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# Activation of RhoA by Lysophosphatidic Acid and $G\alpha_{12/13}$ Subunits in Neuronal Cells: Induction of Neurite Retraction

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Neuronal cells undergo rapid growth cone collapse, neurite retraction, and cell rounding in response to certain G protein–coupled receptor agonists such as lysophosphatidic acid (LPA). These shape changes are driven by Rho-mediated contraction of the actomyosin-based cytoskeleton. To date, however, detection of Rho activation has been hampered by the lack of a suitable assay. Furthermore, the nature of the G protein(s) mediating LPA-induced neurite retraction remains unknown. We have developed a Rho activation assay that is based on the specific binding of active RhoA to its downstream effector Rho-kinase (ROK). A fusion protein of GST and the Rho-binding domain of ROK pulls down activated but not inactive RhoA from cell lysates. Using GST-ROK, we show that in N1E-115 neuronal cells LPA activates endogenous RhoA within 30 s, concomitant with growth cone collapse. Maximal activation occurs after 3 min when neurite retraction is complete and the actin cytoskeleton is fully contracted. LPA-induced RhoA activation is completely inhibited by tyrosine kinase inhibitors (tyrphostin 47 and genistein). Activated $G\alpha_{12}$ and $G\alpha_{13}$ subunits mimic LPA both in activating RhoA and in inducing RhoA-mediated cytoskeletal contraction, thereby preventing neurite outgrowth. We conclude that in neuronal cells, LPA activates RhoA to induce growth cone collapse and neurite retraction through a $G_{12/13}$-initiated pathway that involves protein-tyrosine kinase activity.

## INTRODUCTION

Rho family GTPases control a variety of cellular processes, ranging from cytoskeletal reorganization and cell motility to gene transcription in response to external stimuli (for review, see Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Like Ras, Rho GTPases act as binary switches: they are inactive when bound to GDP and are active in their GTP-bound form. RhoA, the founder member of the Rho subfamily, regulates the actin cytoskeleton in response to G protein–coupled receptor agonists such as the serum-borne phospholipid lysophosphatidic acid (LPA; Moolenaar *et al.*, 1997). The cytoskeletal changes mediated by RhoA vary between cell types. In serum-starved fibroblasts, RhoA induces the assembly of stress fibers and focal adhesions (Ridley and Hall, 1992). In neuronal N1E-115 cells, on the other hand, RhoA induces the formation of a cortical shell of f-actin that mediates cytoskeletal contraction (Kranenburg *et al.*, 1997), which is thought to underlie growth cone collapse, retraction of developing neurites, and rounding of the cell body in response to LPA (Jalink *et al.*, 1993, 1994; Kozma *et al.*, 1997; Kranenburg *et al.*, 1997; van Leeuwen *et al.*, 1997). In vivo, Rho-mediated neurite retraction might occur after nervous system injury, when neurons are suddenly exposed to blood-borne factors such as LPA released by activated platelets (Moolenaar *et al.*, 1997).

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Multiple downstream effectors of RhoA have been identified in recent years (Hall, 1998). Of particular relevance is the Rho-kinase (ROK$\alpha$/ROCK) family of Ser/Thr kinases that mediate both stress fiber formation and cytoskeletal contraction by stimulating myosin light-chain phosphorylation (Leung *et al.*, 1996; Matsui *et al.*, 1996; Amano *et al.*, 1997, 1998; Hirose *et al.*, 1998; Katoh *et al.*, 1998a). It has recently become clear that, in neuronal cells, the Rac and Cdc42 members of the Rho GTPase family oppose RhoA action in that they promote neurite outgrowth and stimulate growth cone motility (Kozma *et al.*, 1997; van Leeuwen *et al.*, 1997).

Insight into the cellular functions of RhoA has been obtained by overexpressing constitutively active and dominant-negative versions of RhoA. Although this overexpression approach has considerably advanced our understanding of RhoA downstream signaling, relatively little is still known about how cell surface receptors couple to activation of RhoA (i.e., RhoA-GTP accumulation). This is mainly because direct monitoring of RhoA activation has proved to be difficult. At present, agonist-induced activation of Rho GTPases is usually assessed in an indirect manner by monitoring changes in f-actin organization and cell morphology.

