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Depletion of lysophosphatidic acid triggers a loss of oriented detyrosinated microtubules in motile fibroblasts

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SUMMARY

We reported earlier that isolated plasma membranes trigger a number of responses comprising contact inhibition of motility, including loss of oriented detyrosinated microtubules (Glu MTs) from the lamella of motile fibroblasts. In this study, we show that the membranes trigger this loss of Glu MTs, not by binding to cells, but by removing an essential component from the medium necessary to maintain oriented Glu MTs. Preincubation of membranes with medium containing serum followed by removal of the membranes by sedimentation rendered the membrane-treated medium capable of triggering the loss of oriented Glu MTs. Membrane activity was inhibited by high concentrations of serum and removal of serum from medium triggered the loss of oriented Glu MTs similar to that triggered by membranes. These results suggest that the membranes trigger the loss of Glu MTs by inactivating factors in serum that are required for the maintenance of oriented Glu MTs. By fractionating serum, we have identified lysophosphatidic acid (LPA) as the principal serum factor that is responsible for supporting oriented Glu MTs. The activity of LPA to maintain oriented Glu MTs upon serum withdrawal was half maximal at 100 nM and no activity was observed with structurally related phospholipids. Serum LPA levels were sufficient to account for the ability of serum to support oriented Glu MTs. Enzymatic degradation of serum LPA strongly reduced the ability of serum to support oriented Glu MTs. That membranes degrade LPA was shown by the ability of membranes to block LPA's ability to maintain oriented Glu MTs, and by direct measurement of the loss of radiolabeled LPA after incubation with membranes in vitro. These results show that isolated plasma membranes trigger the loss of Glu MTs from the lamella of motile cells by degrading serum LPA. Coupled with earlier results showing that membranes trigger a number of contact inhibition responses, our data suggest a new model for contact inhibition of motility in which local degradation of LPA and/or interference with LPA-stimulated signalling pathways initiates a contact inhibition response in colliding cells.

Key words: Contact inhibition, Cell motility, Cytoskeleton, Cell surface, Plasma membrane, Phospholipase

INTRODUCTION

Contact inhibition of motility is a phenomenon originally defined by morphological criteria using cultured fibroblasts, and is thought to be involved in a variety of physiological reactions, such as monolayering and directional motility of cells in culture (Abercrombie and Heaysman, 1954) and tumor metastasis in vivo (Abercrombie, 1979). Contact inhibition was originally described as 'the stopping of the continued locomotion of a cell in the same direction after collision with another cell' (Abercrombie, 1970; Heaysman, 1978). The sequence of events during contact inhibition involves cell-cell adhesion, local paralysis of motility, contraction and withdrawal of the lamellipodium, and re-establishment of motility in a new direction (Abercrombie, 1970; Heaysman, 1978). These cellular reactions are complex and difficult to quantify, and this has contributed to a lack of progress in understanding contact inhibition at a molecular level. The possibility that specific cell surface molecules trigger the events of contact inhibition has been discussed (Heaysman, 1978), but has not been demonstrated to date.

To analyze contact inhibition of motility at a molecular level, we have established a quantitative method for determining the occurrence of contact inhibition reactions by using detyrosinated microtubules (Glu MTs) as a biochemical marker (Nagasaki et al., 1994). Glu MTs arise by post-translational modification of tubulin in stabilized microtubules and represent a subset of MTs in most cells (Gundersen et al., 1987; Bulinski and Gundersen, 1991). In motile fibroblasts, Glu MTs are present in the lamella and are generally oriented toward the direction of movement (Gundersen and Bulinski, 1988; Nagasaki et al., 1992). We have shown that this population of MTs, but not the total population of MTs, is rapidly lost during cell-cell contact (Nagasaki et al., 1992) and also upon treatment of noncontacting motile cells with isolated plasma membranes (Nagasaki et al., 1994). In the latter study, we also showed that the membranes trigger other contact inhibition-like responses in target cells, including altered microfilament distribution and an inhibition of cell movement. Since Glu MTs represent a stable population of MTs among mostly dynamic cellular MTs (designated as Tyr MTs, since they are

comprised predominately of tyrosinated tubulin) (Gundersen et al., 1987; Webster et al., 1987), these observations provided evidence that cell-cell contact triggers rapid and specific alterations in a subset of MTs. The ability to trigger a contact inhibition-like response with isolated plasma membranes suggests that it might be possible to isolate the factor responsible for triggering this reaction.

Using the loss of Glu MTs from the lamella of motile cells as an assay, we have begun to study the molecular mechanism of contact inhibition. We report here that isolated plasma membranes induce the loss of Glu MTs from the lamella of cells, not by binding to target cells, but by inactivating serum factors that normally maintain the Glu MTs. We have identified the serum factor neutralized by membranes as the mitogenic lipid LPA and show directly that the membranes inactivate LPA by degrading it. In addition, we show that removal of LPA from serum by other means is sufficient to trigger the loss of oriented Glu MTs. Our results suggest that contact inhibition of motility may be initiated by interference with LPA signalling, either extracellularly or intracellularly, in contacting cells.

MATERIALS AND METHODS

Materials

NRK fibroblasts were maintained as described previously (Nagasaki et al., 1992). CM Affi-Gel blue resin was purchased from Bio-Rad (Hercules, CA). Horse plasma-derived serum (prepared from platelet-poor plasma), bovine serum albumin (BSA, Cohn fraction V), and fatty acid free BSA were purchased from Sigma (St Louis, MO).

The following lipids were obtained from Sigma except as noted: oleic acid; MOG, 1-monooleoyl-rac-glycerol; LPA, L-α-lysophosphatidic acid (1-oleoyl, obtained from Sigma and Avanti Polar Lipids, Alabaster, AL); PA, L-α-phosphatidic acid (dioleoyl); LPC, L-α-lysophosphatidylcholine (1-oleoyl); LPG, L-α-lysophosphatidylglycerol (1-oleoyl, obtained from Avanti Polar Lipids); LPE, L-α-lysophosphatidylethanolamine (1-oleoyl); LPS, L-α-lysophosphatidyl-L-serine; LPI, L-α-lysophosphatidylinositol; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidyl-L-serine; SPH, D-sphingosine; DHSPH, DL-threo-dihydrosphingosine; SPC, sphingosylphosphorylcholine; PAF, 1-*O*-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor); LPAF, 1-*O*-hexadecyl-sn-glycero-3-phosphocholine (lyso-platelet activating factor); arachidonic acid; 12-hydroxystearic acid. Stock solutions of PAF and LPAF were prepared in water at 5 mg/ml. Stock solutions of LPA, LPG, SPH, DHSPH, arachidonic acid, and 12-hydroxystearic acid were made at 1 mM in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution containing 20 mM Hepes, pH 7.4 (CMFH), and 1 mM fatty acid free BSA. PA, LPC, LPE, LPS, LPI, and OAG were dissolved in 50% ethanol at 5 mg/ml. PC and PS were dissolved at 10 mg/ml in CMFH containing 5% (w/v) octylglucoside.

