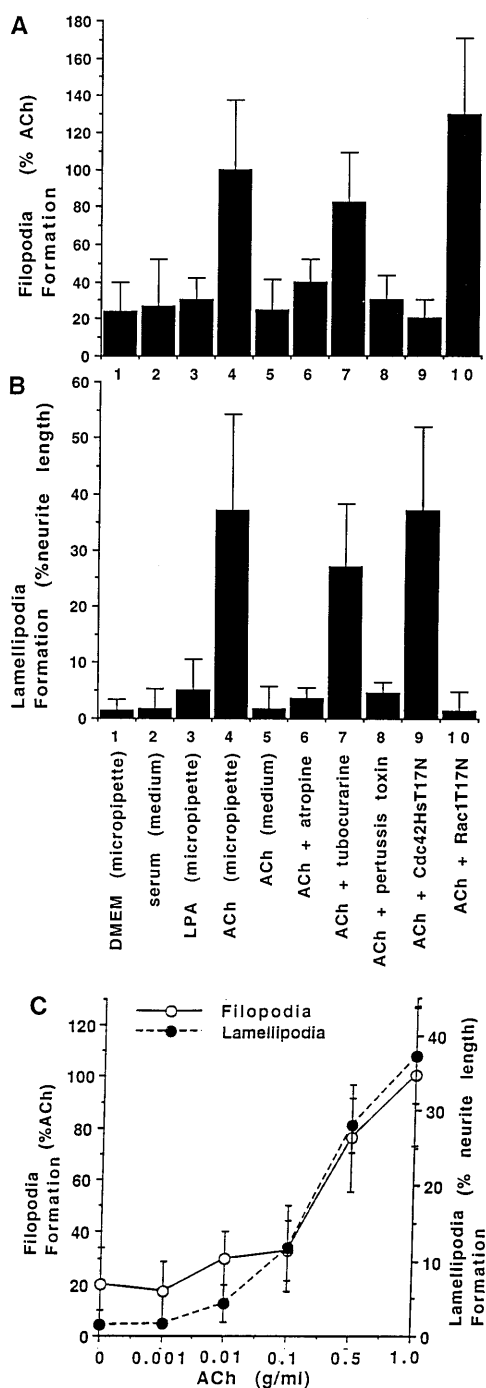


FIG. 7. Quantification of effects of ACh on formation of growth cone filopodia and lamellipodia. The formation of filopodia (A) and lamellipodia (B) was analyzed by time-lapse microscopy. Concentrations of factors were as follows: serum, 10%; LPA, 1  $\mu$ g/ml in the micropipette; and ACh, 1 g/ml in the micropipette. Concentrations of inhibitors in the medium were as follows: atropine, 0.01 g/ml; *d*-tubocurarine, 0.1 mM; and pertussis toxin, 50 ng/ml. All GTPase proteins and mutants were injected at 1 mg/ml. (C) ACh dose-response curve of filopodium and lamellipodium formation. We estimate the concentration of ACh at the growth cone to be approximately 1,000-fold less than that in the micropipette. (A) The y axis indicates the formation of new filopodia relative to that induced by ACh application with the micropipette (100%). (B) The y axis shows the extent of lamellipodial formation, determined by measuring the proportion of neurite length (distance from the tip of the growth cone to the soma) covered by newly induced lamellipodia, with 50% representing the maximum for each side of the neurite. Experiments were carried out over 30-min periods on a minimum of eight cells and repeated two to five times. DMEM, Dulbecco's modified Eagle medium. Standard deviations are shown as error bars.

in fibroblasts, where activation of Rac1 and formation of lamellipodia appear to require activation of Cdc42Hs, in the ACh pathway this GTPase hierarchy is not absolute. Thus, ACh can induce lamellipodial formation even when Cdc42Hs<sup>T17N</sup> is injected, indicating that Cdc42Hs activation is not obligatory for Rac1 activation in neuroblastomas. Low levels of mAChRs are expressed in N1E-115 neuroblastoma cells (17), and this trophic role of ACh acting through mAChRs is in accord with previous reports that transfection of mAChRs into PC12 cells leads to agonist-dependent neurite outgrowth (39).