In the present study, we set out to monitor LPA-induced RhoA activation in a more direct manner and thereby gain further insight into how LPA regulates neuronal morphology, using N1E-115 cells as a model. LPA receptors couple to at least three distinct classes of G proteins: $G\alpha_q$, which activates phospholipase C; $G\alpha_i$, which inhibits adenylyl cyclase, whereas its corresponding $\beta\gamma$ subunits are thought to couple to Ras signaling; and the $G\alpha_{12/13}$ subclass, which has been implicated in Rho activation (Buhl *et al.*, 1995; Fromm *et al.*, 1997; Gohla *et al.*, 1998; Gutkind, 1998; Hart *et al.*, 1998). Our RhoA activation assay is analogous to recently described protocols for measuring Rap1, Ras, and Rac/Cdc42 activation (Taylor and Shalloway, 1996; de Rooij and Bos, 1997; Franke *et al.*, 1997; Manser *et al.*, 1998) and makes use of the selective binding of active, GTP-bound RhoA to the Rho-binding domain of ROK$\alpha$ (Leung *et al.*, 1995). A fusion between this domain and GST allows the specific recovery of activated RhoA on glutathione-Sepharose. Using this assay, we show that LPA rapidly activates endogenous RhoA in N1E-115 cells and that this activation requires tyrosine kinase activity. Moreover, we find that $G\alpha_{12}$ and $G\alpha_{13}$ subunits activate RhoA specifically and completely inhibit neurite outgrowth in a RhoA-dependent manner.

## MATERIALS AND METHODS

### *Cell Lines and Transfection*

COS7 and N1E-115 cells were grown in Dulbecco's modified Eagle's medium containing 10% FCS and antibiotics. COS7 cells were transfected using the DEAE-dextran method, whereas N1E-115 cells were transfected using calcium phosphate precipitates (Gebbink *et al.*, 1997; Kranenburg *et al.*, 1997).

### *RhoA and Cdc42 Activation Assay*

The GST-ROK fusion protein was made by using primers gccggatc-cctactaagtgactctccatc and gcggaattcactgcctatactggaactat in a PCR reaction on the full-length ROK$\alpha$ cDNA (a generous gift from Dr. L. Lim, Institute of Neurology, London, United Kingdom), followed by digestion with *Bam*HI and *Eco*RI and subcloning into pGEX4T3. The *Escherichia coli* Bl21-DE3pLysE strain was transformed with this construct, and expression of the fusion protein was induced by overnight incubation with 0.1 mM isopropyl-1-thio-$\beta$-D-galactopyranoside at room temperature. The fusion protein was prepared by lysing the bacteria in a buffer containing 1% NP-40, 50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol, supplemented with protease inhibitors. The bacterial lysate was then sonicated with 60 1-s pulses, and the lysates were cleared by centrifugation at 10,000 rpm for 15 min. The fusion protein was then recovered by addition of glutathione beads to the supernatant. The beads were washed three times in cell lysis buffer before addition to the cellular lysates. The fusion protein was prepared fresh for every experiment.

Cells were stimulated, washed with ice-cold PBS, and lysed in a buffer containing 50 mM Tris, pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol, supplemented with protease inhibitors. Lysates were cleared by centrifugation (14,000 rpm, 10 min), and the freshly prepared fusion protein, immobilized on glutathione-Sepharose, was added. After 1 h of tumbling at 4°C, beads were washed three times with lysis buffer and analyzed by Western blotting.

### *Western Blotting*

PAA gels were run and blotted onto nitrocellulose filters. The filters were blocked using 5% milk and were subsequently probed with primary antibodies (9E10, anti-myc; 26C4 [Santa Cruz Biotechnology, Santa Cruz, CA], anti-RhoA) and HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark). The 26C4 anti-RhoA is specific for RhoA; it does not recognize Rac or Cdc42 overexpressed in Cos7 cells (our unpublished results). Signals were visualized using the ECL detection system (Amersham, Arlington Heights, IL).

