

# Lysophosphatidic Acid Influences the Morphology and Motility of Young, Postmitotic Cortical Neurons

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Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that produces process retraction and cell rounding through its cognate receptors in neuroblastoma cell lines. Although the expression profile of LPA receptors in developing brains suggests a role for LPA in central nervous system (CNS) development, how LPA influences the morphology of postmitotic CNS neurons remains to be determined. Here we have investigated the effects of exogenous LPA on the morphology of young, postmitotic neurons in primary culture. When treated with LPA, these neurons responded by not only retracting processes but also producing retraction fiber "caps" characterized by fine actin filaments emanating from a dense core. Retraction fiber caps gradually vanished due to the outward spread of regrowing membranes along the fibers, suggesting a role for caps as scaffolds for regrowth of retracted processes. Furthermore, LPA also affects neuronal migration *in vitro* and *in vivo*. Taken together, these results implicate LPA as an extracellular lipid signal affecting process outgrowth and migration of early postmitotic neurons during development.

## INTRODUCTION

Lysophosphatidic acid (LPA), a simple phospholipid, has been shown to elicit diverse cellular responses in

many types of cells (Moolenaar, 1995; Moolenaar *et al.*, 1997; Moolenaar, 1999). These cellular responses are mediated through specific cell-surface G-protein-coupled receptors. A first LPA receptor gene, *lpa<sub>1</sub>* (formerly *ventricular zone gene-1*), was identified from immortalized cortical neuroblast cells (Hecht *et al.*, 1996; Chun, 1999; Chun *et al.*, 1999). Two other LPA receptor genes, *lpa<sub>2</sub>/endothelial differentiation gene-4 (edg-4)* and *lpa<sub>3</sub>/edg-7*, have since been cloned and characterized (An *et al.*, 1998; Bando *et al.*, 1999; Chun, 1999; Fukushima *et al.*, 2001). Activation of LPA<sub>1</sub> and LPA<sub>2</sub>, but not LPA<sub>3</sub> stimulates the small GTPase Rho, followed by activation of Rho-associated kinase (e.g., ROCK) (Fukushima *et al.*, 1998; Ishii *et al.*, 2000; Weiner *et al.*, 2001). This sequential activation, in turn, produces actin rearrangements, leading to the formation of stress fibers consisting of polymerized actin (F-actin) in fibroblasts (Ridley and Hall, 1992) or process retraction in neuroblastoma cells, resulting in complete cell rounding (Jalink *et al.*, 1994).

*lpa<sub>1</sub>* expression is enriched in the ventricular zone (vz), a neurogenic region of the embryonic cerebral cortex (Hecht *et al.*, 1996; Weiner *et al.*, 1998). Although spatial expression of *lpa<sub>2</sub>* and *lpa<sub>3</sub>* in brain has not yet been determined, RT-PCR and Northern blot analyses demonstrate that *lpa<sub>2</sub>* is expressed during earlier development [embryonic day 12 (E12) to postnatal day 7 (P7)], while *lpa<sub>3</sub>* is expressed during later development (E18 to P7) (Contos and Chun, 2000; Contos *et al.*, 2000b). This temporal pattern suggests a role for LPA signaling in neuronal development, including neuro-

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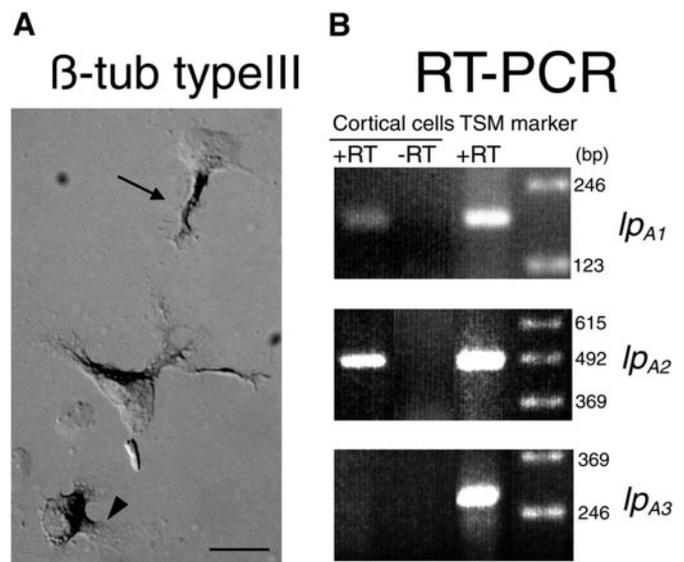
genesis, neuronal differentiation, and morphological changes of neuroblasts and postmitotic neurons.

Indeed, our recent reports have demonstrated that LPA signaling is involved in the regulation of neuroblast morphology during development (Contos *et al.*, 2000a; Fukushima *et al.*, 2000). Neuroblasts undergo actin rearrangement to alter their morphology between fusiform and rounded shapes, and LPA has been shown to stimulate neuroblast rounding (Fukushima *et al.*, 2000). On the other hand, the effects of LPA on newly postmitotic neurons have not yet been determined. Postmitotic neurons are generated in the vz and migrate superficially to form the cortical plate, where they elaborate their complex neuronal processes (both axons and then dendrites) as they differentiate during embryonic and early postnatal periods. The actin cytoskeleton plays important roles in the formation and elongation of these processes as well as in neuronal migration (Jacobson, 1991) and is regulated by many extracellular signals (Culotti and Merz, 1998; Brose and Tessier-Lavigne, 2000). In view of its effects on neuronal cell lines and the expression of LPA receptors in the embryonic brain, LPA could affect the cell morphology, process outgrowth, and/or migration of young, postmitotic neurons. To address this issue, we have examined the effects of LPA in three types of primary cultures derived from the cerebral cortex.

