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Inhibition of Lysophosphatidate- and Thrombin-induced Neurite Retraction and Neuronal Cell Rounding by ADP Ribosylation of the Small GTP-binding Protein Rho

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Abstract. Addition of the bioactive phospholipid lysophosphatidic acid (LPA) or a thrombin receptor-activating peptide (TRP) to serum-starved N1E-115 or NG108-15 neuronal cells causes rapid growth cone collapse, neurite retraction, and transient rounding of the cell body. These shape changes appear to be driven by receptor-mediated contraction of the cortical actomyosin system independent of classic second messengers. Treatment of the cells with *Clostridium botulinum* C3 exoenzyme, which ADP-ribosylates and thereby inactivates the Rho small GTP-binding protein, inhibits LPA- and TRP-induced force generation and subsequent shape changes. C3 also inhibits LPA-induced neurite retraction in PC12 cells. Biochemical analysis reveals that the ADP-ribosylated substrate is RhoA. Prolonged C3 treatment of cells maintained in 10% serum induces the phenotype of serum-starved cells, with initial cell flattening being followed by neurite outgrowth; such C3-differentiated cells fail to retract their neurites in response to agonists. We conclude that RhoA is essential for receptor-mediated force generation and ensuing neurite retraction in N1E-115 and PC12 cells, and that inactivation of RhoA by ADP-ribosylation abolishes actomyosin contractility and promotes neurite outgrowth.

THE Ras superfamily of low molecular weight GTP-binding proteins can be divided into three major subfamilies, i.e., Ras, Rab, and Rho (Bourne et al., 1990; Hall, 1990). The most studied of these proteins, Ras, can cause cell transformation and triggers a serine/threonine protein kinase cascade in growth factor–stimulated cells (e.g., Leevers and Marshall, 1992; Moodie et al., 1993; Warne et al., 1993; Hordijk et al., 1994). In PC12 cells, Ras is an essential component of nerve growth factor (NGF)[1]-induced signaling and differentiation (Noda et al., 1985; Bar-Sagi et al., 1985; Hagag et al., 1985; Qiu and Green, 1991; Thomas et al., 1992; Wood et al., 1992; Li et al., 1992). The Rab proteins, on the other hand, are thought to be involved in intracellular vesicular transport (Balch, 1990) and have been implicated in neurotransmitter release from the presynaptic terminal (Fischer von Mollard et al., 1990; Mizoguchi et al., 1990; Shirataki et al., 1993). The function of the three identified Rho proteins (RhoA, RhoB, and RhoC) have been explored by the use of *Clostridium botulinum* C3 exoenzyme, which ADP-ribosylates Rho in its putative effector domain and thereby inactivates the protein (Aktories et al., 1987; Sekine et al., 1989; Paterson et al., 1990). These studies have revealed that Rho regulates the actin-based cytoskeleton and thereby participates in the control of cell shape, agonist-induced cell adhesion and motility, smooth muscle contraction and cell cycle progression (for review see Hall, 1992; Narumiya and Morii, 1993). Although the Rho proteins are highly expressed in brain and enriched in synaptosomes (Morii et al., 1988; Kim et al., 1989), there are hardly any clues to their neural functions.

We and others recently reported that in serum-starved N1E-115 and NG108-15 neuronal cells, the bioactive phospholipid lyophosphatidic acid (LPA) (Moolenaar, 1994) and a thrombin receptor–activating peptide induce rapid growth cone collapse, neurite retraction, and transient rounding of the cell body, apparently through receptor-mediated contraction of the actin-based cytoskeleton (Jalink and Moolenaar, 1992; Suidan et al., 1992; Jalink et al., 1993; Smalheiser, 1993). Serum, which contains albumin-bound LPA (Tigyi and Miledi, 1992; Eichholtz et al., 1993) also provoked neurite retraction in nerve growth factor (NGF)-

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1. *Abbreviations used in this paper*: LPA, lysophosphatidic acid; MLC(K), myosin light chain (kinase); NGF, nerve growth factor; TRP, thrombin receptor-activating peptide.

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differentiated PC12 cells (Skaper et al., 1983; Dyer et al., 1992; Tigyi and Miledi, 1992) and in serum-starved mouse neuroblastoma cells (Seeds et al., 1970). Furthermore, serum inhibits neurite outgrowth in various types of primary neuronal cultures, an effect presumably caused by albumin-bound LPA (Adler, 1982; Skaper et al., 1983; Ziller and Le Douarin, 1983; Davis et al., 1984; Windebank and Blexrud, 1986; Dyer et al., 1992; Tigyi and Miledi, 1992). These findings support the view that both LPA and thrombin may play a normal physiological role in neural morphogenesis, development, and plasticity and a pathophysiological role during traumatic injury of the nervous system when neurons are acutely exposed to blood-borne factors such as thrombin and LPA, and often undergo irreversible degeneration (Hawkins and Seeds, 1986; Monard, 1988; Grand et al., 1989; Jalink et al., 1993, and references therein).

In previous studies we have explored the mechanisms by which thrombin and LPA exert their dramatic effects on neural cell shape (Jalink and Moolenaar, 1992; Jalink et al., 1993). We have identified the putative LPA receptor by photoaffinity labeling and found that it is prominently expressed in brain as well as in N1E-115 and NG108-15 cells (van der Bend et al., 1992). We have demonstrated that both LPA and thrombin act through their cognate G protein–coupled receptors to rapidly reorganize the cortical actin cytoskeleton via a novel signaling pathway that is independent of prior Ca^{2+} mobilization, protein kinase C activation or altered cAMP levels. Instead, LPA- and thrombin-induced shape changes of N1E-115 cells are coincident with a small but significant increase in $p60^{src}$ tyrosine kinase activity (Jalink et al., 1993). LPA-induced actin reorganization is also observed in fibroblasts: Ridley and Hall (1992) reported that LPA, like serum, triggers the formation of focal adhesions and stress fibers in serum-starved 3T3 cells and that this action is mediated by the Rho protein.

In this paper we have examined the possible role of Rho in agonist-induced shape changes in N1E-115 and PC12 cells. We show that C3 treatment inhibits LPA- and thrombin-induced generation of actomyosin-based contractile forces and thereby prevents neurite retraction and cell rounding. Furthermore, C3 treatment of exponentially growing cells causes prominent neurite outgrowth accompanied by growth arrest. Our results suggest a key role for the RhoA protein in the regulation of neural architecture by extracellular agonists.

Materials and Methods

Materials

L-α-lysophosphatidic acid (1-oleoyl) was obtained from either Sigma Chemical Co. (St. Louis, MO) or Serdary Research Laboratories (London, Ontario), and bovine thrombin was from Sigma Chemical Co. Thrombin receptor–activating peptide (TRP, sequence SFLLRNPDKYEPF; Vu et al., 1991) was synthesized as described (Jalink and Moolenaar, 1992). Recombinant *C. botulinum* C3 exoenzyme was prepared essentially as described (Nemoto et al., 1991). Pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA). NGF was obtained from Collaborative Research Inc. (Bedford, MA) and KT5926 from Calbiochem-Behring Corp. (San Diego, CA). Phallacidin labeled with fluorescent NBD [*N*-(7-nitrobenzo-2-oxa-1,3-diazole)] (NBD-phallacidin) and Indo-1 acetoxymethylester were from Molecular Probes (Eugene, OR). Phenyl-methyl-sulfonyl-fluoride, soybean trypsin inhibitor, and laminin were from Sigma Chemical Co.; leupeptin was from Boehringer Mannheim Gmbtt (Mannheim, Germany); aprotinin from Fluka Chemie (Brussels, Belgium); trichloroacetic acid and EGTA were from Merck (Darmstadt, Germany); and DME, penicillin, and streptomycin from GIBCO BRL Life Technols Inc. (Gaithersburg, MD). [^{32}P]NAD and [^{32}P]orthophosphate were obtained from Amersham. Culture disposables were from Becton Dickinson & Co. (Plymouth, England).