### *Morphological Analysis of N1E-115 Cells*

The morphology of transfected N1E-115 cells was assessed as described (Gebbink *et al.*, 1997). In short, cells were transfected with an expression vector encoding $\beta$-galactosidase and expression vectors encoding activated versions of the G protein $\alpha$ subunits. Activated $G\alpha_{12}$ and $G\alpha_{13}$ were kindly provided by Dr. H. Bourne (University of California, San Francisco, CA); activated $G\alpha_q$ was provided by Dr. S. Gutkind (National Institute of Dental Research, Bethesda, MD); and activated $G\alpha_i$ was provided by Dr. S. Hermouet (Institut Biologie des Hôpitale de Nantes). Cells were either scored rounded ("round"), flattened ("flat"), or flattened with neurites the length of at least twice the cell body diameter ("neurite"). The experiments were performed in triplicate and morphologies were scored without prior knowledge of the dishes' identities. The shown percentages are means of at least three independent experiments.

### *Immunofluorescence*

Cells were grown on glass coverslips and were transfected with either pcDNA-$G\alpha_{12}$ or pcDNA-$G\alpha_{13}$. After overnight culturing in serum-free medium, the cells were fixed in 3.7% formaldehyde and were processed for immunofluorescence as described using anti-$G\alpha_{12}$ and $G\alpha_{13}$ antibodies (A20 and S20, Santa Cruz) and rhodamine-conjugated phalloidin to stain f-actin.

## RESULTS AND DISCUSSION

### *A New Rho Activation Assay*

We developed a novel method to measure the activation of RhoA, analogous to the recently described methods to detect Rap1, Ras, and Rac/Cdc42 activation (Taylor and Shalloway, 1996; de Rooij and Bos, 1997; Franke *et al.*, 1997; Manser *et al.*, 1998). The Rho-binding domain of the RhoA effector ROK$\alpha$ (Leung *et al.*, 1995) (residues 420-1137) was fused to GST, and this fusion protein was then used to precipitate Rho proteins from cell lysates. We first tested whether GST-ROK could discriminate between GTP- and GDP-bound forms of RhoA by using RhoA mutants that are either constitutively GTP bound (V14 and L63) or GDP-bound (N19). Cos7 cells were transfected with expression vectors encoding myc-tagged wild-type (wt), activated (L63), or inactive (N19) RhoA as well as effector loop mutants (L63A37 and L63G39). Cell extracts were then prepared and incubated with the GST-ROK fusion protein. Binding of the various Rho proteins to GST-ROK was analyzed by recovery of the fusion protein on glutathione-Sepharose followed by Western blot analysis using an anti-myc tag antibody. Figure 1A shows that GST-ROK binds to activated (L63) and wt versions of RhoA but not to inactive N19RhoA or to the (GTP-bound) effector domain mutants. None of the RhoA proteins bound to GST alone. Thus, the interaction of GST-ROK with RhoA depends on GTP loading as well as on an intact effector domain.

We next tested whether GST-ROK could also be used to recover the GTP-bound forms of Rac and Cdc42. An in vitro interaction between ROK and Rac has been reported (Lamarche *et al.*, 1996). We found that both Rac1 and Cdc42 are efficiently pulled down by GST-ROK. However, although GST-ROK binds Cdc42 in a GTP-dependent manner, its binding to Rac1 appears to be nucleotide-independent (Figure 1B). Thus, GST-ROK is a useful tool to measure activation of RhoA and Cdc42, but not Rac. As shown in Figure 1, A and B, we consistently observed that in COS cells basal RhoA activity is much higher than basal Cdc42 activity (compare wt with V12 and V14/L63 lanes).