The possibility of a hierarchy of GTPase activation in fibroblasts leading from Cdc42Hs to Rac1 and then to RhoA has been suggested (36). There is good evidence for Cdc42Hs activation leading to Rac1 activation (26, 36) and also that activation of Rac1 can separately lead to RhoA activation (40). In the latter case this is thought to involve arachidonic acid metabolites resulting from possible activation of phospholipase A<sub>2</sub> (38). However, there is little evidence for Cdc42Hs activation leading eventually to RhoA activation. In fibroblasts, there is an inverse relationship between the cellular content of bradykinin-induced actin microspikes and LPA-induced stress fibers, indicating competition between the mediating Cdc42Hs and RhoA pathways (26). Both bradykinin treatment and Cdc42Hs microinjection lead to a loss of Rho-type focal complexes and to the formation of Cdc42Hs- and Rac1-type focal complexes (3), which is consistent with such competition. Competition is further indicated by the report that filopodia are induced more quickly following coinjection of Cdc42Hs with C3 exoenzyme than if C3 is absent (36). We have not observed stress fiber formation even 24 h after microinjection of vectors expressing high levels of Cdc42Hs (27). Furthermore, Rac1-dependent ruffling does not always lead to subsequent stress fiber formation, for instance, following treatment with phorbol esters (40).

In neuroblastoma cells, the ACh- and LPA-mediated pathways appear to underpin different and opposing effects on actin morphology, leading either to neurite development or to neurite retraction. These opposing effects suggest that in this instance a hierarchy exists between Cdc42Hs and Rac1 only. Firstly, microinjection of Cdc42Hs leads to subsequent Rac1-dependent lamellipodial formation, and secondly, microinjection of C3 exoenzyme appears to stimulate Cdc42Hs followed by Rac1. However, following stimulation of Cdc42Hs (by either ACh treatment or microinjection of C3 exoenzyme or of Cdc42Hs itself) we do not observe Rho-type neurite retraction in these cells, even over a period of 2 h. We conclude that the hierarchy Cdc42Hs > Rac1 > RhoA does not occur in either fibroblasts or neuroblastoma cells. Rather, our view is that there is competition between RhoA- and Cdc42Hs-dependent pathways such that activation of one pathway occurs at the expense of the other at a given time, perhaps reflecting the utilization of shared components.

Our observations suggest that an interplay between Cdc42Hs, Rac1, and RhoA may govern the fine balance between neurite retraction, increased filopodium and/or lamellipodium activity at the growth cone, and neurite outgrowth, reflecting the different morphological effects of cells responding to varied environmental cues. These cues may include not only exposure separately to trophic factors such as LPA and ACh but also exposure to a combination of factors whose local concentrations determine the morphological outcome for the neurite. Because ACh can block LPA-induced retraction, switching between activation of Cdc42Hs and Rac1 or RhoA may be a mechanism to regulate either extension or retraction of the growth cone. Depending on the concentration gradients