## RESULTS

### *Effects of LPA on the Morphology of Single Cortical Cells*

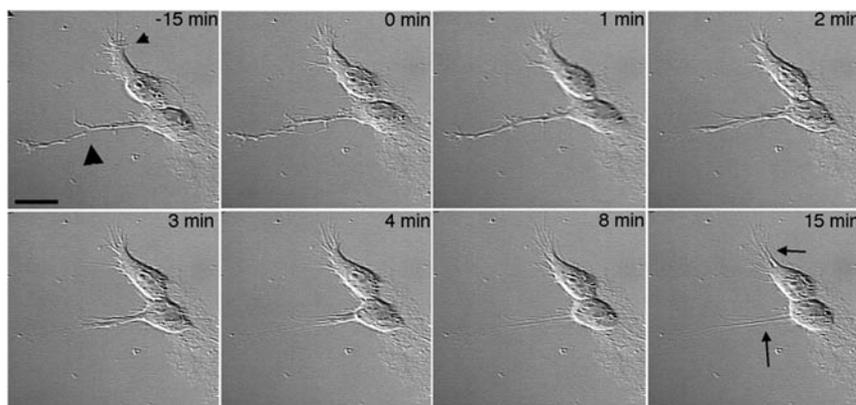
To examine the cellular effects of LPA on newly postmitotic cortical neurons, we employed dissociated cell cultures consisting of single cells from cerebral cortices of E12 mice. At this age *in vivo*, the vast majority of cells have the capacity to proliferate. However, when cultured under serum-free conditions, most single cells of neuronal lineage have been shown to become postmitotic and commence differentiation (Temple and Davis, 1994; Ghosh and Greenberg, 1995; Fukushima *et al.*, 2000). At 24 h after plating in our culture, cortical cells showed short processes and/or extending membranes (lamella) (Figs. 1A and 2), as documented in previous reports (Temple and Davis, 1994; Ghosh and Greenberg, 1995; de Lima *et al.*, 1997). When these cells were immunostained for  $\beta$ -tubulin type III, a marker for early postmitotic neurons (Menezes and Luskin, 1994), immunoreactivity was observed in the majority of viable cells (determined by F-actin staining, see Ex-



**FIG. 1.** Expression of LPA receptor genes in dissociated cortical cultures from E12 mice. (A) E12 cortical cells were cultured in serum-free medium for 24 h, fixed, and stained for  $\beta$ -tubulin type III. Arrow, process; arrowhead, extending membrane. (B) RT-PCR analyses for LPA receptor gene expression. Total RNAs from cultured cortical cells or a TSM cell line (Chun and Jaenisch, 1996) were subjected to RT-PCR to detect *lpa<sub>1</sub>*, *lpa<sub>2</sub>*, or *lpa<sub>3</sub>* (+RT); -RT, negative control that was not reverse transcribed. Cortical cultures employed in this study consist of single cells expressing  $\beta$ -tubulin type III, *lpa<sub>1</sub>*, and *lpa<sub>2</sub>*. Bar = 10  $\mu$ m.

perimental Methods and Table 1) both in cell bodies and in short processes (Fig. 1A, arrow). By contrast, nonneuronal populations such as meningeal cells were easily distinguishable from neuronal cells by their flat, diagonal cell morphology and F-actin staining pattern and were found to be a minor population ( $2.1 \pm 0.3\%$  of total viable cells). Another minor population ( $5.3 \pm 1.1\%$  of total cells) was radial glial cells, as determined by immunostaining for the glutamate transporter (GLAST) (Shibata *et al.*, 1997). Because of their distinct morphology, these cells were excluded from the following quantitative analyses. To detect proliferating cells, bromodeoxyuridine (BrdU) was added during the last 6 h of culture. BrdU immunostaining revealed that less than 10% cells were BrdU-positive at 24 h after culturing (Table 1). These results demonstrated that the employed cultures, at 24 h *in vitro*, consisted of a majority (>90%) of young, postmitotic cortical neurons that exited the cell cycle.

LPA receptor gene expression in these cultures was ascertained by RT-PCR analyses using specific primers for the three known LPA receptor genes, *lpa<sub>1</sub>*, *lpa<sub>2</sub>*, and *lpa<sub>3</sub>*. Detectable expression levels for *lpa<sub>1</sub>* and *lpa<sub>2</sub>*, but



**FIG. 2.** Time-lapse recording of differential interference contrast (DIC) images of single cortical cells. E12 cortical cells were cultured in serum-free medium for 24 h, and DIC images were collected in vehicle treatment (–15 to 0 min) and the subsequent LPA treatment (0.5  $\mu$ M, 0 to 15 min). LPA induces retraction of membranes or processes, resulting in retraction fiber caps. Large arrowhead, process; small arrowheads, extending membrane; arrows, retraction fibers. Bar = 10  $\mu$ m.

not *lpa*<sub>3</sub> (Fig. 1B), were observed 24 h after culturing. This was consistent with previous results from Northern blot analysis, which showed the expression of *lpa*<sub>1</sub> and *lpa*<sub>2</sub> in early brain development (Contos *et al.*, 2000b).

To examine the effects of LPA on the morphology of postmitotic cortical cells, individual cells were monitored by time-lapse videomicroscopy during exposure to LPA. An example of two LPA-responsive cells, one with a single process and the other with an extending membrane (lamella), is shown in Fig. 2. In vehicle medium [containing 0.1% fatty-acid-free bovine serum albumin (FAFBSA) without LPA], no remarkable changes in cell morphology were observed (Fig. 2, –15 to 0 min). When the solution was replaced with medium containing 0.5  $\mu$ M LPA, these cells showed rapid morphological changes, including retraction of growing processes

or membranes and rounding of cell bodies. Surprisingly, some portions of process or membrane remained attached to the substrate, producing retraction fiber caps characterized by fine spikes accumulating in one or two areas of the cells (Fig. 2, 0 to 15 min; Fig. 3C).

#### Characterization of Retraction Fiber Caps

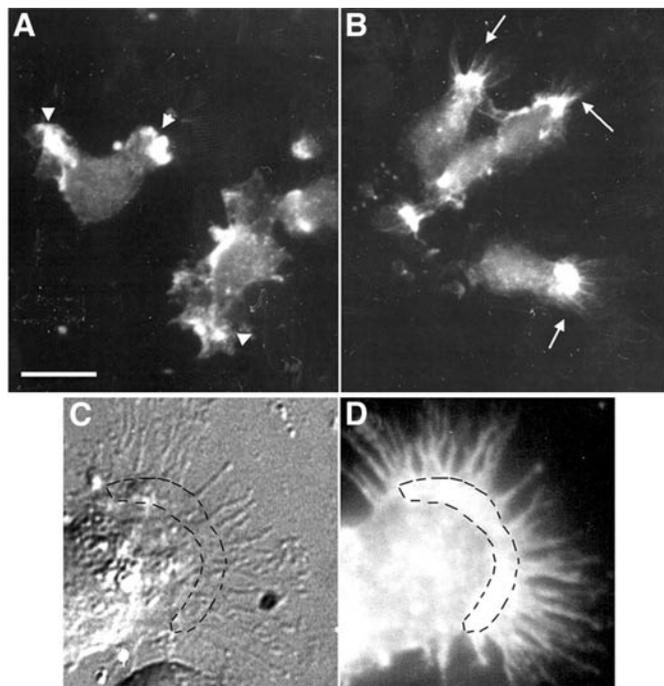
When cells were pretreated with cytochalasin D (1  $\mu$ M for 5 min), an actin polymerization inhibitor, both LPA-induced process/membrane retraction and cap formation were blocked (data not shown), suggesting the involvement of actin polymerization in these morphological changes. Therefore, we examined distribution of F-actin in young, postmitotic neurons using TRITC-labeled phalloidin. Fluorescent labeling was observed throughout control cells, with particularly enriched F-actin staining within lamellipodia (the edges of lamellae; Fig. 3A, arrowheads). In LPA-treated cells with caps, F-actin was not only present in the fine fibers (Fig. 3B) but also strongly concentrated at the base of the caps (Figs. 3C and 3D). This result suggested that an enhancement of actin polymerization at the base of the caps accompanied process/membrane retraction.