Cell Culture

N1E-115 mouse neuroblastoma cells (Amano et al., 1972) and NG108-15 glioma × neuroblastoma hybrid cells (Hamprecht et al., 1985) were routinely grown in DMEM supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), and 10% FCS. For morphological assays, cells (passage 8 to 25) were seeded at a density of 1–2 × 10^4/cm^2 in plastic 35-mm dishes and 24–48 h later they were exposed to serum-free DME for 20–24 h. PC12 cells were seeded at 10^5 cells/35-mm dish and grown in DME supplemented with 10% FCS and 5% horse serum. For neurite retraction studies, PC12 cells were cultured in serum-free chemically defined medium (Skaper et al., 1983) containing NGF (25 ng/ml) for 2 d. Under these conditions, 70–80% of the cells developed neurites of 15–30 μm in length (cf. Dyer et al., 1992).

Cell Fractionation and [^{32}P]ADP Ribosylation

Cells were grown in 10-cm culture dishes for 24 h, washed twice with PBS, scraped with a rubber policeman in ice-cold 20 mM Tris/HCl (pH 8.0) in the presence of protease inhibitors (0.4 mM PMSF, 20 μM leupeptin, 0.005 trypsin inhibiting U/ml aprotinin, 2 μg/ml soybean trypsin inhibitor), and homogenized with 30 strokes (tight pestle) in a 10-ml Douncer. Cell debris was discarded by centrifugation (500 *g* for 10 min) at 4°C. The supernatants were centrifuged at 25,000 *g* for 30 min to separate the cytosolic from the crude membrane fraction. Protein was determined with the Bradford assay (Bio-Rad Laboratories, Cambridge, MA; see Bradford, 1976). For ADP ribosylation assays, membrane or cytosolic fractions (~30 μg protein) were heat inactivated at 65°C for 5 min and then taken up in reaction buffer containing: 90 mM Tris/HCl (pH 8.0), 2.6 mM $MgCl_2$, 1 mM EDTA, 10 mM thymidine, 10 mM dithiothreitol, 1 mM ATP, 100 μM GTP, 10 μCi/ml [^{32}P]NAD. The reaction was started by addition of C3 exoenzyme (5 μg/ml) or pertussis toxin (1 μg/ml). After 60 min at 37°C, the reaction was stopped by addition of ice-cold TCA (10% wt/vol). TCA was removed by centrifugation (15,000 *g* for 15 min) and washing the pellets with ether. After gel electrophoresis (using 12% polyacrylamide), ADP-ribosylated proteins were visualized by autoradiography (Kodak XAR film). For two-dimensional gel analysis, the isoelectric focusing gels contained pH 3.5–10 ampholines (LKB Instruments, Bromma, Sweden) and the second dimension gels 12% acrylamide.

Morphological Analysis

Agonist-induced shape changes of N1E-115 or NG108-15 cells were scored as described previously (Jalink and Moolenaar, 1992). In brief, low-density cultures in 35-mm dishes were shifted to serum-free DME for 20–30 h to obtain a nearly homogeneous population of flattened cells in which 90–95% of the cells have short filopodia and 5–10% of the cells have developed neurites of >25 μm in length. Alternatively, cells were maintained for 4–5 d in serum-free DME to obtain a more fully differentiated cell population in which virtually all cells have well-developed, long neurites. Agonist-induced shape changes were monitored using an inverted microscope (Nikon Diaphot) equipped with phase-contrast optics and a video recording system. Experiments were performed at 37°C in bicarbonate/5% CO_2-buffered DME, pH 7.3. Cells were photographed prior to and 3 min after addition of agents using a Sony UP-850 videoprinter. To determine dose–response relationships, cell morphology was assessed semi-quantitatively at 3 min after agonist addition. Relative shape change ("% shape change") was defined as follows (Jalink and Moolenaar, 1992; Jalink et al., 1993): 0%, no detectable change in any cell (20–40 single cells within a microscopic field); 100%, complete rounding up of all flattened cells; 50%, all cells display partially ("half") rounded shape. Intermediate values were estimated by interpolation. In large series of experiments, assessment of cell rounding from photomicrographs by a second person with no prior knowledge of the experimental protocol, yielded data that did not deviate by more than 10%. Data are presented as means ± SEM ($n > 3$).

Cell viability before and after treatment with the various agents was not detectably affected as determined by trypan blue exclusion. However, after prolonged serum deprivation (6–7 d), differentiated N1E-115 cells begin to die by apoptosis (Kruman et al., 1993).

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Force Generation Assay

Various methods have been described to demonstrate contractile forces in individual cells. Shear forces generated by migrating fibroblasts can cause visible wrinkling of ultrathin sheets consisting of polymerized dimethylpolysiloxane (Harris et al., 1980; Baorto et al., 1992). Alternatively, bending of highly flexible microneedles has been used to measure forces generated by growing neurites (Dennerl et al., 1988). However, our efforts to adopt either of both methods were unsuccessful. N1E-115 cells were found to be incapable of inducing visible wrinkles in laminin-coated layers of polymerized dimethylpolysiloxane (viscosity: 600 Stokes), presumably because of insufficient cell–substratum adhesiveness. Using the flexible needle approach for monitoring the build-up of contractile forces, results were highly variable between different experiments and neurites were often damaged by the microneedle. Moreover, the latter technique is not readily compatible with the focal application of agonists from a micropipette.

We therefore developed an alternative method to monitor the generation of contractile forces in the neurite shafts. In this new assay, we either selected cells with "loose" neurites that adhere to the dish only at the cell soma and the growth cone, or else we deliberately detached neurites from the substratum using a sharp microneedle fixed on a micromanipulator. A thin plastic suction pipette (~25-μm diam) was then positioned at one side of the neurite shaft and a local, continuous flow of medium was then generated by suction to provoke bending of the loose neurite. A second pipette (~2-μm tip size) containing agonist was positioned opposite to the suction pipette, close to the neurite under study. Agonists were pressure applied and neurite morphology was monitored for a period of at least 10 min. This technique has the advantage of local perfusion: agents are rapidly removed by the suction pipette, thus allowing successive experiments to be carried out on different neurite-bearing cells in the same dish. Shape changes were video recorded and photographed using a videoprinter. Statistical significance was calculated using the Kolmogorov/Smirnov parameter-free test for differences between two samples.