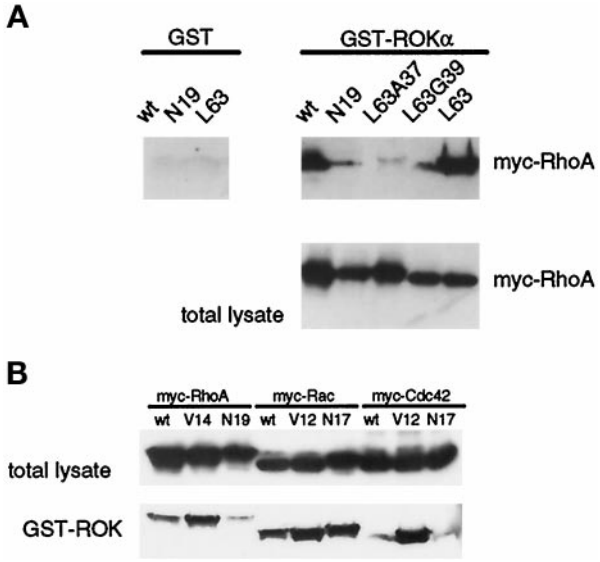


**Figure 1.** A new assay to measure Rho activation. (A) Cos7 cells were transfected with expression vectors encoding myc-tagged versions of either wt, activated (L63), or inactive (N19) RhoA. In addition, two activated RhoA mutants with additional point mutations in the effector loop were used: L63A37 and L63G39. Cell lysates were incubated with GST-ROK, and GST-ROK–associating proteins were analyzed by Western blotting using 9E10 monoclonal anti-myc antibody. GST-ROK binds to wt and L63 Rho but not to N19, L63G39, or L63A37. (B) Cos cells were transfected with myc-tagged versions of wt, activated (V12/V14), or inactive (N17/N19) Rho, Rac, or Cdc42. Binding to GST-ROK was then assessed as in A. GST-ROK discriminates between activated and inactive Rho and Cdc42, but Rac is recovered independent of its activation state.

### *LPA-induced RhoA Activation in Neuronal Cells: Tyrosine Kinase Involvement*

We next used GST-ROK to measure activation of endogenous RhoA in response to external stimuli. To this end, we used neuronal N1E-115 cells, which are highly responsive to LPA (Jalink *et al.*, 1993, 1994; Kranenburg *et al.*, 1997). When exposed to LPA, these cells undergo rapid RhoA-mediated contraction of the actomyosin-based cytoskeleton, leading to growth cone collapse, neurite retraction, and cell rounding (Jalink *et al.*, 1994). As shown in Figure 2A, LPA activates endogenous RhoA within 30 s, which coincides with the rapid onset of growth cone collapse and neurite retraction (Jalink *et al.*, 1993). Maximal RhoA activation is observed at 3 min after addition of LPA, when neurites are retracted and the cell body has adopted a fully rounded morphology. Bradykinin, which stimulates phosphoinositide hydrolysis and $Ca^{2+}$ signaling but not neurite retraction (Jalink and Moolenaar, 1992), fails to activate RhoA in N1E-115 cells (Figure 2A).

In an attempt to measure activation of endogenous Cdc42, we tested several commercially available antibodies. However, although some of them reacted with overexpressed Cdc42 in Cos7 cells, none of them detected endogenous Cdc42 in N1E-115 cells, thus precluding the detection of Cdc42 activation.

Circumstantial evidence suggests that, in 3T3 cells, receptor-mediated Rho activation involves protein-tyrosine kinase activity. This notion is based on the finding that tyrphostin inhibits stress fiber formation induced by LPA but not that induced by activated RhoA (Nobes *et al.*, 1995). Similarly, tyrosine kinase inhibition by either genistein or tyrphostin 47 blocks the rapid effects of LPA on growth cone collapse and