Stock solutions of phospholipase A_2 (PLA_2; Sigma P9279), phospholipase B (PLB; Sigma P8914), and phospholipase D (PLD) from cabbage (Sigma P0282), and PLD from *Streptomyces chromofuscus* (Sigma P8023 or Boehringer 430331) were prepared in CMFH and aliquots were stored at −70°C. Alkaline phosphatase (Sigma P0905) was stored at 4°C.

Plasma membranes from NRK cells were prepared by the method of Raben et al. (1981). The solubilization of membrane components with octylglucoside is described elsewhere in detail (Nagasaki et al., 1994). This solubilized membrane preparation was as active as the intact membranes (see Nagasaki et al., 1994) and was used in place of membranes in some experiments. Protein concentration was determined by BCA protein assay using BSA as a standard.

Assay of oriented Glu MTs in target cells

NRK cells migrating into an experimental wound were used to assess the ability of test compounds to alter the level of oriented Glu MTs in the leading lamella of target cells. The detailed procedures including immunofluorescence detection of Glu and Tyr MTs with Glu and Tyr tubulin specific antibodies are described elsewhere (Nagasaki et al., 1994). Briefly, NRK cells were grown to confluency on acid-washed circular glass coverslips (18 mm diameter) in 10% calf serum (Hyclone, Denver, CO) in DME (Gibco BRL, Grand Island, NY), and subsequent operations were done in wells of 12-well plastic plates. For all other operations on cells, we used horse plasma-derived serum (referred to below as 'serum'). Monolayers of NRK cells that had been confluent for no more than 18 hours were wounded with a jeweler's screw driver by scraping a streak of cells. Five streaks were made in each coverslip. Cell debris were immediately removed by washing with 5% serum in DME and wounded monolayers were incubated in the same medium for one hour at 37°C to allow the generation of oriented Glu MTs in the lamella of cells at the wound margin (see Fig. 1; also Nagasaki et al., 1994). These wounded monolayers were then used to test membrane preparations. In general, the medium bathing the cells was replaced with either fresh 5% serum in DME or serum-free DME with or without various test substances (e.g. treated serum, lipids, etc.). Treated cells were briefly rinsed in Hanks' balanced salt solution at 37°C, fixed in methanol at −10°C, processed for immunofluorescence staining of Glu MTs, and then examined with a ×40 dry objective (Nikon, Neofluar, NA 0.9) on a Nikon Optiphot microscope equipped for fluorescence. To quantify the data, we counted the number of wound edge cells, which contained Glu MTs that were selectively localized in the lamella and extended at least half the way to the leading edge (see Nagasaki, 1994). At least 400 cells were counted per sample.

Treatment of medium with membranes

For the treatment of medium with membranes, 500 μl of DME containing 5% serum was incubated at either 4°C or 37°C for 30 minutes with different concentrations of NRK cell plasma membranes (measured as μg/ml protein; Nagasaki et al., 1994). In some experiments, longer incubations were done. At the end of the incubation, the mixture was centrifuged at 100,000 ***g*** to pellet the membranes, and the supernatant was assayed for the activity to support oriented Glu MTs as described above.

Fractionation of serum with CM Affi-Gel blue column

To fractionate serum with CM Affi-Gel blue, serum was first dialyzed extensively against phosphate buffered saline (PBS) using a membrane with a cutoff of 12 kDa. A 10 ml aliquot of serum in PBS was loaded onto a column of CM Affi-Gel Blue (80 ml bed volume) equilibrated with PBS, and a flowthrough fraction was collected. After thorough washing with PBS, the bound materials were eluted with 1.4 M NaCl in PBS. Both flowthrough and 1.4 M NaCl eluent fractions were concentrated with Centricon-10 (Amicon) and dialyzed extensively against PBS before use.

Treatment of phospholipids with phospholipases and alkaline phosphatase

Phospholipids (1-5 μg) and serum (5-10 μl) were treated with PLA_2 (1 unit), PLB (1 unit), PLD from cabbage (1 unit), PLD from *Streptomyces chromofuscus* (1 unit), or alkaline phosphatase (10 units) in microfuge tubes in a final volume of 10-30 μl in CMFH at 37°C for 2-3 hours. After the enzyme treatment of lipids, the reaction mixture was heated in boiling water for 4 minutes to destroy the enzyme activity before adding to cells for the assay. Boiling had no effect on the activity of lipids or serum (data not shown).

Analysis of phospholipids in serum

Phospholipids were extracted from serum using 1-butanol and separated with a 2D-TLC (Silica Gel 60, Merck) as described by

Eichholtz et al. (1993). The LPA spot was identified by comparison with the migration of authentic LPA (oleoyl) purchased from Sigma. Lipids were detected with iodine vapor. The region corresponding to LPA was scraped and the LPA extracted from silica with chloroform:methanol (1:1). Quantification of LPA was done by determining phosphorus content using inorganic phosphate as a standard (Hess and Derr, 1975).

Analysis of LPA degrading activity of plasma membranes

A 1 μCi sample of [^{3}H]LPA (1-oleoyl [oleoyl-9,10-^{3}H], 56.2 Ci/mmol, kindly provided by Dr Ernst Do, NEN) was incubated at 37°C for 60 minutes with 10 μg of membranes in 150 mM NaCl containing 10 mM Hepes, pH 7.4, in a final volume of 50 μl. As controls, incubations were done at 4°C with membranes and at 37°C without membranes. The lipids were extracted (Bligh and Dyer, 1959), and separated with TLC using chloroform:methanol:acetic acid:H_2O (100:60:16:8) as mobile phase (Eichholtz et al., 1993). The TLC plate was dried under vacuum, coated with En^{3}Hance (Amersham) scintillation spray, and exposed to a Kodak XAR-5 film at −70°C. The exposed film was scanned with a Hewlet Packard color scanner with a transparency adapter and the image was digitized, and printed with a color printer. Migration of LPA, monooleoylglycerol, oleic acid, and phosphatidic acid (dioleoyl) was established by chromatographing authentic standards of each lipid individually and locating them by iodine vapor stain.