F-Actin Staining

Cells grown on circular cover glasses were fixed for 5 min with 3% paraformaldehyde in PBS followed by permeabilization using PBS containing 0.1% Triton X-100 and 1 mM EGTA for 5 min. After rinsing the cells in EGTA-containing PBS, NBD-phallacidin (2 ng/ml) was added for 20 min at room temperature. Cells were viewed using a Bio-Rad/Nikon confocal laser scanning microscope.

$[Ca^{2+}]_i$ Measurements

Serum-deprived cells, attached to glass cover slips (3 × 1 cm), were loaded with indo-1 by exposing them to 5 μM indo-1-acetoxymethylester for 30 min at 37°C. Fluorescence measurements were carried out as described (Jalink et al., 1990).

Activation of $p21^{ras}$

Serum-starved cells in six-well plates (one well, 10 cm^2) were serum starved for 20 h and then exposed to phosphate-free DME supplemented with 200 μCi $[^{32}P]_i$ per well for an additional 4 h. Cells were treated with agonist, lysed in a 1% Triton X-114 Hepes buffer, and $p21^{ras}$ was immunoprecipitated, and then assayed for bound nucleotides by thin layer chromatography as described (van Corven et al., 1993).

Results

ADP Ribosylation of Rho Proteins in N1E-115 Cells by C3 Exoenzyme

When membranes and cytosol from either N1E-115 cells or Rat-1 fibroblasts are ADP-ribosylated by C3 exoenzyme and subjected to SDS-PAGE, a distinct [^{32}P]ADP-ribosylated protein band of ~23 kD is detected (Fig. 1 *A*). N1E-115 cells showed consistently stronger ^{32}P-labeling in the cytosol than in the membrane fraction, in agreement with the reported subcellular distribution of $p21^{rho}$ (Narumiya et al., 1988; Adamson et al., 1992), whereas about equal amounts

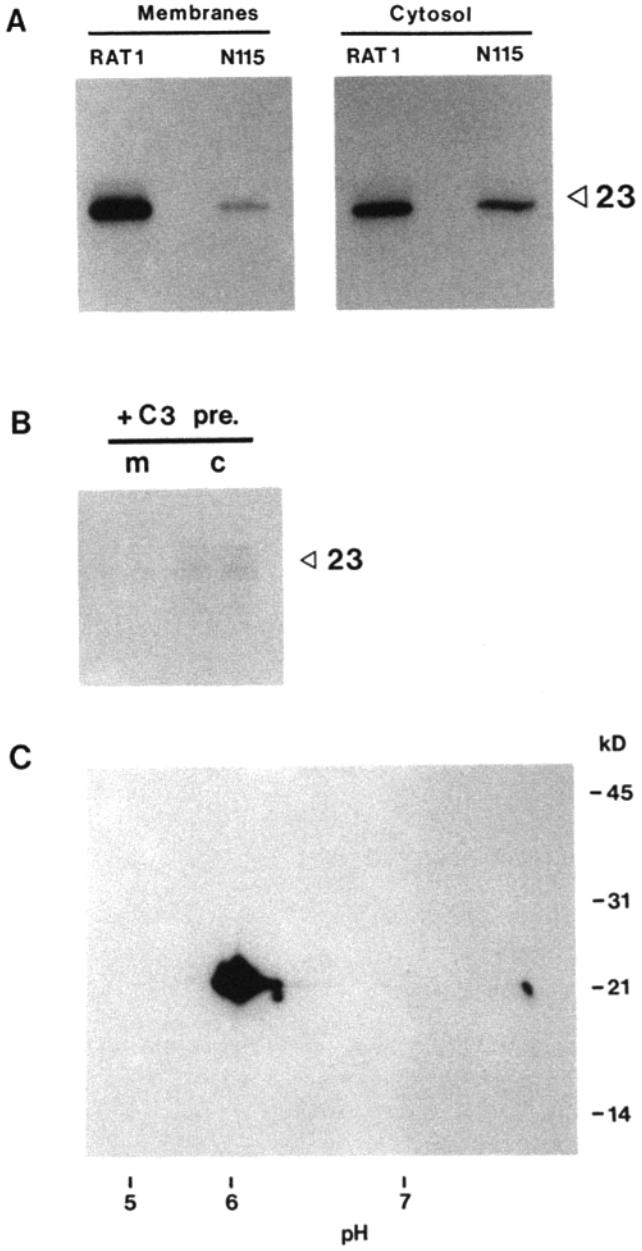

Figure 1. [^{32}P]ADP-ribosylation of 23-kD protein by C3 exoenzyme in Rat-1 and N1E-115 cells. (*A*) Cytosol and crude membranes were ADP-ribosylated using [^{32}P]NAD as described in Materials and Methods. (*B*) In situ ADP-ribosylation in N1E-115 cells by C3 treatment (30 μg/ml; 4-h preincubation) of intact cells. Cells were then homogenized and cytosol and membrane fractions used for ADP ribosylation. (*m*, membranes; *c*, cytosol). (*C*) Two-dimensional polyacrylamide gel analysis of proteins [^{32}P]ADP-ribosylated by C3 in cell lysates from N1E-115 cells. Cell homogenization and ADP-ribosylation of proteins by C3 was carried out as described in Materials and Methods. The reaction mixture was solubilized in sample buffer, separated by IEF (ampholines 3.5–10), and then by SDS-PAGE on 12% acrylamide gels. The small circular spot (~20 kD) at pH 6.3 is also observed in control samples (in the absence of C3) and hence is not a C3 substrate.

of radioactivity were found in the membrane and cytosolic fraction from Rat-1 cells. To identify the C3 substrate(s) in further detail we performed two-dimensional gel analysis of ADP-ribosylated protein extracts from N1E-115 cells (Fig. 1