LPA-induced cap formation was dose-dependent, with an EC<sub>50</sub> of 30 nM (Fig. 4A), comparable to those observed for process retraction in immortalized neuroblast TSM or TR cells or primary neuroblasts endogenously expressing LPA receptors (Hecht *et al.*, 1996; Fukushima *et al.*, 2000; Ishii *et al.*, 2000). The percentage of cells with caps peaked 15 min after LPA exposure and gradually decreased until 2 h even in the presence

**TABLE 1**  
Characterization of Single Dissociated Cortical Cultures

	Percentage population (mean $\pm$ SEM)
$\beta$ -Tubulin type III	92.4 $\pm$ 5.7
BrdU	9.6 $\pm$ 0.6

*Note.* E12 cortical cells were cultured under serum-free conditions and fixed at 24 h after plating. BrdU was pulsed between 18 and 24 h after plating. Cells were double immunostained for  $\beta$ -tubulin type III or BrdU and F-actin, and the percentage of positive cells to total viable cells (determined by F-actin staining, see Experimental Methods) was determined ( $n = 6$ ).

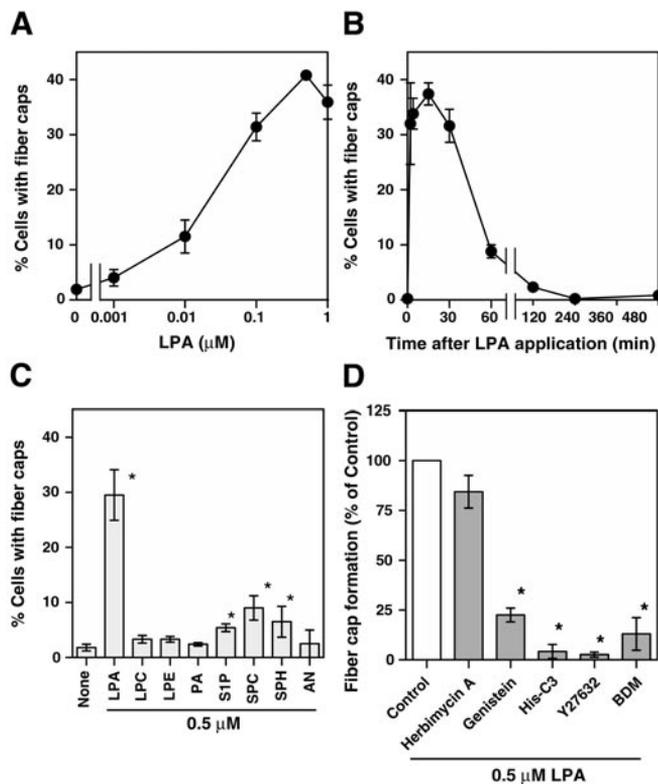


**FIG. 3.** Retraction fiber structures stained for F-actin. E12 cortical cells were cultured for 24 h, treated without (A) or with (B–D) 0.5  $\mu$ M LPA for 15 min, and fixed. Cells were then stained for F-actin (A, B, and D) and photographed. (C) Differential interference image of the same cell in D. Arrowhead, lamella; arrows, retraction fibers; broken lines indicate the base of the retraction fiber cap. F-Actin is present in the retraction fibers and more enriched at the base of this cap. Bar = 10  $\mu$ m in A and B and 4  $\mu$ m in C and D.

of LPA (Fig. 4B). In addition, a second LPA exposure 2 h after the first failed to fully induce cap formation (data not shown). These results demonstrated that process retraction mechanisms were desensitized 15 min after LPA exposure and that desensitization continued for at least 2 h, during which time the caps disappeared. This transient response contrasts with those observed for actin polymerization reactions in fibroblasts or Schwann cells (Weiner *et al.*, 2001) (data not shown). In these cells, thick stress fibers or circular bundles of actin filaments, respectively, are produced in response to LPA and maintained for 3 to 6 h after LPA exposure.

Cap formation was not induced by other closely related lipids, including lysophosphatidyl choline (LPC), lysophosphatidyl ethanolamine (LPE), and phosphatidic acid (PA; Fig. 4C). On the other hand, sphingosine-based signaling lipids showed detectable albeit weak effects on cap formation (Fig. 4C). These included sphingosine 1-phosphate (S1P), sphingosylphosphoryl choline (SPC), and sphingosine (SPH). No activity was detected for the cannabinoid lipid agonist, anandamide.

LPA induces actin rearrangement in neuroblastoma through activation of both tyrosine kinase and Rho signaling (Moolenaar, 1995; Moolenaar *et al.*, 1997). The involvement of these pathways in LPA-induced cap formation was examined (Fig. 4D). Pretreatment of cells



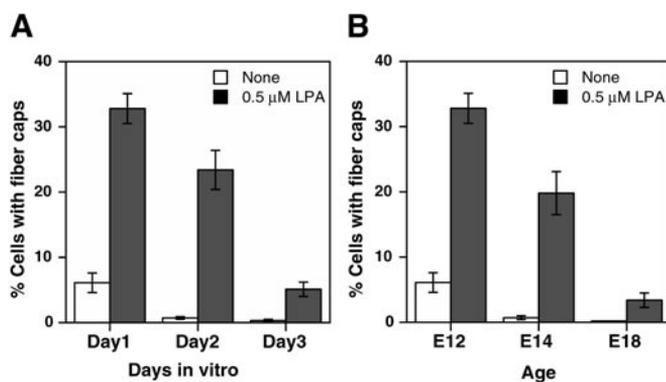
**FIG. 4.** Effects of LPA on fiber cap formation. (A) Dose–response relationship of LPA to fiber cap formation. E12 cortical cells were cultured for 24 h in serum-free medium and subsequently treated with varying concentrations of LPA for 15 min. Cells were stained for F-actin and the percentage of cap-bearing cells to total viable cells (F-actin-positive cells) was determined. Data are means  $\pm$  SEM ( $n = 6$ ). (B) Time course for LPA-induced fiber cap formation. Cortical cells cultured for 18 h were treated with 0.5  $\mu$ M LPA and fixed at the indicated time. Data were determined as described above ( $n = 6$ ). (C) Effects of structurally related lipids on fiber cap formation. Cortical cells cultured for 24 h were treated with 0.5  $\mu$ M lipids for 15 min and fixed. Data were determined as described above ( $n = 6$ ). \* $P < 0.05$  vs none. (D) Effects of specific pharmacological inhibitors on LPA-induced fiber cap formation. Cortical cells were cultured for 24 h, pretreated with various inhibitors (5 min except for his-C3), and treated with 0.5  $\mu$ M LPA for 15 min. His-C3 was added as cells were plated. The used concentrations were 1  $\mu$ M for herbimycin A, 50  $\mu$ M for genistein, 30  $\mu$ g/ml for C3, 2  $\mu$ M for Y-27632, and 20 mM for BDM. Data were determined as described above and represent the percentage of control (=LPA alone) ( $n = 3$ –6). \* $P < 0.05$  control vs treatment. LPA-induced cap formation is dose-dependent, transient, and blocked by inhibitors of tyrosine kinase, Rho, and myosin pathways.