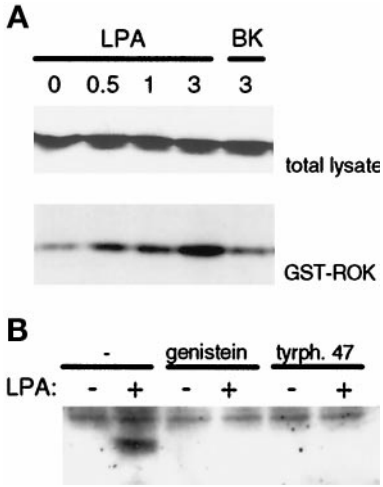


**Figure 2.** LPA activates endogenous RhoA in N1E-115 cells. (A) N1E-115 cells were cultured overnight in serum-free medium and were subsequently stimulated with LPA (1 $\mu$M) or bradykinin (1 $\mu$M) for the indicated periods. Cell lysates were prepared and incubated with GST-ROK. The activation state of RhoA was then assessed by anti-RhoA Western blotting. (B) RhoA activation depends on tyrosine kinase activity. Cells were pretreated with either genistein (25 $\mu$M, 30 min) or tyrphostin 47 (150 $\mu$M, 30 min) before stimulation with LPA (3 min). The activation state of RhoA was then assessed as in A.

neurite retraction in N1E-115 cells (Jalink *et al.*, 1993), whereas the inactive tyrphostin-1 had no effect (our unpublished results). To examine whether tyrosine kinase activity acts upstream or downstream of RhoA, we measured LPA-induced RhoA activation in the absence and presence of genistein or tyrphostin 47. Figure 2B shows that pretreatment of N1E-115 cells with either compound prevents LPA-induced activation of RhoA. Thus, tyrosine kinase activity links LPA receptors to RhoA activation in neuronal cells.

### *$G\alpha_{12/13}$ Subunits Activate RhoA and Induce Cytoskeletal Contraction*

LPA signals through multiple heterotrimeric G proteins to evoke its cellular responses. The $G_{12/13}$ subclass has been implicated in Rho activation and signaling (Buhl *et al.*, 1995; Fromm *et al.*, 1997; Gohla *et al.*, 1998; Hart *et al.*, 1998). We transfected various expression vectors encoding activated (GTPase-deficient) versions of $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_i$, and $G\alpha_q$, together with wt RhoA into Cos cells. The activation state of RhoA was determined using GST-ROK. We found that both $G\alpha_{12}$ and $G\alpha_{13}$ activate RhoA, whereas $G\alpha_i$ had no effect (Figure 3). Activated $G\alpha_q$ caused massive cell death (our unpublished results; also see Xu *et al.*, 1993) and hence could not be used in RhoA activation assays.

When transfected into N1E-115 cells, activated $G\alpha_{12}$ and $G\alpha_{13}$, like LPA or active RhoA (Jalink *et al.*, 1993; Gebbink *et al.*, 1997; Kranenburg *et al.*, 1997), induced

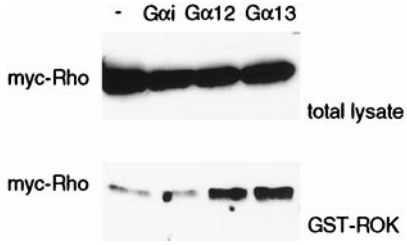


**Figure 3.** Activation of RhoA by $G\alpha_{12}$ and $G\alpha_{13}$. Cos7 cells were cotransfected with expression vectors encoding activated $G\alpha_i$, $G\alpha_{12}$, $G\alpha_{13}$, or a control vector, together with an expression vector encoding myc-tagged wt RhoA. Twenty-four hours after transfection, the cells were cultured overnight in serum-free medium, and the activation state of RhoA was assessed as in Figure 1.

cell rounding, thereby preventing cell flattening and neurite outgrowth (Figure 4A). The rounded cells expressing activated $G\alpha_{12}$ and $G\alpha_{13}$ display a contracted cortical cytoskeleton (Figure 4B), indicating that cell rounding induced by these subunits is not secondary to loss of cell adhesion attributable to dissolution of the cytoskeleton. We did not observe the formation of actin stress fibers in N1E-115 cells expressing either $G\alpha_{12}$ or $G\alpha_{13}$. We tested the sensitivity of $G\alpha_{12}$- and $G\alpha_{13}$-induced cell rounding to both genistein and tyrphostin 47. Although we observed a slight inhibition in $G\alpha_{13}$-expressing cells (consistent with findings by Gohla *et al.* [1998] and Katoh *et al.* [1998b]), interpretation of these results was obscured by increased cell death and shape changes in control cells (our unpublished results).