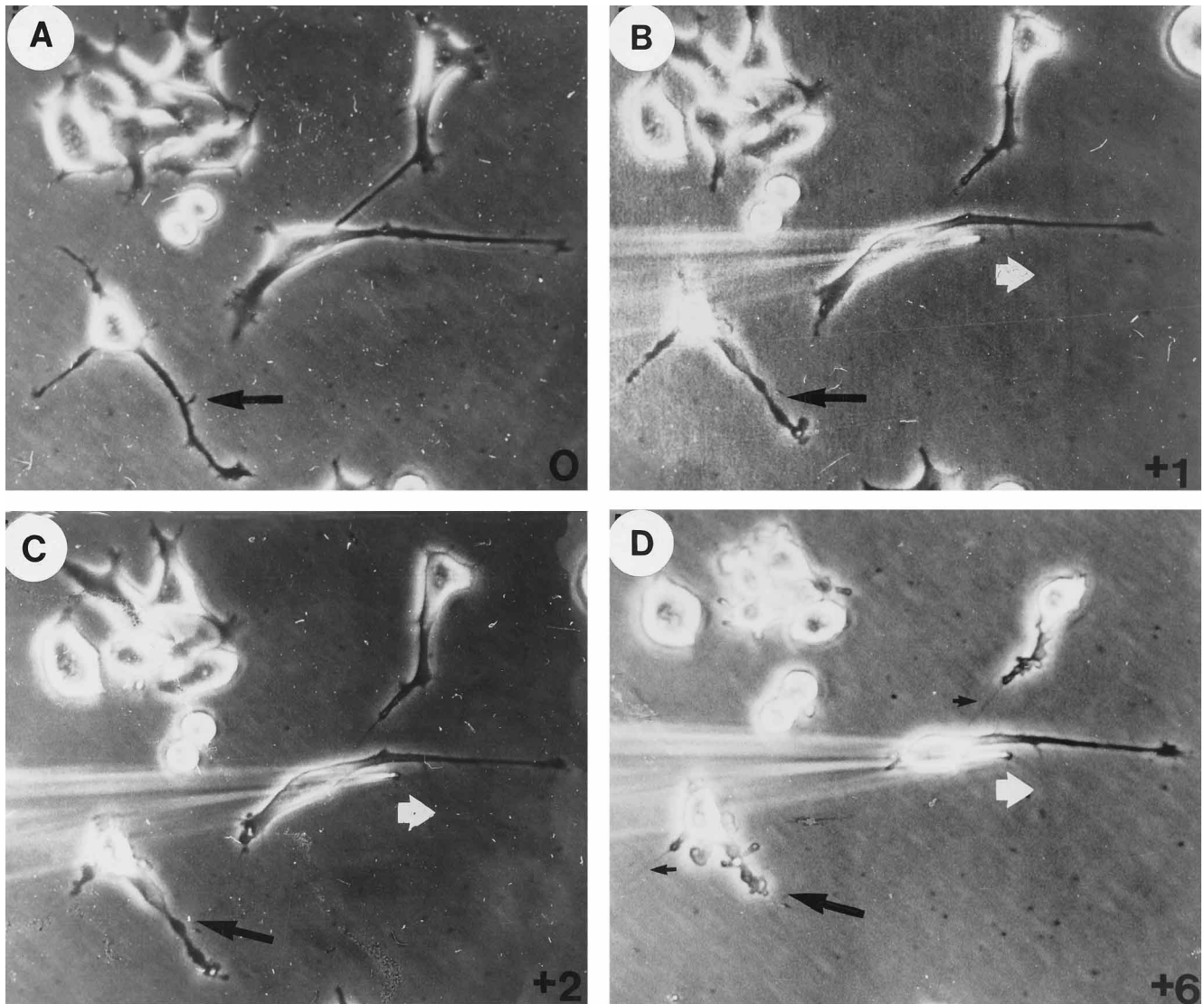


FIG. 8. ACh inhibits LPA-induced neurite retraction. ACh was applied just prior to addition of LPA ( $1 \mu\text{g/ml}$  in medium), shown at time zero (A) and 1 (B), 2 (C), and 6 (D) min after LPA addition. Note the complete retraction of neurites not subjected to ACh treatment, indicated by large black arrows. White arrows indicate the direction of the ACh gradient emanating from the tip of the pipette shown in the photographs. Immediately in front of the pipette tip is the neurite protected from retraction by ACh. Retraction fibers from other receding growth cones are indicated by small black arrows.

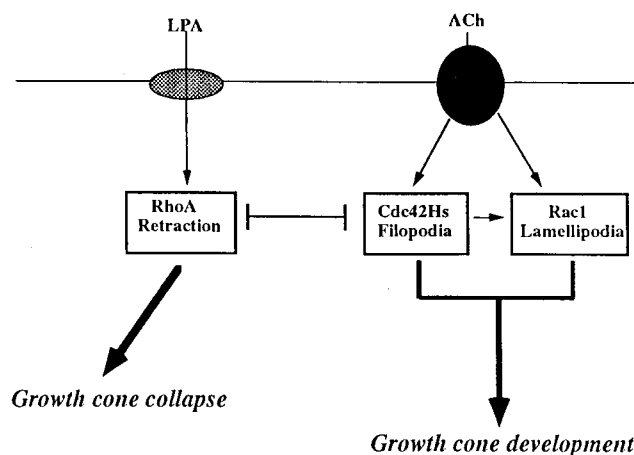


FIG. 9. Possible role for Rho family GTPases in signal transduction pathways leading to neurite development or collapse. It is proposed that binding of ACh to mAChRs will lead to stimulation of GTP-GDP exchange activity for Cdc42Hs and Rac1. The increase in GTP-bound forms of the GTPases results in stimulation of formation of filopodia and lamellipodia. ACh-mediated activation of Rac1 occurs independently of Cdc42Hs activation. However, as microinjection of Cdc42Hs leads to Rac1-dependent lamellipodium formation, it is also possible that there is some lamellipodium formation derived from Cdc42Hs-driven activation of Rac1. Neurite outgrowth will result if consolidation of structures occurs, for instance, by recruitment of tubulin to the developing growth cone (19, 46, 49). Factors which stimulate activity of Rho, such as LPA (22, 40), will promote growth cone and neurite collapse. The presence of one factor can lead to inhibition of the effects promoted by a second. Thus, ACh can protect an axon or dendrite from LPA-induced retraction, and conversely, LPA can inhibit ACh-induced neurite development. In vivo, local concentrations of these opposing factors can determine either neurite growth or retraction, facilitating neuronal plasticity.

of extracellular factors, RhoA would be stimulated to cause retraction, or it would be blocked and Cdc42Hs and Rac1 would be stimulated, causing increased morphological complexity (Fig. 9). In the development of the nervous system, this could well be one means by which axons guide themselves towards their eventual targets.

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