RESULTS

Isolated plasma membranes trigger the loss of oriented Glu MTs by inactivating medium components

We previously showed that plasma membranes prepared from NRK fibroblasts trigger contact inhibition-like responses in migrating target cells, including loss of oriented Glu MTs from the leading lamella (Nagasaki et al., 1994). We have now examined in detail the mechanistic basis for how membranes trigger the loss of oriented Glu MTs. As a quantitative measure for this study, we determined the percentage of wound edge cells that contained oriented Glu MTs in their lamella. The determination of the number of cells with oriented Glu MTs provides a rapid assay that is highly sensitive, concentration dependent, and reproducible (Nagasaki et al., 1994).

Because contact inhibition requires cell-cell interaction, our first thought was that the membranes trigger contact inhibition by directly interacting with receptors on the surface of the target cells. To test this notion, we first determined whether the isolated membranes had to be present in the assay medium continuously to trigger the loss of oriented Glu MTs. We preincubated membranes with assay medium (5% serum in DMEM) at 37°C for 60 minutes and then removed the membranes by centrifugation. Unexpectedly, this membrane-pretreated medium was capable of triggering the loss of Glu MTs by itself. Results from a typical experiment are shown in Fig. 1. The oriented array of Glu MTs in the lamella of cells at the edge of a wound, the target cells, is shown in Fig. 1b. After incubation with membrane-treated medium, the target cells exhibited a loss of oriented Glu MTs (Fig. 1f), very similar to that in cells incubated directly with membranes (Fig. 1d; see also Nagasaki et al., 1994). In most treated cells, a small number of Glu MTs are still observed, but they are clustered around the nucleus, rather than extending into the lamella (Fig. 1). As observed with the direct addition of membranes, the effect of membrane-treated medium was specific for Glu MTs since little change was noted in the distribution or the number of Tyr MTs (Fig. 1a,c,e), which roughly represent the total population of MTs (Gundersen et al., 1987).

Quantification of the number of cells containing oriented Glu MTs showed that pretreatment of medium with membranes caused a loss of oriented Glu MTs comparable to that caused by directly adding the membranes to cells (Fig. 2). Since the membrane-pretreated medium was freed of membrane particles by centrifugation, these results indicate that membranes do not need to be in direct contact with the cells to trigger the loss of oriented Glu MTs. When the preincubation of membranes with medium was done at 4°C instead of 37°C, the loss of oriented

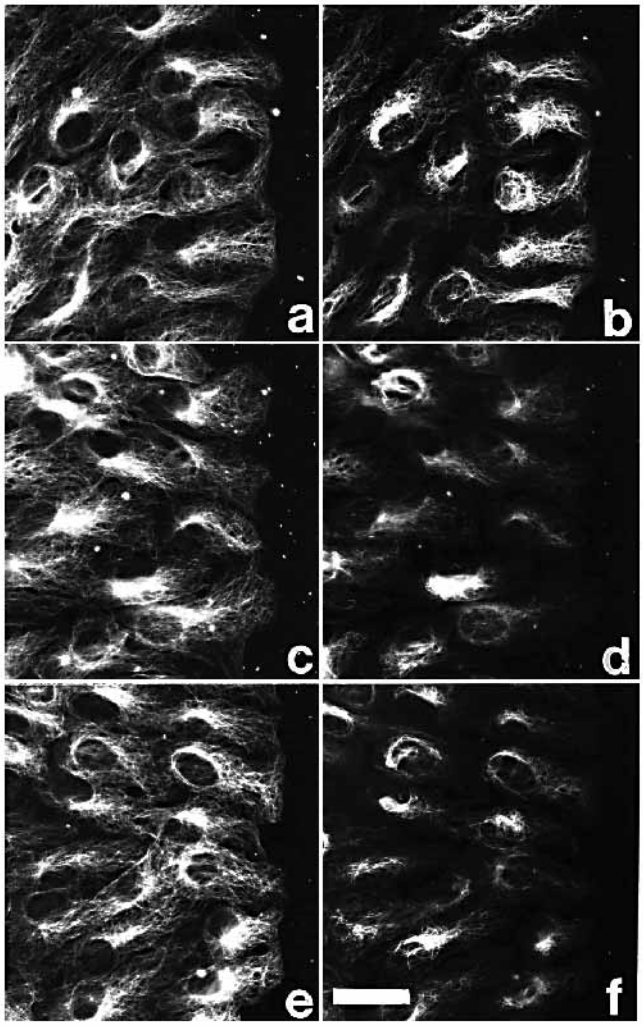


Fig. 1. Pretreatment of serum containing medium with membranes induces the loss of oriented Glu MTs in target cells. A confluent monolayer of NRK cells was wounded, and cells at the edge of the wound were allowed to generate oriented MTs in the presence of 5% serum as described in the text. These cells were incubated with test samples for 60 minutes before fixation and double immunofluorescence staining of Tyr MTs (a,c,e) and Glu MTs (b,d,f). Control cells incubated with 5% serum/DME alone (a,b); cells incubated with 5% serum/DME containing 7 μg/ml of plasma membranes (c,d); cells incubated with 5% serum/DME that had been pretreated with 7 μg/ml of plasma membranes, followed by removal of the membranes by centrifugation (e,f). Bar, 20 μm.

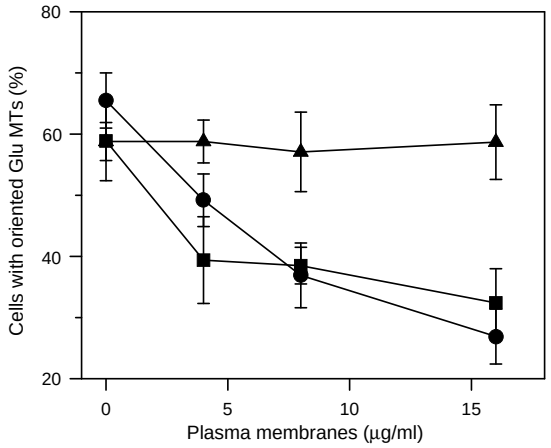


Fig. 2. Comparison of membrane-pretreatment of medium and direct membrane addition in inducing the depletion of oriented Glu MTs. NRK cells were treated as described in the legend to Fig. 1, and the percentage of cells at wound margins containing oriented Glu MTs was determined. Cells incubated with indicated concentrations of plasma membranes (●, average of eight experiments); cells incubated in medium that had been pretreated with the indicated concentrations of plasma membranes at 37°C (■, average of five experiments) or at 4°C (▲, average of two experiments).