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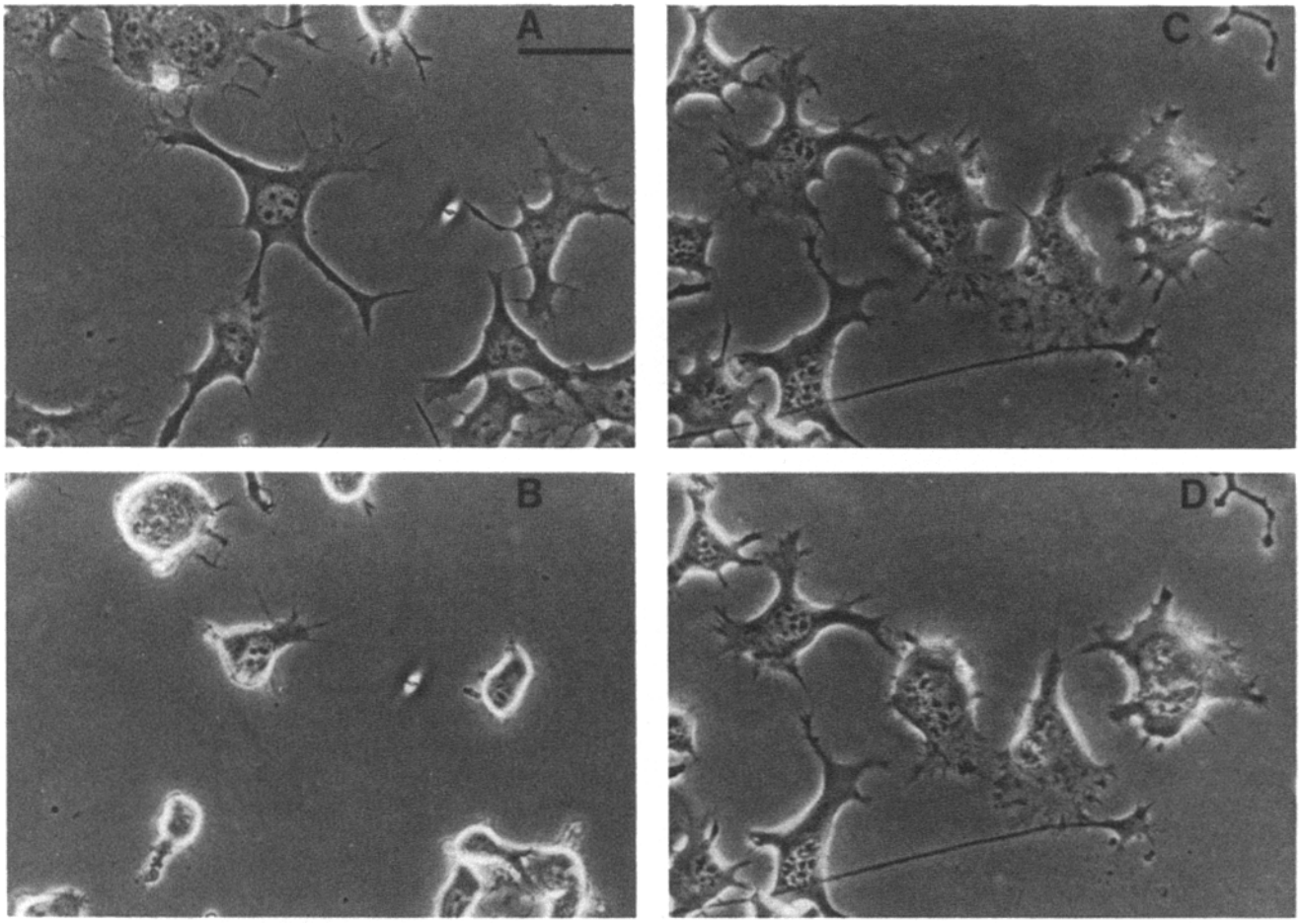

Figure 2. Effect of C3 on agonist-induced shape changes in N1E-115 cells. (*A*) Morphology of N1E-115 cells maintained in serum-free medium for 24 h. (*B*) Response of the cells in *A* to treatment with LPA (1 μM) for 3 min. (*C*) Serum-deprived cells pretreated with C3 (30 μg/ml) for 3 h. (*D*) Response of the cells in *C* to treatment with LPA (1 μM) for 3 min. Note absence of morphological changes in C3-treated cells. Similar results were obtained with NG108-15 cells. Bar, 40 μm.

C). This analysis reveals that the major 21–23 kD C3 substrate migrates between pH 5.9 and 6.2, which identifies this protein as being RhoA (pI 6.1, as opposed to 5.3 for RhoB and 6.8 for RhoC; Yeramian et al., 1987; Nemoto et al., 1992). Overexposure of the film reveals an additional minor C3 substrate, migrating at pH >8 and with an apparent molecular mass of 22 kD (Fig. 1 *C*). This minor spot most likely is the Rho-related Rac protein, which has a pI of 8.5 (Didsbury et al., 1989) and serves only as a very poor substrate for C3 ADP-ribosyltransferase in vitro (Just et al., 1992; Ridley and Hall, 1992); when introduced into living cells, C3 specifically inhibits Rho-dependent, but not Rac-dependent events (Ridley and Hall, 1992). From these results we conclude that the RhoA protein is by far the predominant C3 substrate in N1E-115 cells.

When N1E-115 cells were pretreated with 30 μg/ml C3 for 4 h and then homogenized and fractionated followed by ADP ribosylation, the amount of Rho protein available for subsequent in vitro [^{32}P]ADP-ribosylation by C3 is strongly reduced (Fig. 1 *B*). Thus, RhoA has undergone almost complete ADP-ribosylation in situ during several hours of C3 treatment.

Effect of C3 on Agonist-induced Shape Changes

Addition of LPA, serum (which contains 1–5 μM LPA; Eichholtz et al., 1993), thrombin or TRP (Vu et al., 1990) to serum-deprived N1E-115 or NG108-15 cells causes immediate, dramatic morphological changes (Jalink et al., 1992, 1993; see also Suidan et al., 1992). Within seconds of agonist addition, growth cones collapse, developing neurites retract and flattened cell bodies start to round-up (Fig. 2, *A* and *B*). Rounding up of somata is usually complete within 1 min, while complete retraction of lengthy neurites can last for up to 30 min in the continuous presence of agonist; shrunken neurites often undergo visible degeneration, whereas rounded cell bodies gradually reflatten in the continuous presence of agonist (Jalink et al., 1992, 1993).

Pretreatment of serum-starved N1E-115 cells with C3 for several hours markedly inhibits LPA- and thrombin-induced shape changes (Fig. 2, *C* and *D*) which correlates with in situ ADP-ribosylation of RhoA (Fig. 1 *B*). The effect of C3 is dose and time dependent, with almost complete inhibition observed at 30 μg/ml C3 after a 3-h incubation (Fig. 3). Inhibition was not reversed by washing the C3-treated cells. Of

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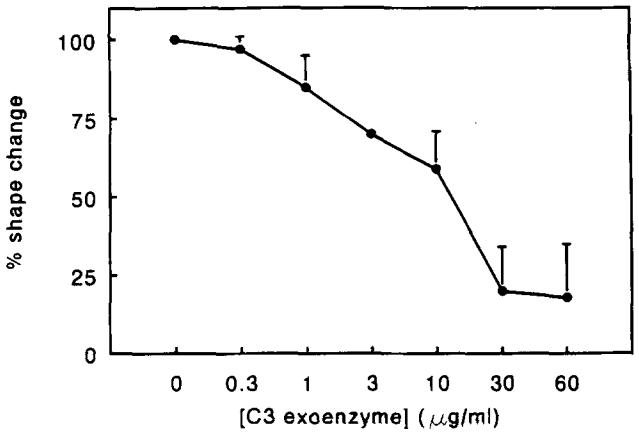

Figure 3. Dose dependence of shape change inhibition by C3 in N1E-115 cells. Cells were treated for 3 h with the indicated concentrations of C3, exposed to LPA for 3 min and the ensuing shape change assessed as described in Materials and Methods.

note, there are no significant differences in cell shape between control cells and cells treated with C3 for 3 h in serum-free medium (cf. Fig. 2, *A* and *C*). This contrasts to the behavior of fibroblasts, which rapidly round up after introduction of C3 (Chardin et al., 1988; Paterson et al., 1990).

Serum and LPA also promote neurite retraction in primary neurons and NGF-treated PC12 cells, respectively (see Introduction for references). Generally, PC12 cells respond more slowly to LPA and serum than N1E-115 cells, with the first signs of neurite retraction being detectable after 5–10 min rather than seconds (Dyer et al., 1992; Tigyi and Miledi, 1992; Jalink, K., unpublished observations). Preincubation of NGF-treated PC12 cells with C3 (30 μg/ml for 3 h) largely inhibits LPA- and serum-induced neurite retraction (not shown), demonstrating that the effects of C3 on agonist-induced neurite retraction is not restricted to N1E-115 cells. From these results we conclude that RhoA function is essential for agonist-induced neurite retraction and cell rounding.