with genistein, a broad tyrosine kinase inhibitor, significantly inhibited LPA-induced cap formation. By contrast, herbimycin A, a tyrosine kinase inhibitor that potently inhibits SRC kinase (Uehara *et al.*, 1986; Fukazawa *et al.*, 1990), failed to inhibit cap formation. When the cells were pretreated with C3 exoenzyme (his-C3; Fukushima *et al.*, 1998), which inactivates Rho, the effect of LPA on cap formation was nearly abolished. Cells pretreated with Y-27632, a ROCK inhibitor (Uehata *et al.*, 1997; Hirose *et al.*, 1998), also failed to form caps in response to LPA. We further tested BDM, a myosin ATPase inhibitor (Cramer and Mitchison, 1995), since myosin activation is important for actin polymerization and cell contractility downstream of Rho (Chrzanowska-Wodnicka and Burridge, 1996). Pretreatment of cells with this compound also inhibited LPA-induced cap formation. Taken together, these results demonstrated that tyrosine kinase(s), Rho, and myosin mediated LPA-induced cap formation in young postmitotic neurons.

### Characterization of LPA-Responsive Cells

We next examined the relationship between LPA-induced cap formation and neuronal maturation. When cortical cultures from E12 mice were grown for 1 to 3 days *in vitro*, the population positive for MAP2 (MAP2a and MAP2b), a phenotypic marker for mature neurons, increased (53.7% at day 1, 76.6% at day 2, and 92.4% at day 3). Although LPA-induced caps were observed in all cultures examined (Fig. 5A), the percentage of LPA responsive cells decreased with time. We employed cultures from different ages (E12, E14, and E18) to examine LPA-induced cap formation. While the MAP2 positive population increased with the age of these cultures (data not shown), there was a concomitant reduction in the population of LPA responsive cells with age (Fig. 5B). These results suggested that the population of LPA responsive cells decreased as neuronal maturation progressed. However, because both MAP2-positive and -negative cells responded equally to LPA with cap formation (34.9 and 40.7%, respectively), there was likely no specific correlation between LPA-induced cap formation and phenotypic maturation (i.e., MAP2 expression). Rather, morphological maturation (e.g., process length) might affect the ability of cells to respond to LPA.

No LPA-induced caps were observed in GLAST-positive cells. Likewise, when cultures were treated with BrdU during the last 6 h of culture and exposed to LPA, only 4.7% of BrdU-positive cells showed fiber cap formation. By contrast, 52.5% of BrdU-negative cells produced caps in response to LPA. Therefore, LPA-induced

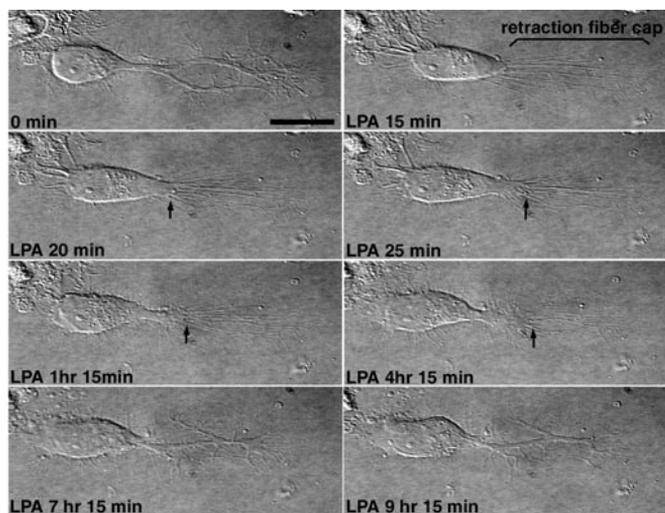


**FIG. 5.** Relationship between LPA-induced retraction fiber caps and neuronal maturation. (A) Cap formation in cultures grown for different periods. E12 cortical cells were cultured for 1, 2, or 3 days and treated without or with 0.5  $\mu$ M LPA for 15 min. Data were determined as described in the legend to Fig. 4 and represent means  $\pm$  SEM ( $n = 6$ ). (B) Cap formation in cultures prepared from different aged mice. E12, E14, or E18 cortical cells were cultured for 24 h and treated without or with 0.5  $\mu$ M LPA. Data were determined as described above ( $n = 6$ ). The LPA responsive population is reduced with maturation.

cap formation was likely to be limited to young, postmitotic neurons, but not to proliferative-dissociated cells.

### Reoutgrowth of Processes after Cap Formation

Only a few reports describe a role of retraction fibers. One well-documented example is retraction fibers observed in epithelial cells undergoing cell division (Mitchison, 1992; Cramer and Mitchison, 1993). After cytokinesis, these cells extend plasma membranes outward along these fibers and recover their interphase morphologies. Thus, these fibers have been suggested to be involved in reestablishment of cell shape (Cramer and Mitchison, 1993). Because LPA-induced caps vanished with time in cortical neurons (Fig. 4B), it was possible that membranes regrew toward the tops of the fibers, as in epithelial cells. To address this issue, cortical cells were treated with LPA and monitored over 9 h (Fig. 6). At 15 min after LPA exposure, long retraction fibers were produced. At 20 min, membranes at the base of the fibers appeared to initiate regrowth toward the top of the fibers. Over time, these membranes slowly continued to grow, while the fiber tips were retained in their original positions. Between 4 and 7 h, neurons recovered thick processes, different in shape from those seen before LPA exposure. Compared with immediate (4–15 min) retraction and cap formation induced by LPA, the regrowth step was slower (over



**FIG. 6.** Time-lapse recording of differential interference contrast (DIC) images of single cortical cells with retraction fibers. E12 cortical cells were cultured in serum-free medium for 24 h and treated with 0.5  $\mu$ M LPA. DIC images were collected over 9 h after LPA exposure. Arrow, regrowing membrane. A process regrows along with fibers. Bar = 10  $\mu$ m.

hours). However, no regrowth of membranes was observed where fiber caps were not present. Similar recovery was observed in cultures in which LPA was removed by washout 15 min after LPA exposure (data not shown). Combined with the results in Fig. 4B, these observations demonstrated that the mechanisms utilized for fiber cap formation were inactivated at 15 min after LPA exposure, resulting in regrowth of membranes and processes, and also implicated the caps as scaffolds for process regrowth.