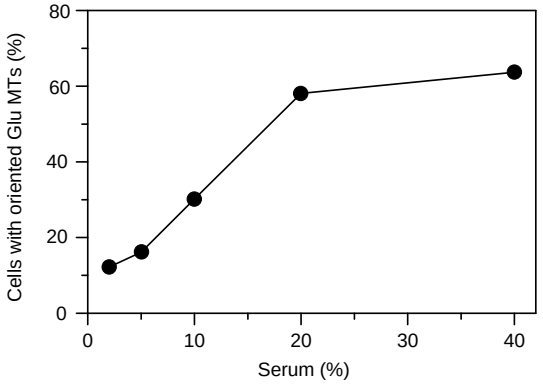


Fig. 3. Serum blocks the Glu MT reducing activity of plasma membranes in a concentration dependent manner. Wounded monolayers of NRK cells were incubated with 18 μg/ml solubilized NRK plasma membranes in the presence of indicated concentrations of serum in DME. The percentage of cells at the wound margin containing oriented Glu MTs was then determined. Data are representative of three independent experiments.

Glu MTs was abolished (Fig. 2), suggesting that the depletion of Glu MTs may require enzyme activity.

The ability of membrane-treated medium to deplete oriented Glu MTs from the leading edge of the cell may be due to: (1) membrane components that were released into the medium; (2) serum components that were activated by the membranes; or (3) membrane-mediated removal or inactivation of serum components that were required for the maintenance of oriented Glu MTs. We think the first possibility is unlikely since we had previously found that the activity was not released from membranes with high salt or protease treatments (Nagasaki et al., 1994). Also, the membranes recovered from preincubation with medium still retain the capability of depleting Glu MTs from cells (data not shown). To distinguish between possibilities 2 and 3, we determined the effect of excess serum on the activity of the membranes. If possibility 2 is correct, excess serum would have no effect or perhaps increase the membrane activity. If possibility 3 is correct, excess serum would block the membrane activity. As shown in Fig. 3, as the concentration of serum was increased, the ability of solubilized membranes to initiate the loss of Glu MTs was decreased. Identical results were obtained with intact membranes (data not shown). This result is consistent with the idea that membranes remove or inactivate a serum factor that is necessary for maintaining oriented Glu MTs (possibility 3), and does not support the idea that membranes activate latent serum components (possibility 2).

To test possibility 3 further, we examined the effect of completely removing all serum components on the oriented Glu MTs. Exposure of cells to serum-free medium led to a rapid loss of extended Glu MTs (Fig. 4), which was similar to that observed in membrane-treated cells (see Fig. 1). As with the membrane-treated cells (Fig. 1c,d), the removal of serum specifically affected the oriented Glu MTs (Fig. 4d), with much less effect on the Tyr MTs (Fig. 4c). The loss of oriented Glu MTs after treatment with serum-free medium occurred within 30 minutes (Fig. 5), which was similar to that observed with membrane-treated cells (see Fig. 5 of Nagasaki et al., 1994). Change of medium alone (to fresh serum containing medium) did not cause a loss of oriented Glu MTs (Fig. 5). Taken together, these results strongly support the idea that membranes induce the loss of oriented Glu MTs by inactivating serum factors that are necessary for maintaining oriented Glu MTs.

LPA is capable of supporting oriented Glu MTs in motile NRK cells

To identify the factor(s) in serum that were inactivated by membranes, we assayed preparations for the ability to maintain oriented Glu MTs upon serum removal. NRK cells at the wound edge were allowed to generate oriented Glu MTs in the presence of serum, and then the medium was switched to serum-free medium. At this point, test samples were added to the serum-free medium to see if they prevented the loss of oriented Glu MTs (Figs 4 and 5). Once we identified factors with this assay, we could then test whether the factors were also inactivated by membranes.

To determine the type of activity present in serum, we initially subjected serum to a number of biochemical treatments. The ability of serum to maintain oriented Glu MTs was resistant to dialysis (12 kDa cutoff), heat and acid treatment, but was labile to alkaline treatment (data not shown). When we fractionated serum with a CM Affi-Gel blue column, the flowthrough fraction contained less than 2% of the applied activity and the 1.4 M NaCl eluent (see Materials and Methods) contained the majority of the recovered activity. As expected, the 1.4 M NaCl eluent contained serum albumin as its major component, so we tested the ability of commercially available BSA to support oriented Glu MTs. However, purified BSA prepared from an initial ethanol precipitation (95% pure, Cohn fraction V; Sigma) was not active at concentrations as high as 30 mg/ml.

Since the albumin fraction of serum prepared chromatographically contained the Glu MT supporting activity but

albumin purified by the Cohn's procedure, which might remove lipids, was inactive, we tested whether lipids were responsible for the activity of serum to maintain oriented Glu MTs. Among many lipids tested, we found that LPA and PA could support the oriented Glu MTs in motile NRK cells when serum was withdrawn (Fig. 4f, Table 1). The level of Glu MTs and their asymmetric distribution in the leading lamella of LPA-treated wound edge cells (Fig. 4f) was remarkably similar to that in cells treated with serum (Fig. 4b).

The activity of LPA was observed at concentrations as low as 100 nM and reached maximum at approximately 500 nM (Fig. 6). We did not observe any deleterious effect of LPA on the target cells up to 400 μM, which was the highest concentration we tested. We also found that LPA was active either as a carrier-free molecule or as a 1:1 molar complex with fatty acid free BSA (data not shown). PA exhibited a low level of activity, requiring 50-100 times the concentration of LPA to elicit an equivalent effect. We do not know whether this is due to intrinsic activity of PA or to trace amounts of LPA in the commercial PA as has been reported previously (Jalink et al., 1990). All other lipids we examined were inactive at the concentrations >50 times the effective concentration of LPA (Table 1).