Cytoskeletal contraction was not observed with activated $G\alpha_i$, whereas activated $G\alpha_q$ again induced cell death (our unpublished results). Yet, it seems highly unlikely that active $G\alpha_q$ would promote RhoA activation for several reasons. First, bradykinin, which couples to $G\alpha_q$-mediated phosphoinositide hydrolysis in these cells, does not activate RhoA (Figure 2), nor does it induce neurite retraction (Jalink and Moolenaar, 1992). Second, in neuronal PC12 cells activated $G\alpha_q$ promotes rather than prevents neurite outgrowth (Heasley *et al.*, 1996), and finally, $G\alpha_q$ promotes the disassembly of stress fibers in fibroblasts (Buhl *et al.*, 1995), opposite to what is observed with activated RhoA and $G\alpha_{12/13}$.

To assess whether the contractility in neuronal cells induced by $G\alpha_{12/13}$ requires RhoA activity, we cotransfected dominant-negative RhoA (N19) or a control vector with either $G\alpha_{12}$ or $G\alpha_{13}$. Figure 4C shows that N19RhoA largely restores the normal morphology of N1E-115 cells, consistent with $G\alpha_{12/13}$ acting via RhoA to induce cytoskeletal contraction.

### *Concluding Remarks*

In conclusion, we have developed a new Rho activation assay to show that, in neuronal cells, LPA rapidly