#### LPA-Induced Cap Formation in Cortical Neurons from *lpa*<sub>1</sub>-null Mice

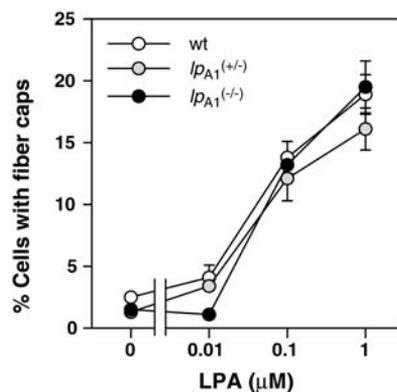
In our cultures, both *lpa*<sub>1</sub> and *lpa*<sub>2</sub>, but not *lpa*<sub>3</sub>, were expressed. Since we have recently generated mice lacking *lpa*<sub>1</sub> by a gene-targeting technique (Contos et al., 2000a), cortical neurons prepared from *lpa*<sub>1</sub><sup>(-/-)</sup> mice were used to examine the role of *lpa*<sub>1</sub> in LPA-induced fiber-cap formation. Cortical cells were prepared from individual E12 mice generated by crossing *lpa*<sub>1</sub><sup>(+/-)</sup> mice and analyzed 24 h after plating. LPA treatment resulted in the cap formation in the cortical neurons from all genotypes with a similar dose-response relationship (Fig. 7). Expression of *lpa*<sub>2</sub> and *lpa*<sub>3</sub> mRNA in cultures from *lpa*<sub>1</sub><sup>(-/-)</sup> mice was unchanged relative to *lpa*<sub>1</sub><sup>(+/-)</sup> and *lpa*<sub>1</sub><sup>(+/+)</sup> controls (Contos et al., 2000a, and data not

shown). These results demonstrated that *lpa*<sub>1</sub> was not necessary for LPA-induced cap formation in cortical, young neurons.

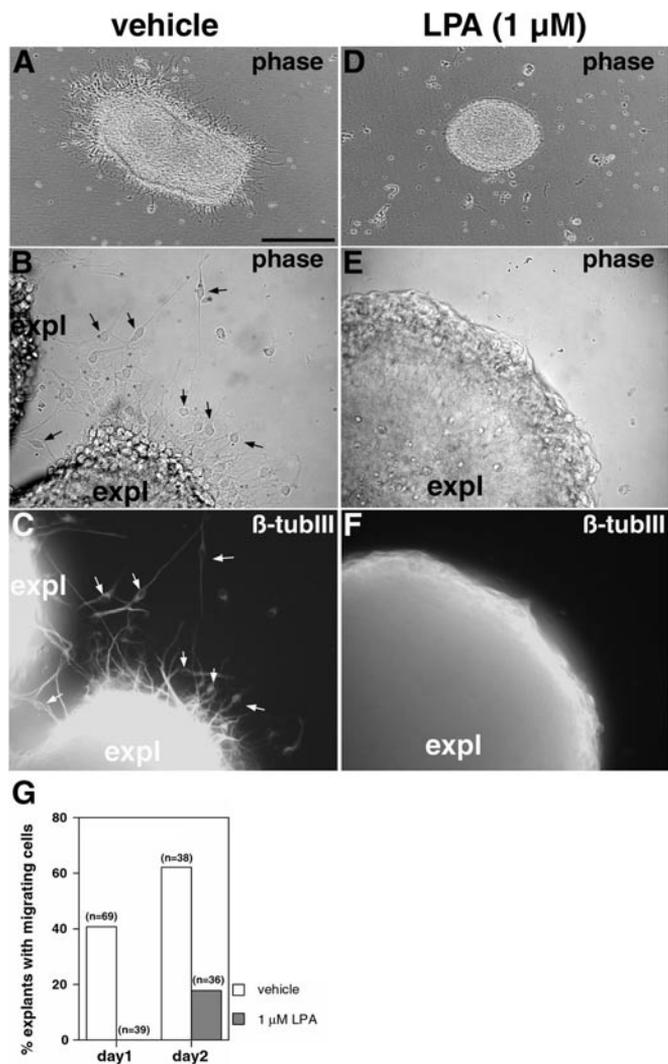
#### Effects of LPA on Neuronal Migration in Cortical Explant Cultures and Whole-Brain Cultures

Finally, we examined how LPA influences young neurons in cortical tissues using two types of cultures: cortical explant cultures consisting of small pieces of cortical tissues of E13 mice and whole-brain cultures that use intact whole brain of E14 mice (see Experimental Methods). In cortical explant cultures, cells radially migrated away from explants 15 h after plating in the absence of LPA (Figs. 8A, 8B, and 8G). Most migrating cells expressed  $\beta$ -tubulin type III, indicating that this culture was a useful model for examining neuronal migration (Fig. 8C). Some MAP2-positive neurons were also observed outside explants (data not shown). When LPA was added during culturing, there were few migrating cells outside explants (Figs. 8D, 8E, and 8G). This was not due to cell death in cortical tissues because cells located at the edge of explants showed  $\beta$ -tubulin type III expression (Fig. 8F) and because neuronal migration occurred 15 h after removal of LPA by replacing culture medium (data not shown).

Whole-brain cultures are also a useful model system to examine how cells are affected by extracellular signals within intact tissues (Fukushima et al., 2000). The cultured brains were fixed, sectioned, and stained for  $\beta$ -tubulin type III. In the control brain, most  $\beta$ -tubulin



**FIG. 7.** LPA-induced fiber cap formation in cortical neurons from *lpa*<sub>1</sub><sup>(-/-)</sup> mice. E12 cortical cells were prepared from wild-type (wt), *lpa*<sub>1</sub><sup>(+/-)</sup>, or *lpa*<sub>1</sub><sup>(-/-)</sup> embryos and LPA-induced cap formation analyzed. Data were determined as described in the legend to Fig. 4 and are the mean  $\pm$  SEM ( $n = 3-6$ ). No difference in LPA-induced cap formation is observed among wt, *lpa*<sub>1</sub><sup>(+/-)</sup>, and *lpa*<sub>1</sub><sup>(-/-)</sup>.