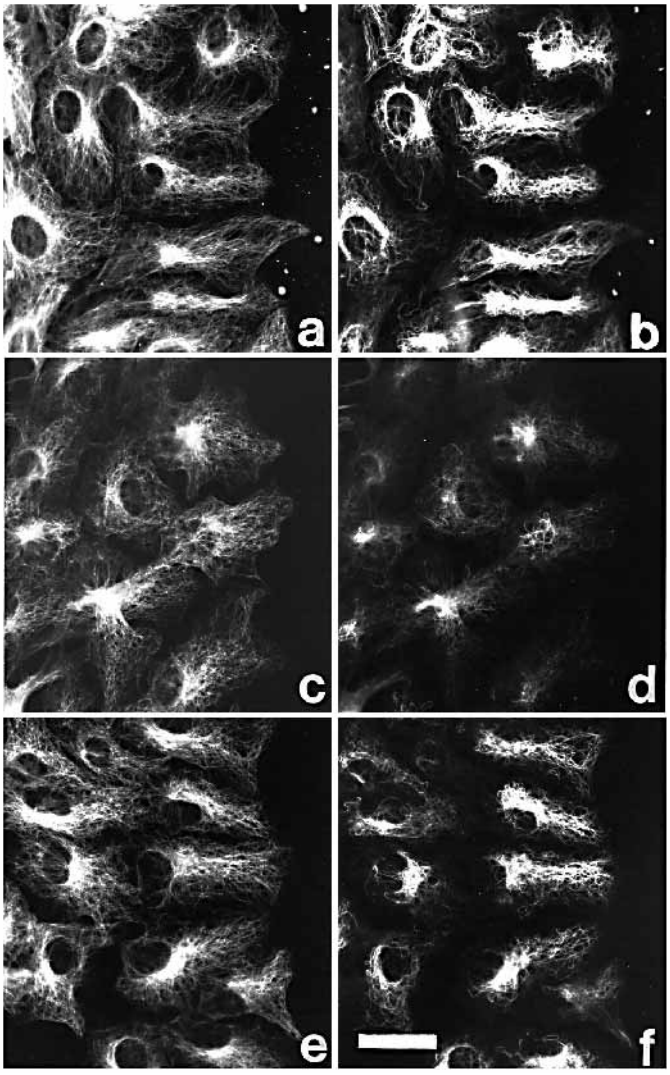


Fig. 4. Serum or LPA is necessary to maintain oriented Glu MTs in NRK cells. Confluent monolayers of NRK cells were wounded and incubated in 5% serum for 60 minutes. These cells were rinsed with serum-free medium, and further incubated in either 5% serum (a,b), serum-free medium (c,d), or 430 nM LPA (e,f). After a one hour incubation at 37°C, cells were fixed and processed for double immunofluorescence staining of Tyr MTs (a,c,e) and Glu MTs (b,d,f). Bar, 20 μm. Differences in the cell density in the examples shown were not responsible for the differences in the levels of oriented Glu MTs.

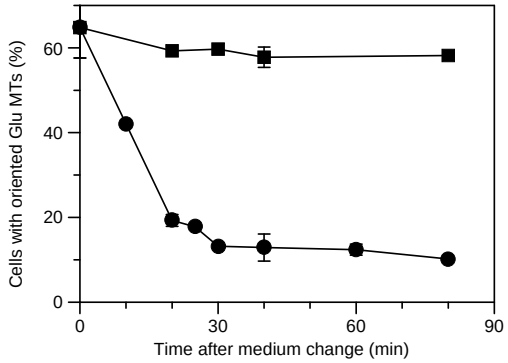


Fig. 5. Time course of depletion of oriented Glu MTs upon removal of serum. Wounded monolayers of NRK cells were incubated in 5% serum/DME for 60 minutes. Then, the monolayers were exposed to either fresh medium containing 5% serum (■) or serum-free medium (●). Cells were fixed at indicated time points and the percentage of wound-edge cells with oriented Glu MTs was determined. Data are representative of two independent experiments with similar results.

Effects of LPA analogs on oriented Glu MTs

To test whether a lipid structurally similar to LPA or a trace amount of a contaminating compound in the commercial LPA preparation was responsible for maintaining oriented Glu MTs,

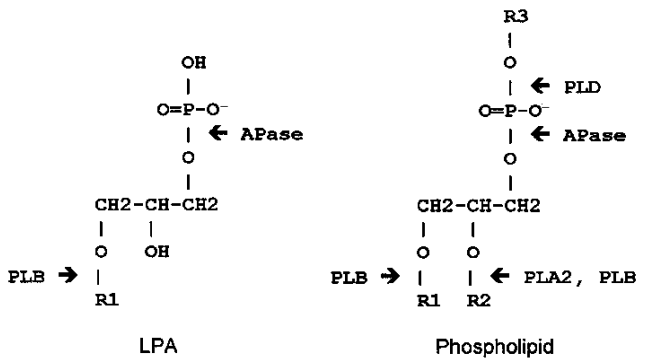
Table 1. Activity of lipids to maintain oriented Glu MTs in motile NRK cells after serum removal

Lipid	Concentration (μM)	Oriented Glu MTs
LPA	0.1	+
	0.3	++
	1.0	+++
PA	2.0	–
	20	++
LPC, LPE, LPS, LPI, LPG	10	–
PC, PS	20	–
OAG*	5	–
SPH, DHSPH*	8	–
PAF, LPAF*	10	–
Arachidonic acid	100	–
12-hydroxystearic acid	100	–

Lipids were tested for their ability to support oriented Glu MTs in NRK cells at a wound edge after serum was removed from the assay medium as described in Materials and Methods. The activity of lipids is expressed relative to that of 2% serum, which gave the maximal level of oriented Glu MTs under our assay conditions, and is designated (+++). Lower levels of Glu MTs are indicated by (++) for ~50% of maximal response, (+) for a just detectable response, and (–) for no response over that observed with serum-free medium (i.e. oriented Glu MTs were lost from target cells). For lipids without effect, the highest concentrations tested are shown.

*These lipids were detrimental to the normal morphology of cells at higher concentrations.