Effects of C3 on the Actin Cytoskeleton

We next examined the effects of C3 on the actin cytoskeleton in serum-starved N1E-115 cells. Like most neuronal cells, N1E-115 and NG108-15 cells do not possess actin stress fibers extending throughout the cytoplasm but instead show f-actin staining in the cell periphery only, particularly in filopodia and growth cones (Jalink and Moolenaar, 1992; Jalink et al., 1993). Fig. 4 shows the distribution of f-actin in control N1E-115 cells, LPA-treated cells, and cells pretreated with 30 μg/ml C3 for 3 h and then challenged with LPA. As shown earlier for thrombin (Jalink and Moolenaar, 1992), LPA triggers the rapid formation of a "contractile ring" of f-actin just underneath the plasma membrane (Fig. 4 *B*). C3-treated cells remain flattened and resist LPA action, but it is seen that they display somewhat reduced f-actin staining in the filopodia and the cell cortex when compared to control cells (cf. Fig. 4, *A* and *C*).

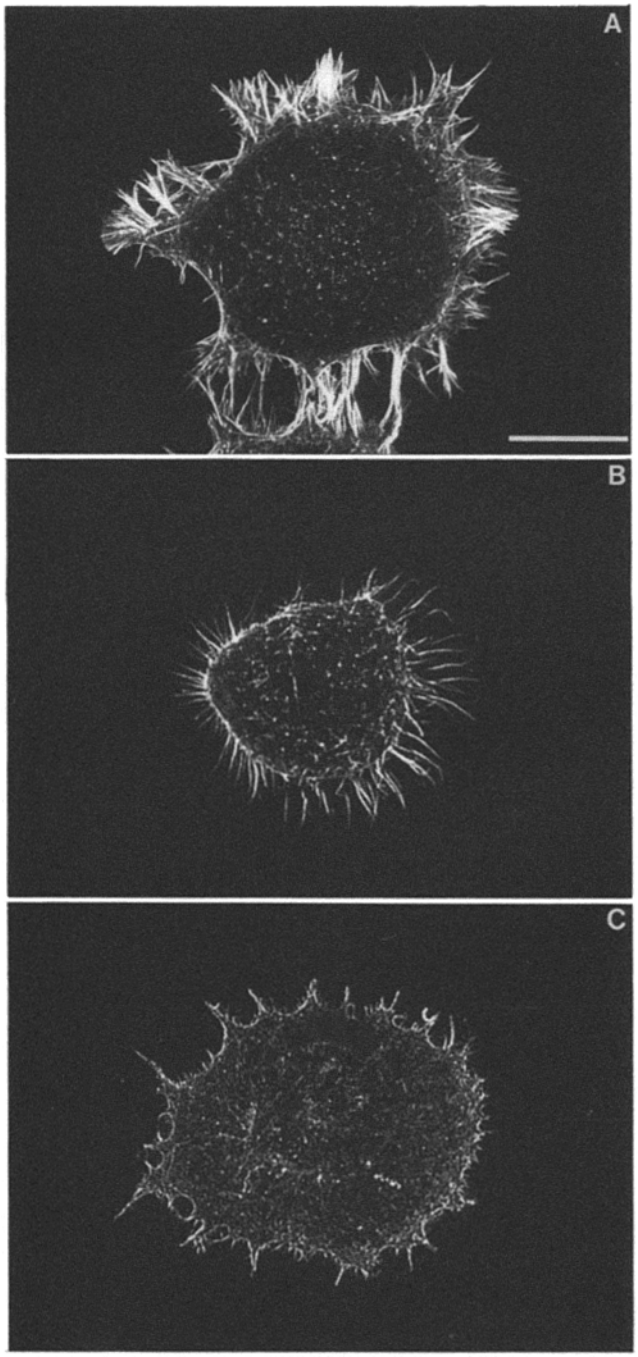

Figure 4. F-actin staining in serum-starved N1E-115 cells. (*A*) Flattened cell deprived from serum for 24 h. (*B*) Rounded, "contracted" cell treated with LPA for 3 min. (*C*) Cell pretreated with C3 for 3 h; subsequent treatment with LPA or thrombin left morphology unaltered (not shown). Note diminished f-actin staining in C3-treated cell as compared to control cell (*A*). Cells were fixed, stained with NBD-phallacidin and viewed by confocal microscopy as described in Materials and Methods. Bar, 20 μm.

C3 Blocks Generation of Actomyosin-based Contractile Forces

We previously suggested that LPA- and thrombin-induced cell rounding and neurite retraction is secondary to contraction of the cortical actin cytoskeleton (Jalink and Moolenaar,

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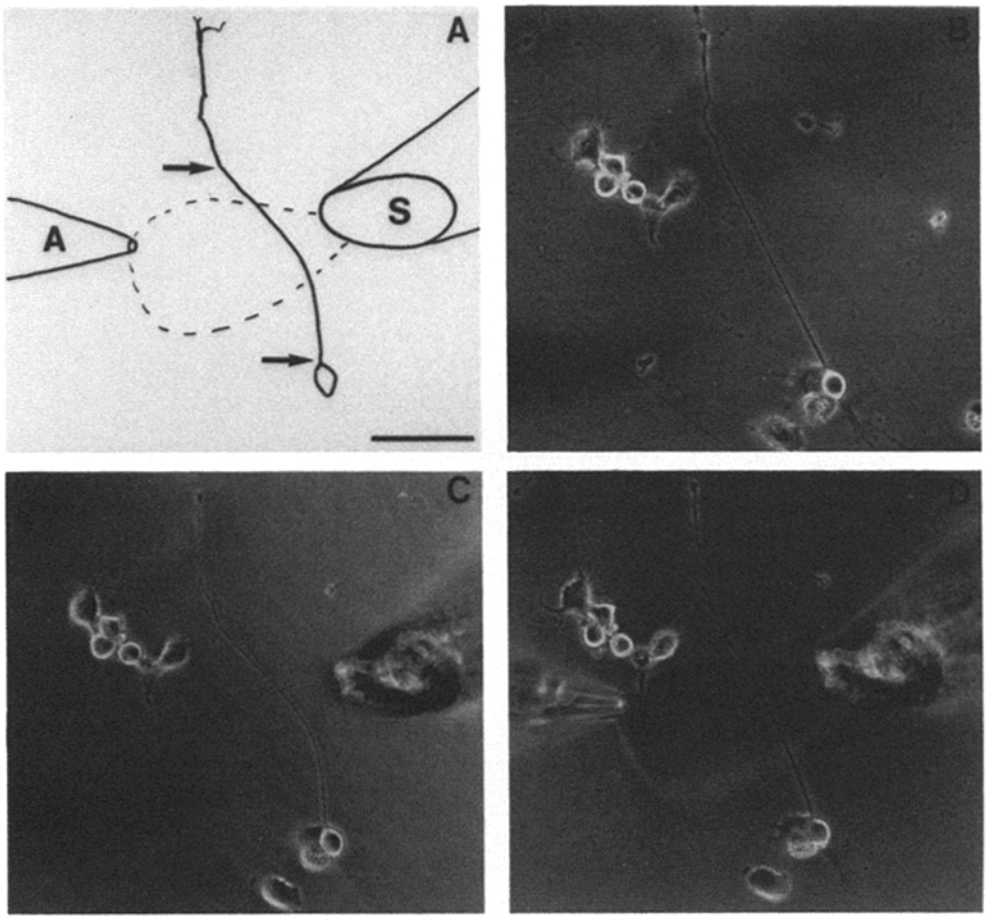