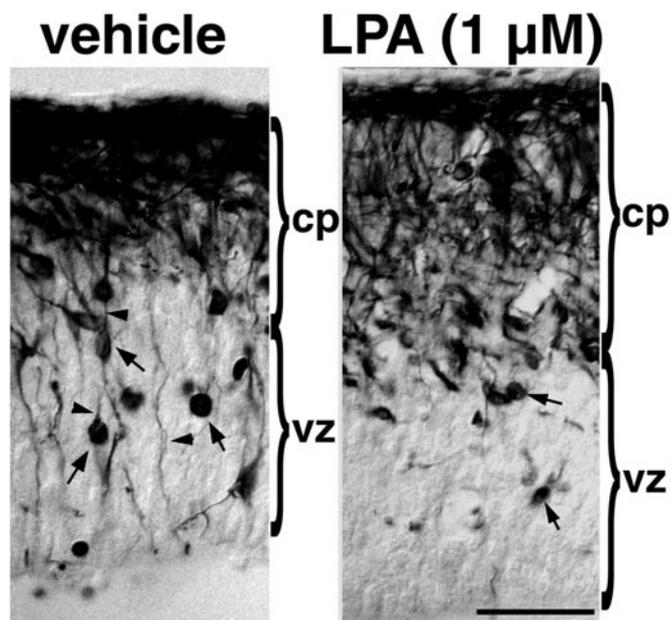


**FIG. 8.** Neuronal migration in cortical explant cultures. Small pieces of E13 cortical tissues were cultured for 15 h and stained for  $\beta$ -tubulin type III. Typical tissues cultured in the absence (A–C) or presence of 1  $\mu$ M LPA (D–F) were shown. (A, B, D, and E) Phase-contrast micrographs; (C and F)  $\beta$ -tubulin type III staining of tissues shown in B and E, respectively. Arrows, typical  $\beta$ -tubulin type III-positive neurons migrating away from explants (expl). (G) Effects of LPA on the number of explants with migrating cells. Cortical explants were cultured for 15 h (day 1) or 40 h (day 2). The number of explants with more than 10 cells outside within the range of 50  $\mu$ m distance from explants was counted, and the percentage to total number of explants ( $n$ ) was expressed. LPA inhibits neuronal migration. Bar = 200  $\mu$ m in A and D and 50  $\mu$ m in B, C, E, and F.

type III staining was observed in fibers and cells within the vz, a neurogenic region, as well as in the cortical plate, a postmitotic region (Fig. 9). The fibers within the vz perhaps corresponded to leading processes of migrating neurons and descending processes from cortical

plate neurons (Menezes and Luskin, 1994). In the LPA-treated brain, the numbers of  $\beta$ -tubulin type III-positive cells and fibers in the vz decreased (Fig. 9). In addition, there was a marked difference in  $\beta$ -tubulin type III staining within the cortical plate between control and LPA treatment, although cell morphology of single neurons could not be identified in this culture. We cannot exclude the possibility that LPA influenced neuronal differentiation within the vz in the whole-brain cultures where the effects of LPA on processes were observed. However, combined with the results from the explant cultures (Fig. 8), LPA likely affected neuronal motility related migration *in vivo*.

LPA-induced process retraction in dissociated cultures was transient (Figs. 2 and 4), whereas LPA-induced morphological changes and inhibition of neuronal migration are relatively long lasting (Figs. 8 and 9). This could be explained by modification of cell adhesion in brain tissues: LPA could strengthen the cell–cell or cell–substratum adhesion within the developing cortex, as has been demonstrated for Schwann cells (Weiner *et al.*, 2001).



**FIG. 9.** Neuronal migration in whole-brain cultures. Whole-brain samples from E14 embryos were cultured with vehicle or 1  $\mu$ M LPA for 14 h and fixed. Thin sections were prepared and stained for  $\beta$ -tubulin type III. Arrows, typical migrating neurons positive for  $\beta$ -tubulin type III; arrowheads, leading or descending processes positive for  $\beta$ -tubulin type III; cp, cortical plate; vz, ventricular zone. LPA affects neuronal migration and morphology including their processes. Bar = 25  $\mu$ m.

## DISCUSSION

In this study we have examined how LPA affects the morphology of young, postmitotic cortical neurons. We have shown that (1) LPA induces process/membrane retraction through Rho/actomyosin pathways, resulting in the formation of retraction fiber caps, which may play a role as scaffolds for regrowing processes/membranes; and (2) LPA inhibits neuronal migration. These data implicate LPA as a regulatory signal not only for process outgrowth but also for migration of young, postmitotic neurons.

### *LPA Induces the Formation of Retraction Fiber Caps in Young, Postmitotic Neurons*

A variety of neuronal cell lines have previously been shown to respond to LPA with neuritic process retraction. Examples include the peripheral nervous system-derived N1E-115, NG108-15, NGF-treated PC12, SY-SH5Y (Jalink *et al.*, 1993; Tigyi *et al.*, 1996; Sayas *et al.*, 1999), and the central nervous system- (CNS) derived TSM or TR (Chun and Jaenisch, 1996; Hecht *et al.*, 1996; Ishii *et al.*, 2000). These studies suggested a role for LPA in the regulation of process formation and elongation in the nervous system. However, the effects of LPA on the morphology of primary neurons, particularly those from the CNS, have not been determined. In the present study, we employed acutely dissociated cell cultures from embryonic mice, which consist of young postmitotic neurons that initiate process outgrowth (Temple and Davis, 1994; Ghosh and Greenberg, 1995).

As expected, LPA induced retraction of growing processes/membranes in these young neurons. In addition, LPA also produced unexpected, F-actin-based retraction fiber caps with an EC<sub>50</sub> similar to those observed for process retraction in other neuronal cell lines (Jalink *et al.*, 1993; Hecht *et al.*, 1996; Ishii *et al.*, 2000). Such LPA-induced cap formation has not been reported to be associated with process retraction in neuroblastoma cells, which instead produce complete cell rounding in response to LPA (Jalink *et al.*, 1993; Jalink *et al.*, 1994; Hecht *et al.*, 1996; Tigyi *et al.*, 1996; Ishii *et al.*, 2000). On the other hand, we have recently demonstrated that a population of proliferative neuroblasts from cortical vz also responds to LPA by forming many fine actin retraction fibers (Fukushima *et al.*, 2000). These fibers in neuroblasts also seemed to result from retraction of extending membranes. Thus, LPA-induced retraction fibers are likely structures observed only in primary neuroblast and young, postmitotic neurons, which ex-

tend processes or lamella. However, membrane retraction in neuroblasts was always accompanied by marked cellular and nuclear migration (Fukushima *et al.*, 2000), which was not significantly observed in the present study. This result may indicate similar, but partially distinct mechanisms for retraction fiber formation between postmitotic neurons and proliferative neuroblasts.