Table 2. Effect of hydrolytic enzymes on the ability of phospholipids to support oriented Glu MTs



	Level of oriented Glu MTs				
	Treatment of lipids				
Phosopholipids	Control	PLA2	PLB	PLD	Alkaline phosphatase
LPA	+++	+++	–	+++	–
PA	–	++	–	–	ND
LPC, LPS, LPI, LPE, PAF, LPAF	–	ND	ND	+++	ND
Serum	+++	+++	+	ND	–

Phospholipids and serum were treated with phospholipases and alkaline phosphatase as described in Materials and Methods, and assayed for their ability to support oriented Glu MTs in wound edge cells. All lipids were treated and assayed at 2 μg/ml (2-4 μM, concentrations determined before the enzyme treatment). serum was assayed at 5% (v/v) in DME. The levels of oriented Glu MTs were then scored as in Table 1.

we subjected LPA and other lipids to treatment with phospholipases A_2, B, and D, and alkaline phosphatase (Table 2). The activity of LPA was destroyed by PLB, but not by PLA_2 or PLD, consistent with structural requirements of the active species for an acyl group at an sn1 position, but not at the sn2 position, and for no head group. The phosphate at the sn3 position appeared necessary since alkaline phosphatase also destroyed the activity of LPA. PA did not support oriented Glu MTs at low concentrations, yet PA treated with PLA_2, which generates LPA, showed increased ability to support oriented Glu MT (Table 2). Furthermore, lysophospholipids with a polar head group (LPC, LPS, LPI, LPE, PAF, LPAF), which by themselves were inactive, were capable of maintaining oriented Glu MTs after PLD treatment (Table 2). For each of these lipids, PLD is expected to remove the head group and generate LPA. Together, these results are consistent with the idea that LPA, rather than a contaminant in the LPA preparation, is responsible for the activity.

LPA is the major serum factor responsible for maintaining oriented Glu MTs

The ability of LPA to support oriented Glu MTs was labile to alkaline treatment (0.1 N NaOH), but resistant to treatment with heat (100°C, 5 minutes), and acid (1 N AcOH). These properties were identical to those exhibited by the factor in serum (data not shown), suggesting that LPA may be the serum component that was responsible for maintaining oriented Glu MTs. To test this idea, we treated serum with PLB or alkaline phosphatase, both of which were capable of inactivating LPA (Table 2). We found that treatment of serum with PLB and alkaline phosphatase, but not by PLA_2, dramatically reduced the activity of serum (Table 2). The effect of PLB or alkaline phosphatase to neutralize the serum activity was abolished when the enzyme preparations were preincubated in boiling water for 4 minutes (data not shown), showing that the enzymatic activity of PLB or alkaline phosphatase was required to abolish the activity of serum to maintain oriented Glu MTs. These results strongly suggest that LPA is one of the major serum factors involved in the maintenance of oriented Glu MTs in motile NRK cells.

To determine whether serum contains sufficient LPA to account for the Glu MT maintenance activity of serum, we determined the LPA content of serum by extracting phospholipids, separating them by 2D-TLC, and then quantifying the lipid migrating at the LPA position. We found that serum contained 12±4 μM LPA (n=2), which is in the range reported previously by others; 66 μM of LPA in rat plasma that had been incubated at 37°C for 48 hours (Tokumura et al., 1986), and 1-5 μM LPA in human serum (Eichholtz et al., 1993). Thus, at the concentration of serum that yielded a half maximal response (0.5-2% serum, v/v), there would be 60-240 nM LPA present in the medium, which is comparable to the concentration of pure LPA that induced a half maximal response (200-300 nM, Fig. 6). These results are consistent with the idea that LPA is one of the major factors present in serum that is responsible for supporting oriented Glu MTs.

Plasma membranes cause loss of oriented Glu MTs by inactivating serum LPA

We showed that high concentrations of serum can neutralize the ability of plasma membranes to cause the loss of oriented Glu MTs (Fig. 3). Since LPA appears to be the major component of serum that supports the presence of oriented Glu MTs, we hypothesized that the ability of plasma membranes to trigger depletion of oriented Glu MTs may be due to removal of LPA from the medium. To test this idea, we preincubated purified LPA with plasma membranes, removed the membranes by sedimentation, and then assayed the treated LPA for its ability to support oriented Glu MTs. We found that

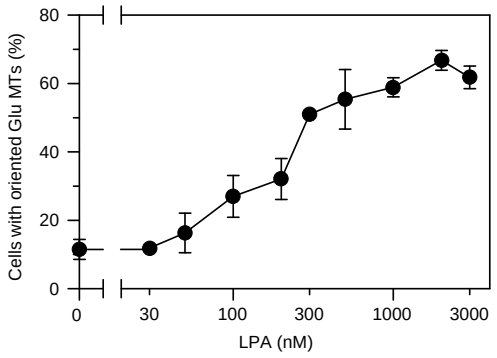


Fig. 6. Concentration dependence of LPA in maintaining oriented Glu MTs. Target NRK cells containing oriented Glu MTs were transferred to serum-free medium with the indicated concentrations of LPA and incubated for 60 minutes before fixation, immunofluorescence staining, and determination of oriented Glu MTs. Similar results were obtained in over 10 independent experiments.

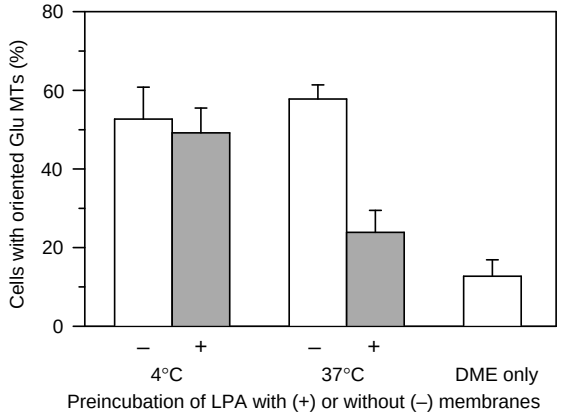


Fig. 7. Plasma membranes destroy the ability of LPA to maintain oriented Glu MTs in a temperature dependent manner. LPA (2 μM) was preincubated with 8 μg/ml membranes for 40 minutes at either 4°C or 37°C, and the membranes were removed by centrifugation. Then, the resultant supernatant was assayed for the ability to support Glu MTs in wound-edge cells which had been transferred to serum-free medium. A bar at the far right shows the level of oriented Glu MTs in cells incubated with DME alone. Average and standard deviation from four independent experiments are shown.