Figure 5. Agonist-induced contraction of detached neurites. (*A*) Schematic illustration of experimental procedure. A flow of test medium was generated by a suction pipette (*S*) positioned close to a loose neurite (detached from the dish between the arrows), causing the neurite to bend. Agonists were applied from a micropipette (*A*). (*B*) Detached neurite under control conditions. (*C*) Neurite bending provoked by flow of medium. (*D*) Contraction (shortening) of bent neurite in response to flow of LPA-containing medium from micropipette. Note pear-shaped contours of LPA-containing medium (also schematically illustrated in *A*). Bar, 40 μm.

1992; Jalink et al., 1993). We sought to directly monitor the build-up of contractile forces in neurites using a new assay (see Materials and Methods). In this assay, the shaft of a long neurite was mechanically detached from the dish and the "loose" part of the neurite was then subjected to a continuous flow of medium in such a manner as to bend it (Fig. 5). Application of medium containing either LPA or TRP causes rapid shortening of the neurite, indicative of contractile force generation (Fig. 5). Contraction of bent neurites was observed within min after application of LPA (5 μM) or TRP (250 μM) in every experiment ($n = 14$ and $n = 10$, respectively). In contrast, after pretreating the cells with C3 (30 μg/ml) for at least 3 h, LPA and TRP were no longer capable of shortening loose neurites (LPA, 8 nonresponders in 10 experiments carried out in five different cultures; $P < 0.002$; TRP, 8 nonresponders in 9 experiments in three different cultures; $P < 0.001$). As expected, cells treated with the actin-disrupting agent cytochalasin D (5 μM) also fail to shorten their neurites in response to agonists.

Actin-based contractility in both muscle and nonmuscle cells is driven by the motor protein myosin. Normally, myosin activity is regulated by myosin light chain kinase (MLCK) and various other protein kinases and phosphatases (reviewed by Sellers, 1991). We used the selective MLCK inhibitor KT5926 (Nakanishi et al., 1990) to test the possible involvement of MLCK in the observed contractions. KT5926 appears to be highly selective for MLCK when compared to other protein kinases tested (Nakanishi et al., 1990). Treatment of serum-deprived N1E-115 cells with KT5926 blocks LPA-and TRP-induced shape changes in a dose-dependent manner (IC_{50} ~0.5 μM; Fig. 6). The concentration of KT5926 required for full inhibition (2–5 μM) is similar to the dose required to suppress MLC phosphorylation in intact platelets (Nakanishi et al., 1990). These results suggest that the RhoA-dependent contractile response to agonists in N1E-115 cells requires MLCK activity.

Lack of Effect of C3 on Agonist-induced Ca^{2+} Mobilization and $p21^{ras}$ Activation

We next tested whether C3 treatment affects other early responses to LPA and thrombin. Both agonists evoke a rapid, phospholipase C–mediated Ca^{2+} transient in their target cells (Jalink et al., 1990, 1993; Jalink and Moolenaar, 1992). In cells pretreated with C3 (30 μg/ml for either 3, 24, or 96 h), there were no detectable differences in shape and kinetics of the LPA- and TRP-induced Ca^{2+} signal under conditions where agonist-induced shape changes were fully blocked (Fig. 7).

Apart from phospholipase C activation and subsequent Ca^{2+} mobilization, LPA, and thrombin also induce rapid activation of $p21^{ras}$ in a pertussis toxin–sensitive manner, at least in fibroblasts (van Corven et al., 1993). In serum-starved N1E-115 cells, basal $p21^{ras}$-GTP levels were found

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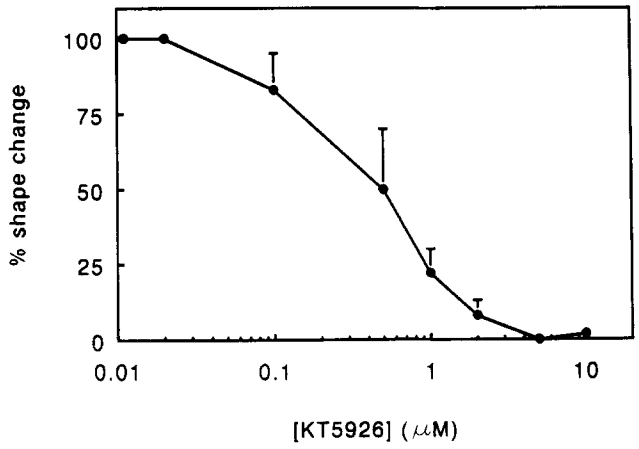

Figure 6. Effect of KT5926 on LPA-induced Shape Changes. N1E-115 cells were treated for 3 h with the indicated concentrations of KT5926 (stock dissolved in DMSO), exposed to LPA (1 μM) for 3 min, and the ensuing shape change assessed as described in Materials and Methods.

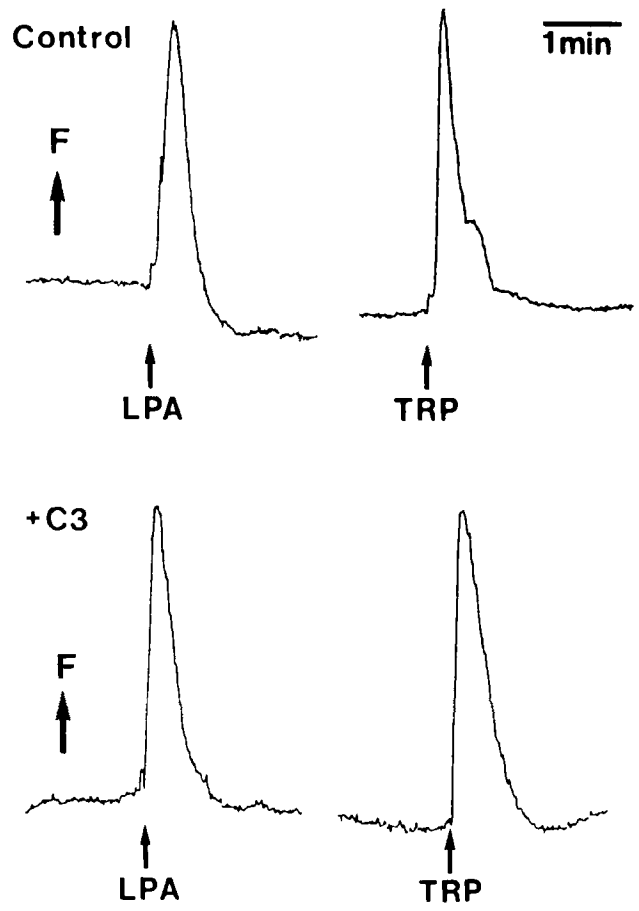

Figure 7. Effects of C3 on agonist-induced Ca^{2+} mobilization. Time course of changes in cytoplasmic free Ca^{2+} induced by LPA and TRP in control and C3-pretreated NE-115 cells, as indicated. (*F*) Ca^{2+}-dependent indo-1 fluorescence. For experimental details see Materials and Methods.