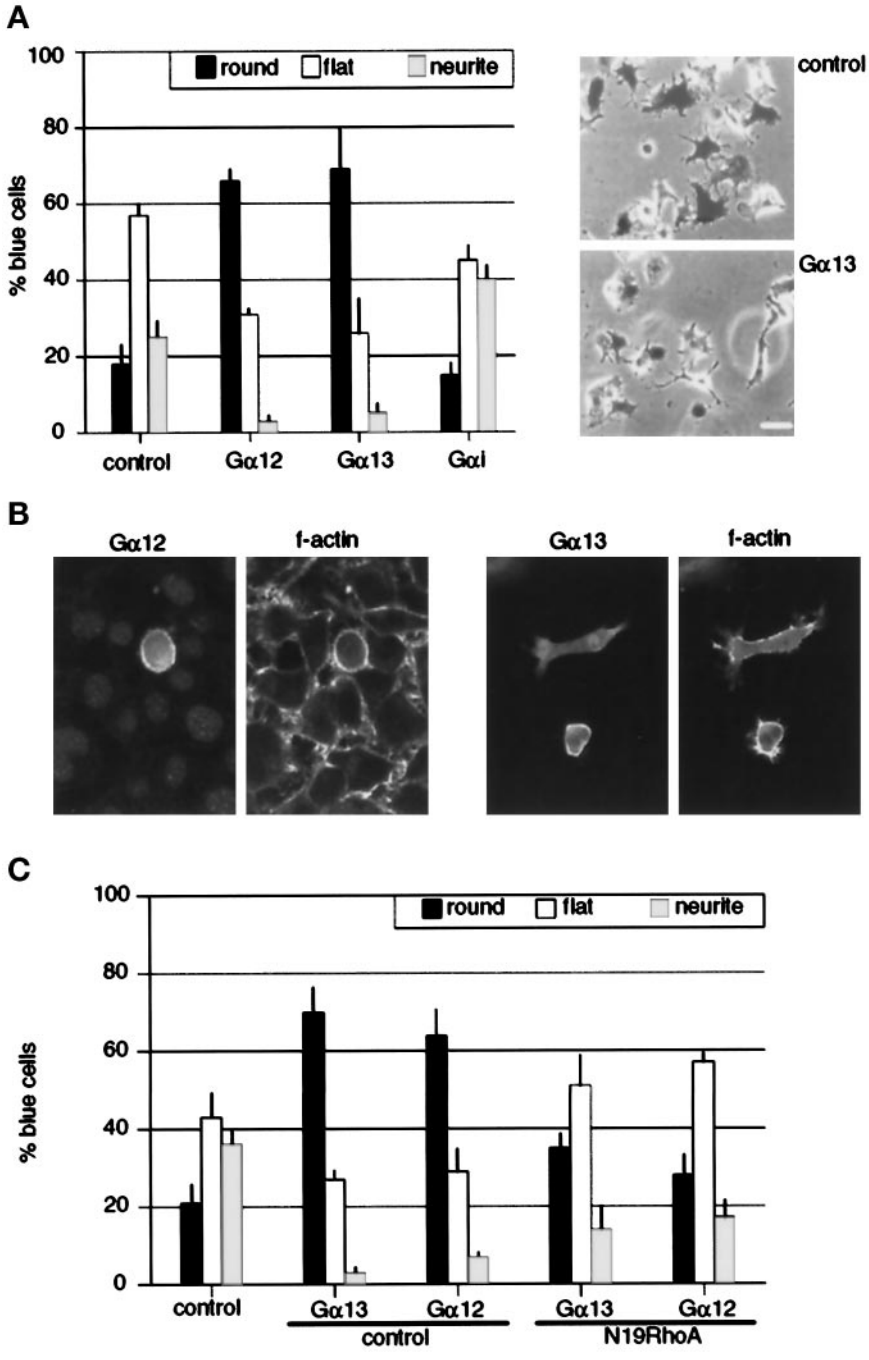


**Figure 4.** $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_i$, inhibit neurite outgrowth and induce cell rounding. (A) N1E-115 cells were transfected with either a control vector or expression vectors encoding activated $G\alpha_{12}$, $G\alpha_{13}$, or $G\alpha_i$, together with an expression vector encoding $\beta$-galactosidase. Cells were cultured in serum-free medium overnight, and morphologies were assessed as described. (B) Analysis of the actin cytoskeleton in $G\alpha_{12}$- and $G\alpha_{13}$-expressing cells. Rhodamine-phalloidin staining reveals that the cortical cytoskeleton in these cells is completely contracted, forcing the cells to round up. (C) Cells were transfected with $G\alpha_{12}$ and $G\alpha_{13}$ together with either a control vector or a vector encoding (dominant-negative) N19RhoA. Morphologies were then assessed as in A: $G\alpha_{12}$- and $G\alpha_{13}$-induced inhibition of neurite outgrowth and cytoskeletal contraction are RhoA dependent.

activates RhoA through $G\alpha_{12/13}$ and an unidentified protein-tyrosine kinase. The finding that tyrosine kinase activity is required for RhoA activation is important, because it implies that the recently reported in vitro interaction between $G\alpha_{13}$ and a Rho-specific exchange factor (p115-RhoGEF; Hart *et al.*, 1998) is not sufficient for efficient RhoA activation in intact cells. Recent studies by Gohla *et al.* (1998) and Katoh *et al.* (1998b) suggest that there is a differential requirement for tyrosine kinase activation in the induction of RhoA signaling by $G\alpha_{12}$ and $G\alpha_{13}$. A major challenge for further studies is to identify the tyrosine kinase involved in RhoA activation by $G\alpha_{12/13}$ in neuronal cells. Both the EGF receptor and Tec family tyrosine kinases have been implicated in Rho activation (Gohla *et al.*, 1998; Mao *et al.*, 1998). However, neither of these tyrosine kinases is highly expressed in neuronal N1E-115 cells. Further studies should reveal how $G\alpha_{12/13}$,

Rho exchange factor(s), and tyrosine kinase(s) interact to promote RhoA activation in neuronal cells exposed to LPA. The presently described assay should serve as a useful tool in these studies.

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