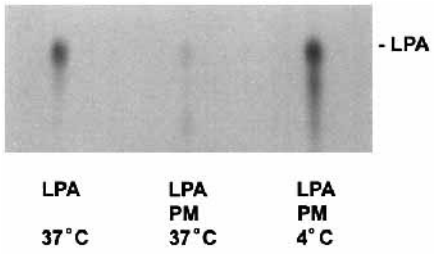


Fig. 8. Degradation of [^{3}H]LPA by incubation with membranes. [^{3}H]LPA was incubated with membranes and a lipid fraction was extracted and separated by TLC as described in the Materials and Methods. Shown is the autoradiogram of the TLC plate at the position of oleoyl-lysophosphatidic acid (LPA). Lanes shown are, from the left to the right, LPA incubated without membranes at 37°C, LPA incubated with membranes at 37°C, and LPA incubated with membranes at 4°C.

pretreatment of 2 μM LPA with membranes at 37°C decreased the ability of LPA to maintain oriented Glu MTs (Fig. 7). The level of oriented Glu MTs after membrane treatment was similar to that observed after treatment with serum free medium ('DME only', Fig. 7). When preincubation was done at 4°C, there was no significant change in the ability of LPA to maintain oriented Glu MTs (Fig. 7).

The ability of membranes to block the activity of LPA was neutralized when high concentrations of LPA were present in the assay medium (data not shown) as was the case with the serum (Fig. 3). We also found that suboptimal concentrations of membranes blocked LPA if a longer preincubation was used (data not shown). The temperature and time dependence of LPA inactivation by the membranes suggested that the membranes were enzymatically degrading LPA rather than simply binding it. To test this directly, we incubated [^{3}H]LPA with membranes and then analyzed the lipids by TLC. Fig. 8 shows that LPA is degraded to other lipid species by incubation with membranes at 37°C. At 4°C, little degradation of LPA was observed. We also found that the ability of membranes to degrade LPA was destroyed if the membranes were boiled before incubation with LPA (data not shown). These results show that membranes are capable of directly degrading LPA, thus explaining the ability of membranes to trigger the loss of Glu MTs from the leading lamella.

DISCUSSION

LPA signaling and maintenance of MT stability

We previously showed that the depletion of Glu MTs provides a reliable and quantifiable marker for contact inhibition of motility (Nagasaki et al., 1994). Our assay for contact inhibition involves an assessment of whether Glu MTs are present and extended in the lamella of cells undergoing directional migration. Our results show that without serum LPA, these extended Glu MTs are lost from the cells (Fig. 4). Some Glu MTs are still present in the LPA-deprived cells, but these are frequently clustered around the nucleus. Thus, these experiments show that LPA is necessary to maintain the normal extended distribution of Glu MTs in cells. Since Glu MTs are known to be stable MTs in cells (Webster et al., 1987; Kreis, 1987; Gundersen and Bulinski, 1988; Gundersen et al., 1994), this raises the possibility that the stability of Glu MTs is regulated by LPA.

Although extended Glu MTs are depleted in the LPA-deprived cells, there are still a small number of Glu MTs clustered around the nucleus. Therefore, we cannot directly distinguish between loss of extended Glu MTs due to depolymerization (the Glu MTs around the nucleus would represent incompletely depolymerized Glu MTs) and loss of extended Glu MTs due to translocation of the Glu MTs to a perinuclear site. In other studies, we have observed the centripetal movement of entire MTs from the leading edge into the cell interior (Mikhailov and Gundersen, 1995), so such a redistribution of Glu MTs would not be without precedent. Nonetheless, we favor the idea that LPA is involved in regulating MT stability since the relative level of Glu MT immunofluorescence is reduced after LPA withdrawal. Thus, removal of LPA may directly interfere with MT stability and lead to a depolymerization of Glu MTs.

The possibility that LPA is an important regulator of MT dynamics is an intriguing one given all the intracellular signalling pathways that are known to be stimulated by LPA. Intracellular signaling by LPA is assumed to be initiated at the cell surface by an LPA receptor, which has not been purified or cloned. However, evidence for a specific, G-protein-coupled LPA receptor has been obtained by photoaffinity probes (van der Bend et al., 1992) and from binding studies (Shiono et al., 1993; Thomson et al., 1994). The intracellular signaling pathways stimulated by LPA include: changes in cAMP via the heterotrimeric G-protein, Gi, activation of protein kinase C, rapid change of intracellular calcium levels, and alterations in actin cytoskeleton mediated by the small GTP-binding protein, rho (reviewed by Moolenaar et al., 1992; Durieux and Lynch, 1993; Ridley, 1995). Which of these pathways is involved in maintaining oriented Glu MTs will need to be tested in future experiments. It is worth noting that LPA has now been show

to affect both actin filaments and MTs, so alterations in LPA signaling could account for the diverse cytoskeletal responses exhibited by cells undergoing contact inhibition of motility (see below).

Plasma membranes contain a phospholipase activity that degrades LPA

Our data show that the plasma membranes trigger a loss of Glu MTs by degrading serum LPA. Numerous lines of evidence suggest that this activity is due to a membrane-associated enzyme: (1) the membrane activity is heat and acid sensitive (Nagasaki et al., 1994); (2) low concentrations of membranes can achieve a maximal response with prolonged incubation; (3) preincubation of LPA with membranes (or membrane extracts) blocked the LPA-induced maintenance of Glu MTs at 37°C, but not at 4°C (Fig. 7); and (4) incubation of [^{3}H]LPA with membranes at 37°C, but not at 4°C, resulted in the loss of [^{3}H]LPA (Fig. 8). In preliminary experiments, we have found that the membrane activity is inhibited by phenylmethylsulfonyl fluoride treatment (data not shown), suggesting that the responsible enzyme contains an active site serine residue as is found in a number of esterases.

A number of membrane localized enzymes capable of degrading LPA have been reported. PLB activities have been detected in plasma membrane fractions (Sekharam et al., 1991; Pind and Kuksis, 1991). A PLB activity would inactivate LPA by degrading it to the fatty acid and the glycerol phosphate, each of which was inactive in our assay (see Table 2). The protease resistant nature of our membrane activity (Nagasaki et al., 1994) is reminiscent of PLB from intestinal brush border membranes, which is solubilized from the membranes by papain digestion (Pind and Kuksis, 1991). Another candidate enzyme is the ecto-LPA phosphatase that was recently identified on the surface of keratinocytes (Xie and Low, 1994) and neutrophils (Perry et al., 1993). These studies suggest that phospholipases are present on the surface of many cells and support the possibility that LPA levels are regulated locally by cell surface enzymes.

Implication for contact inhibition of motility

Historically, contact inhibition has been viewed as a cell-cell interaction and response behavior (Heaysman, 1978). The responses are thought to be triggered by an interaction of a ligand on one cell surface with a receptor on the other cell surface. However, our data with membranes and with serum removal suggest that a loss of oriented Glu MTs, one of the contact inhibition reactions in fibroblasts, is triggered when a continuous signal arising from a serum cytokine, LPA, is interrupted.