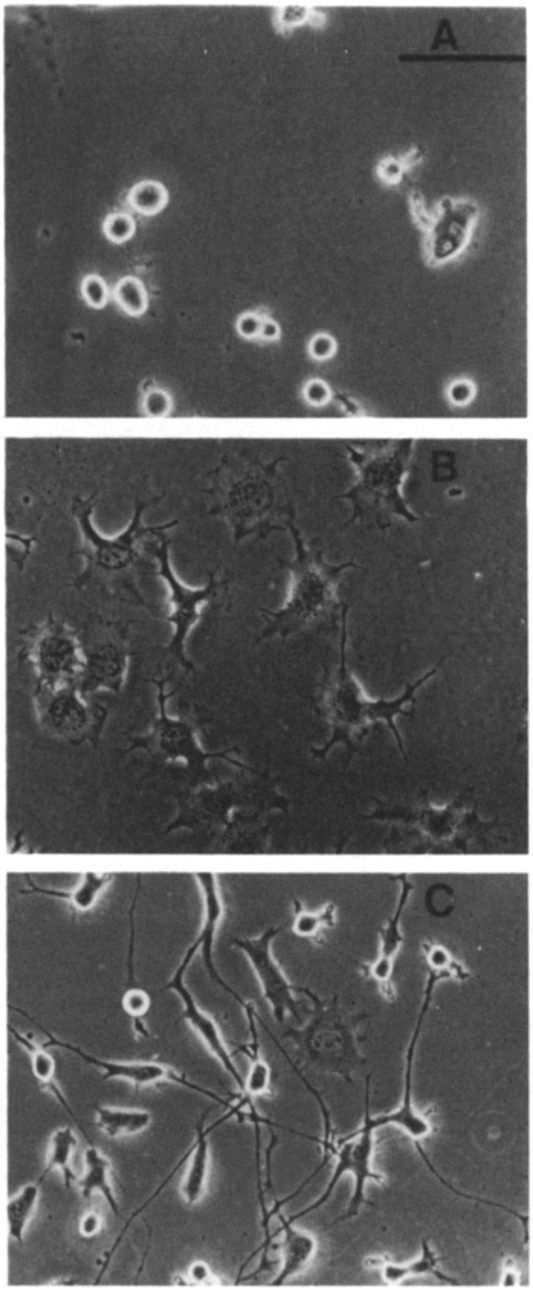

Figure 8. Effects of C3 on the morphology of N1E-115 cells in serum-containing medium. (*A*) Exponentially growing cells in the presence of 10% serum. (*B*) Cells treated with 30 μg/ml C3 for 3 h in serum-containing medium. Note extensive flattening of cell bodies and formation of short neurite-like extensions. (*C*) Neurite formation in cells maintained in 10% serum and 30 μg/ml C3 for 4 d. At that time, C3 was still active since C3-containing medium could be re-used several times to induce neurite outgrowth in other cultures. Bar, 40 μm.

to be as low as in quiescent normal fibroblasts, i.e., around 5% of total $p21^{ras}$ (van Corven et al., 1993; and results not shown). However, for unknown reasons, LPA-induced $p21^{ras}$ activation was much more difficult to detect in N1E-115 cells than in fibroblasts. To examine whether ADP-ribosylation of Rho might affect $p21^{ras}$-GTP accumulation, we therefore turned to Rat-1 fibroblasts. We verified that C3 (30 μg/ml,

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4 h pretreatment) fully inhibits LPA-induced stress fiber formation in these cells, as it does in 3T3 cells (Paterson et al., 1990; Ridley and Hall, 1992; Jalink, J., unpublished observations). We found that in C3-treated cells, LPA stimulates $p21^{ras}$-GTP accumulation to the same extent as in control cells (not shown). It thus appears that Rho function is required neither for agonist-induced Ca^{2+} mobilization nor for $p21^{ras}$ activation.

C3 Treatment Causes Cell Flattening and Neurite Outgrowth in the Presence of Serum

In the presence of 10% FCS, exponentially growing N1E-115 cells are round and refractile with neurite outgrowth being fully suppressed (Fig. 8 *A*; Jalink and Moolenaar, 1992). Given the inhibitory effect of C3 on cell rounding and neurite retraction in serum-starved cells, we reasoned that C3 treatment of rounded, undifferentiated cells maintained in 10% FCS might induce cell flattening and morphological differentiation (i.e., the "serum-starved" phenotype). Indeed, cells treated for a few hours with C3 (30 μg/ml) in the presence of FCS become progressively more flattened, very similar to the phenotype induced by serum removal (Fig. 8 *B*; cf. Fig. 2 *A*). Maximal cell flattening was observed after 24 h using 30 μg/ml C3. Cell flattening was also induced by the MLCK inhibitor KT5926 in a dose- and time-dependent manner (EC_{50} ~0.5 μM at 24-h pretreatment), suggesting that the phosphorylation state of myosin light chain is critical for maintaining the rounded, 'contracted' cell shape observed in serum-containing medium (not shown).

Prolonged C3 treatment of N1E-115 cells for several days in the continuous presence of 10% FCS causes prominent neurite outgrowth accompanied by growth arrest (Fig. 8 *C*), again reminiscent of the differentiated phenotype induced by long-term serum starvation. When C3-differentiated N1E-115 cells are exposed to LPA or thrombin, no shape changes were detectable even when monitored over a period of several hours (not shown). Treatment of PC12 cells with C3 for several days also results in neurite outgrowth and growth arrest (Nishiki et al., 1990; Jalink, K., unpublished observations); we found that the growth cones and neurites of C3-differentiated PC12 cells were completely resistant to LPA (not shown).

Discussion

The mechanisms by which neural cells regulate their complex morphology are poorly understood. Molecules as diverse as extracellular matrix components, growth factors, neurotransmitters, bioactive phospholipids, and secreted proteases, as well as their inhibitors, can exert dramatic effects on neural architecture, ranging from stimulation of neurite outgrowth to induction of growth cone collapse, neurite retraction, and even neurodegeneration. Rapid withdrawal of developing neurites and their growth cones evoked by extracellular molecules is fundamental to nervous system development and neural plasticity. Furthermore, reversal and inhibition of neurite outgrowth is also of considerable clinical relevance: for example, axons in the central nervous system fail to regenerate after traumatic injury and this failure might well be due to inhibitory factors formed at the sites of injury. Several studies point to an important role for thrombin and LPA in provoking neurite retraction and suppressing neural differentiation (Monard, 1988; Jalink and Moolenaar, 1992; Suidan et al., 1992; Tigyi and Miledi, 1992; Jalink et al., 1993, and references therein). Both thrombin and LPA are rapidly produced during injury and blood clotting, but they may also play a role in normal nervous system functioning. In cultures of primary neurons and neural cell lines, thrombin, LPA, and serum (which contains LPA) cause rapid retraction of developing neurites and, at least in neuroblastoma cells, transient rounding of the cell soma. Thrombin and LPA act through their cognate G protein-coupled receptors but the ensuing morphological effects appear to be independent of known second messenger systems (Jalink and Moolenaar, 1992; Suidan et al., 1992; Jalink et al., 1993).