Interestingly, the tips of retraction fibers remained attached at the distal end of extending processes/membranes. Although these fibers contain F-actin, higher levels of F-actin are accumulated at the base of retraction fibers. On the other hand, control (nontreated) cells contain F-actin-enriched lamellipodium (leading edge of extending membranes). These results suggest that actin microfilaments in retracted portions of processes/membranes are rearranged so that the filaments accumulate at the base of retraction fibers, whereas actin in retraction fibers remain unchanged. In epithelial cells, actin filaments are radially oriented along retraction fibers of dividing cells and randomly in lamellipodium of control (nondividing) cells (Cramer and Mitchison, 1995). If actin filaments in the neurons employed here are organized similarly to those in epithelial cells, radial actin filaments may be unaffected by LPA while other filaments may break in response to LPA (e.g., un-cross-linking). These issues will be addressed in future studies.

The mechanisms through which fiber tips remain stationary are still unclear. Because LPA has been known to stimulate the assembly of focal adhesion (Ridley and Hall, 1992), we attempted to detect any component of focal adhesion at the tips of retraction fibers. However, no significant accumulation of focal adhesion kinase or vinculin, both of which are major components of focal adhesion complex, was observed within fibers (data not shown). Therefore, there may be some unknown mechanisms by which the tips remain attached to the substrate.

Our present results indicate that young, postmitotic neurons, but not older neurons, respond to LPA with process/membrane retraction and fiber cap formation. The decrease of the LPA responsive population over the course of neuronal maturation is in agreement with a reduction in the expression levels of *lpa1* and *lpa2* over time *in vitro* (data not shown). However, dorsal root ganglion neurons with long neurites have also been demonstrated to show growth cone collapse without remarkable process retraction or cell rounding in response to LPA (Jin and Strittmatter, 1997; Saito, 1997). Recently, we have also observed that LPA induces growth cone collapse without process retraction in mature cortical neurons cultured for 5 days (Fukushima *et al.*, 2002). This response is mediated through Ca<sup>2+</sup>-

alpha-actinin interactions. Therefore, cellular responses to LPA may vary with neuronal maturation.

After retraction fibers are produced, regrowing membranes at the base of caps spread toward fiber tips. This observation implicates a role of caps in regrowth of processes and reshaping of neurons, which might influence pathfinding of growing neurites or migrating neurons. A similar role for retraction fibers has been previously suggested for epithelial cells (Cramer and Mitchison, 1993). However, activation of myosin is required for fiber cap formation in neurons (Fig. 4D), whereas epithelial cells do not utilize myosin for retraction fiber formation (Cramer and Mitchison, 1997). In addition, the myosin ATPase inhibitor BDM disrupted preexisting retraction fibers produced by LPA in neurons (data not shown), suggesting continuous activation of myosin while caps are present. By contrast, BDM protects epithelial cells from membrane respreading, resulting in stabilization of retraction fibers (Cramer and Mitchison, 1995, 1997). Moreover, retraction fibers of epithelial cells are produced during mitosis, independent of LPA. Therefore, the mechanisms for membrane retraction and spreading must differ between neurons and epithelial cells.

### ***LPA<sub>1</sub> Does Not Mediate LPA-Induced Cap Formation***

*lpa<sub>1</sub>* is unlikely to be involved in LPA-induced cap formation, based on the results from *lpa<sub>1</sub><sup>(-/-)</sup>* mice. This result is consistent with the fact that *lpa<sub>1</sub>* is undetectable in the embryonic cortical plate, which consists of young postmitotic neurons (Hecht *et al.*, 1996; Weiner *et al.*, 1998). Rather, *lpa<sub>2</sub>* appears to be expressed in cortical cells during development (McGiffert *et al.*, unpublished data) and is prominently detectable by RT-PCR assays in our cultures. In addition, *lpa<sub>3</sub>* is not expressed in our cultures nor coupled to a Rho pathway (Ishii *et al.*, 2000). Therefore, *LPA<sub>2</sub>* or other lysophospholipid receptors [e.g., S1P<sub>1</sub>, which is a member of the S1P receptor family and acts as a low-affinity LPA receptor (Lee *et al.*, 1998), or yet-unidentified receptors] may mediate the effects of LPA observed here. Resolution of these issues awaits production and characterization of *lpa<sub>2</sub>* mutant mice and double (*lpa<sub>2</sub>* and *s1p<sub>1</sub>*) mutant mice.

### ***LPA Signaling Influences Neurite Outgrowth and Neuronal Migration***

In the developing brain, postmitotic neurons generated in the vz utilize their leading processes to migrate toward cortical plate (Walsh and Goffinet, 2000). There-

after they begin to extend neurites (axon and dendrites) to form synaptic connections. Both neuronal migration and neurite outgrowth require intrinsic and extrinsic signals for pathfinding of the leading processes and growing neurites before they reach their final destination. Many factors have been proposed as potential extrinsic signals in different systems. These signals are attractants or repellents and influence the direction of extension of growth cones or lamellae, which are associated with cell motility and migration (Song and Poo, 1999). Such changes require cytoskeletal rearrangements induced by the interactions between the signal and its receptor. In the present study, we found that LPA also influenced the morphology and motility of extending neurites and leading processes of neurons possibly through actin cytoskeletal rearrangements and further affected neuronal migration. Retraction of leading processes might be a stop or refinement signal for neuronal migration. Clearly, further analyses using alternative assays should be necessary to clarify the relationship between retraction fibers and neuronal migration and the precise mechanisms by which LPA regulates neuronal migration. Nonetheless, the LPA receptor gene (*lpa<sub>2</sub>*) is expressed in developing neurons and LPA itself is secreted from neurons (Fukushima *et al.*, 2000). Taken together, the present results implicate LPA as a biologically relevant signal in neuronal development.

## **EXPERIMENTAL METHODS**

### ***Pharmacological Inhibitors***

Herbimycin A, genistein, and 2,3-butanedione 2-monoxime (BDM) were purchased from Calbiochem. Histidine-tagged C3 exoenzyme (his-C3) was prepared as described previously (Fukushima *et al.*, 1998). Y-27632 was from Bio-Mol.