In this study we have used recombinant bacterial C3 exoenzyme to explore the possible role of the Rho small GTP-binding protein in the action of LPA and thrombin. The C3 ADP-ribosyltransferase is very specific for Rho proteins and is at least two orders of magnitude less active on the closely related CDC42 and Rac proteins (Just et al., 1991; Ridley and Hall, 1992). We show that C3 causes in situ ADP-ribosylation of the RhoA protein and thereby inhibits thrombin- and LPA-induced neurite retraction and soma rounding in serum-starved N1E-115 cells; moreover, we found that C3 treatment blocks LPA-induced neurite retraction in PC12 cells. In particular, our analysis reveals that RhoA function is essential for the generation of contractile, actomyosin-based forces in the cortical cytoskeleton. Conversely, ADP-ribosylation of Rho in serum-exposed rounded cells causes cell flattening, probably as a result of reduced cytoskeletal tension, followed by prominent neurite outgrowth and growth arrest, very similar to what is observed after long-term serum starvation of N1E-115 cells. These findings suggest that RhoA functions in a receptor-linked signal transduction cascade that regulates actomyosin contractility which, in turn, controls both the retraction and outgrowth of neurites.

A signaling role for RhoA in cytoskeletal organization was recently put forward by Hall and co-workers who showed that in serum-starved 3T3 cells, ADP-ribosylated Rho inhibits serum- and LPA-induced formation of actin stress fibers and focal adhesions, whilst the active GTP-bound form of RhoA promotes actin stress fiber formation (Paterson et al., 1990; Ridley and Hall, 1992). Although ADP-ribosylation of Rho causes flattened fibroblasts to round up with loss of attachment (Chardin et al., 1989; Paterson et al., 1990), just opposite to what is observed in N1E-115 cells (Figs. 2 and 8), our results are not necessarily incompatible with the fibroblast model, in which LPA triggers Rho-dependent formation of stress fibers. It is known that actin stress fibers are under a state of isometric contraction and that this contraction contributes to their formation (Burridge, 1981); moreover, serum stimulation of quiescent fibroblasts causes stress fibers to contract (Giuliano et al., 1992). On the other hand, the present results are not readily compatible with LPA promoting cell–substratum adhesion, as occurs in serum-starved fibroblasts due to Rho-dependent focal adhesion assembly (Ridley and Hall, 1992).

In platelets, ADP-ribosylation of RhoA inhibits thrombin-induced aggregation (Morii et al., 1992); and in a B cell line, ADP-ribosylation of RhoA prevents phorbol ester-induced cell aggregation, apparently by affecting the process of integrin "activation" rather than interfering with actin polymerization (Tominaga et al., 1993). In the latter cell types, RhoA

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was suggested to function in the phospholipase C–protein kinase C–signaling pathway. In neuronal cells, however, agonist-induced neurite retraction and cell rounding are causally unrelated to the phospholipase C/Ca^{2+}/protein kinase C cascade (Jalink and Moolenaar, 1992; Jalink et al., 1993), which strongly argues against a role for RhoA in this classic second messenger system. Instead, the available evidence supports a model in which LPA and thrombin trigger at least three separate signaling pathways involving activation of phospholipase C, $p21^{ras}$ and $p21^{rho}$, respectively. Cross-talk between Ras- and Rho-dependent signaling pathways has recently been proposed (Settleman et al., 1992).

How may RhoA function in neurite retraction and, when ADP-ribosylated, promote neurite outgrowth? RhoA-dependent contractility is abolished by a selective inhibitor of MLCK, KT5926, suggesting a critical role for the phosphorylation state of myosin light chain. Interestingly, increased myosin phosphorylation accompanies the contraction of actin stress fibers in serum-stimulated fibroblasts (Giuliano et al., 1992). In astrocytes, a decrease in myosin light chain phosphorylation (by KT5926) has been suggested to cause destabilization of the cortical actin cytoskeleton which, in turn, promotes process outgrowth (Baorto et al., 1992). But perhaps the most appealing analogy emerges from smooth muscle physiology. In smooth muscle, several agonists can increase G-protein–mediated MLC phosphorylation and contractile force at constant cytosolic [Ca^{2+}] (Kitazawa et al., 1991). ADP-ribosylated RhoA was reported to block GTP-induced enhancement of smooth muscle contraction and active RhoA.GTP could overcome this inhibition (Hirata et al., 1992). Taken together, the results suggest that RhoA and its putative downstream effector(s) somehow regulate receptor-linked actomyosin contractility independently of prior Ca^{2+}-mobilization, possibly by altering the balance between MLC kinase and phosphatase activity (cf. Kitazawa et al., 1991), and thereby direct neurite behavior. Our preliminary results indicate that LPA-induced neuronal shape changes are accompanied by enhanced phosphorylation of a 20-kD protein with a pI close to that of the 20-kD myosin light chain (Eichholtz, T., and W. H. Moolenaar, data not shown). Thus, the available evidence supports a model in which basal Rho activity is necessary to maintain actomyosin-based neurite tension and cell shape; increased Rho activity (Rho-GTP accumulation) then triggers neurite contraction, whereas inactive (ADP-ribosylated) Rho somehow shuts off the contractile function of the cortical actomyosin system and thereby promotes neurite outgrowth. Direct biochemical studies should help to unravel the Rho-dependent events that control actomyosin contractility in neural cells.

Since RhoA in N1E-115 cells apparently does not function in one of the classic signal transduction pathways, it is tempting to speculate that RhoA is activated by LPA and thrombin in a manner similar to that proposed for $p21^{ras}$ activation, i.e., through a putative, intermediate tyrosine kinase (van Corven et al., 1993). Indeed, not only are the morphological actions of LPA and thrombin blocked by protein tyrosine kinase inhibitors, neurite retraction and cell rounding are also accompanied by a rapid increase in $p60^{src}$ tyrosine kinase activity (Jalink and Moolenaar, 1992; Jalink et al., 1993). On the other hand, recent results suggest that in 3T3 cells Rho is essential to couple the LPA receptor to enhanced protein tyrosine kinase activity (Kumagai et al., 1993). The putative link between tyrosine kinase activation, RhoA function, and actomyosin contractility should be explored in further biochemical studies. Although a signaling role for RhoA in linking the receptors for LPA and thrombin to the actomyosin system is supported by the available data, it should be emphasized that our results do not eliminate the formal possibility that ADP-ribosylated Rho causes subtle modifications in the actomyosin system and thereby prevents it from proper functioning. Determination of the relative levels of guanine nucleotides bound to RhoA and microinjection of active RhoA.GTP will help to distinguish between these possibilities.

In conclusion, the present results add a new aspect of RhoA function to the rapidly expanding list of Rho-dependent cytoskeletal events in that we have identified RhoA as an essential component in the control of actin-based forces and neuronal cell shape by LPA and thrombin. How the receptors for LPA and thrombin couple to RhoA-dependent actomyosin contractility and thereby regulate neural architecture is a major challenge for future experiments.

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