### ***Cell Cultures***

Embryos were obtained from timed-pregnant Balb/c mice (Harlan-Sprague-Dawley) with morning of vaginal plug designated day 0 (E0). Dissociated cell cultures were carried out essentially as described previously (Dubin *et al.*, 1999; Fukushima *et al.*, 2000). Briefly, the cerebral cortices from E12 mice were dissected and the meninges were carefully removed. Tissues were transferred to culture medium (Opti-MEM, Gibco-BRL) containing 20 mM glucose and 55  $\mu$ M  $\beta$ -mercaptoethanol and penicillin/streptomycin and gently triturated with

a fire-polished Pasteur pipette until tissues were mostly dissociated into single cells. Cells were seeded onto 12-mm glass coverslips (3000–6000 cells/cm<sup>2</sup>) pre-coated with Cell-Tak (2 µg/cm<sup>2</sup>, Collaborative Research) and cultured at 37°C under 5% CO<sub>2</sub>. For time-lapse recording, 40-mm coverslips were used. A similar dose-response and time course for cap formation was also observed when coverslips were coated with poly-L-lysine plus laminin (1 µg/cm<sup>2</sup>) or fibronectin (1 µg/cm<sup>2</sup>) (data not shown). Therefore, we used Cell-Tak as a substrate in all experiments. For cortical explant cultures, the cortices from E13 were cut into small pieces (200–500 µm thickness) by using surgical blades (No. 11, Feather). Three to seven pieces were placed on Cell-Tak-coated coverslips and cultured as mentioned above. Whole-brain cultures were performed as described previously (Fukushima et al., 2000).

### **Immunocytochemistry and Immunohistochemistry**

Cultured cells or explanted tissues were fixed with 4% paraformaldehyde. Whole brains were fixed, frozen, and sagittally cut into 20-µm sections. Cells, tissues, or sections were permeabilized, blocked, and incubated with anti-tubulin type III antibody (1:250, Sigma), followed by the incubation with a biotinylated secondary antibody (6 µg/ml, Vector). They were further incubated with ABC solution (Vector) and diaminobenzidine or Alexa488-avidin (1 µg/ml, Molecular Probes). For double-labeling of tubulin type III or MAP2 [with anti-MAP2 (2a+2b) antibody at 1:100, Sigma] and F-actin, cells were incubated with biotinylated secondary antibody, followed by the incubation with FITC-avidin (2 µg/ml, Vector) and TRITC-phalloidin (0.1 µg/ml, Sigma). For double-labeling of BrdU and F-actin, fixed cells were incubated with FITC-phalloidin (0.1 µg/ml, Sigma), followed by the incubation with peroxidase-labeled anti-FITC IgG (1 µg/ml, Chemicon). Bound antibodies were visualized using tyramide signal amplification (TSA) kit (DuPont) according to the manufacturer's protocol. Cells were further treated with 2 M HCl to denature DNA, neutralized, and incubated with anti-BrdU rat IgG (1:10 dilution, Harlan), followed by the incubation with biotinylated anti-rat IgG antibody (6 µg/ml, Vector) and then TRITC-avidin (1 µg/ml, Vector). To detect viable cells, fixed cells were subjected to *in situ* end-labeling (ISEL) of 3'-end DNAs using a cell death detection kit (Roche Biochemicals) and then stained for F-actin. This double-labeling revealed that all F-actin-stained cells were viable (data not shown). For quantitative analyses, the experiments were performed in duplicate, and 100–200 viable (i.e., F-actin

positive) cells were counted from 10–20 randomly selected fields per coverslips under fluorescent microscopy with a 40X objective lens.

### **RT-PCR Analysis**

Total RNAs were prepared by using Trizol, treated with RNase-free DNase, and reverse-transcribed with oligo(dT)<sub>12–18</sub> and Superscript reverse transcriptase (all purchased from Gibco-BRL). The resultant cDNAs (0.1 µg) were amplified by PCR for *lpa* family members. The used primers were follows: *lpa*<sub>1</sub>: 5'-CAACGAGAAC-CCTAATGG-3' (sense) and 5'-CTCTGGCTCCCCAC-CTT-3' (antisense), producing a 183-bp product; *lpa*<sub>2</sub>: 5'-CGAGACCATCGGTTTCTTCTATA-3' (sense) and 5'-TGTGCAGGTAGCAACCCAGA-3' (antisense), producing a 471-bp product; *lpa*<sub>3</sub>: 5'-CCCATTTACAG-TAGGAGTTA-3' (sense) and 5'-CTTGCAGTTCAGGC-CGTCCAG-3' (antisense), producing a 261-bp product. The cycling protocol was 60 s at 94°C; followed by 35 cycles of 20 s at 94°C, 40 s at 55°C, and 40 s at 72°C; followed by 7 min at 72°C at the end of cycling to complete extension.

### **Time-Lapse Video Microscopy**

The 40-mm coverslips were mounted onto a heat-controlled perfusion apparatus (Biopetechs) set at 37°C (the chamber volume was 700 µl) and observed with an inverted microscope (Axiovert 135, Carl Zeiss) using a 63X or 100X oil-immersion objective lens. Single cells were observed for at least 20 min to confirm that cells were healthy, and then the experiment was started. Replacement of culture medium containing 0.1% FAFBSA (Sigma) was manually performed with a syringe (approximately 2 ml/min). The differential interference contrast images were collected with a cooled charge-coupled device color camera (DEI-47, Carl Zeiss). Images were collected on a Power Macintosh G3 running Scion Image software (Scion Corp.) and modified using Adobe Photoshop (Adobe Systems Inc.).

### **Lipid Treatment**

Oleoyl-LPA, -LPC, -LPE and -PA (all purchased from Avanti Polar Lipids) stock solutions were made up in distilled water at 10 mM, stored at –20°C, and used within a week. S1P, SPC, and SPH (all purchased from BioMol) and anandamide (from RBI) solutions were in 0.01% FAFBSA.

## *lpa*<sub>1</sub> Mutant Mice

For the experiment with *lpa*<sub>1</sub> mutant mice, embryos were generated by mating adult male *lpa*<sub>1</sub><sup>(+/-)</sup> and adult female *lpa*<sub>1</sub><sup>(+/-)</sup> (Contos *et al.*, 2000a). Genotype of embryonic mice was determined by PCR as described previously (Contos *et al.*, 2000a).

## Statistical Analysis

An analysis of variance followed by a post hoc test was applied to data to determine statistical significance by using the statistical software, StatView 4.5 (Abacus Concepts).

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