It is possible that cells require a constant and localized stimulus from serum LPA to maintain an active, polarized morphology to continue directed migration. In the absence of extracellular LPA, or of intracellular signals emanating from the putative LPA receptor, cells would lose the polarization necessary for directed migration. Cells may be able to redirect their migration (i.e. turn) if portions of the cell that are not in contact with other cells still exhibit LPA signalling. In this sense, the loss of LPA signalling is analogous to the tumbling response exhibited by chemotaxing bacteria; without the intracellular signals elicited by LPA, the cells becomes depolarized and this allows the cell to repolarize toward a new site that still maintains LPA signalling. In support of this, we have found in preliminary studies that depletion of LPA causes other contact inhibition reactions, such as decreased cell locomotion, decreased lamellipodial motility, and loss of filamentous actin cytoskeleton in motile NRK cells (data not shown).

Our results presented in this report have not ruled out the possibility that there is another serum factor (other than LPA) whose depletion triggers all of the cellular reactions associated with contact inhibition. It is now possible, however, to test if LPA is the only serum factor for contact inhibition by specifically removing LPA from serum, such as with PLB, and examining if the LPA-depleted serum will trigger all of the contact inhibition-associated reactions. Regardless of the precise mechanism, our identification of the LPA signaling pathway as a potential target for contact inhibition should facilitate further dissection of contact inhibition of motility at a molecular level. Since the data in this study have been obtained from a reconstituted system, it will also be important in the future to determine whether LPA signaling is abrogated in actual colliding cells and whether this occurs as a result of depleting external LPA or of interfering with internal LPA-generated signals.

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REFERENCES

Abercrombie, M. and Heaysman, J. E. M. (1954). Social behaviour of cells in tissue culture, II. 'Monolayering' of fibroblasts. *Exp. Cell Res.* **6**, 293-306.

Abercrombie, M. (1970). Contact inhibition in tissue culture. *In Vitro* **6**, 128-142.

Abercrombie, M. (1979). Contact inhibition and malignancy. *Nature* **281**, 259-262.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917.

Bulinski, J. C. and Gundersen, G. G. (1991). Stabilization and post-translational modification of microtubules during cellular morphogenesis. *BioEssays* **13**, 285-293.

Durieux, M. E. and Lynch, K. R. (1993). Signalling properties of lysophosphatidic acid. *Trends Pharmacol. Sci.* **14**, 249-254.

Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W. H. (1993). The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* **291**, 677-680.

Gundersen, G. G., Khawaja, S. and Bulinski, J. C. (1987). Postpolymerization detyrosination of α-tubulin: a mechanism for subcellular differentiation of microtubules. *J. Cell Biol.* **105**, 251-264.

Gundersen, G. G. and Bulinski, J. C. (1988). Selective stabilization of microtubules oriented toward the direction of cell migration. *Proc. Nat. Acad. Sci. USA* **85**, 5946-5950.

Gundersen, G. G., Kim, I. and Chapin, C. J. (1994). Induction of stable microtubules in 3T3 fibroblasts by TGF-beta and serum. *J. Cell Sci.* **107**, 645-59.

Heaysman, J. E. (1978). Contact inhibition of locomotion: a reappraisal. *Int. Rev. Cytol.* **55**, 49-66.

Hess, H. H. and Derr J. E. (1975). Assay of inorganic and organic phosphorus in the 0.1-5 nanomole range. *Anal. Biochem.* **63**, 607-613.

Jalink, K., van Corven, E. J. and Moolenaar, W. H. (1990). Lysophosphatidic acid, but not phosphatidic acid, is a potent $Ca^{2(+)}$-mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action. *J. Biol. Chem.* **265**, 12232-12239.

Kreis, T. E. (1987). Microtubules containing detyrosinated tubulin are less dynamic. *EMBO J.* **6**, 2597-2606.

Mikhailov, A. V. and Gundersen, G. G. (1995). Centripetal transport of microtubules in motile cells. *Cell Motil. Cytoskel.* **32**, 173-186.

Moolenaar, W. H., Jalink, K. and van Corven, E. J. (1992). Lysophosphatidic acid: a bioactive phospholipid with growth factor-like properties. *Rev. Physiol. Biochem. Pharmacol.* **119**, 47-65.

Nagasaki, T., Chapin, C. J. and Gundersen, G. G. (1992). Distribution of detyrosinated microtubules in motile NRK fibroblasts is rapidly altered upon cell-cell contact: implications for contact inhibition of locomotion. *Cell Motil. Cytoskel.* **23**, 45-60.

Nagasaki, T., Liao, G. and Gundersen, G. G. (1994). Isolated plasma membranes induce the loss of oriented detyrosinated microtubules and other contact inhibition-like responses in migrating NRK cells. *J. Cell Sci.* **107**, 3413-3423.

Perry, D. K., Stevens, V. L., Widlanski, T. S. and Lambeth, J. D. (1993). A novel ecto-phosphatidic acid phosphohydrolase activity mediates activation of neutrophil superoxide generation by exogenous phosphatidic acid. *J. Biol. Chem.* **268**, 25302-25310.

Pind, S. and Kuksis, A. (1991). Further characterization of a novel phospholipase B (phospholipase A2 – lysophospholipase) from intestinal brush-border membranes. *Biochem. Cell Biol.* **69**, 346-357.

Raben, D., Lieberman, M. A. and Glaser, L. (1981). Growth inhibitory protein(s) in the 3T3 cell plasma membrane. Partial purification and dissociation of growth inhibitory events from inhibition of amino acid transport. *J. Cell. Physiol*. **108**, 35-45.

Ridley, A. J. (1995). Rho-related proteins: actin cytoskeleton and cell cycle. *Curr. Opin. Genet. Dev.* **5**, 24-30.

Sekharam, K. M., Patel, J. M. and Block, E. R. (1991). Plasma membrane-specific phospholipase A1 activation by nitrogen dioxide in pulmonary artery endothelial cells. *Toxicol. Appl. Pharmacol.* **107**, 545-554.

Shiono, S., Kawamoto, K., Yoshida, N., Kondo, T. and Inagami, T. (1993). Neurotransmitter release from lysophosphatidic acid stimulated PC12 cells: involvement of lysophosphatidic acid receptors. *Biochem. Biophys. Res. Commun.* **193**, 667-673.

Thomson, F. J., Perkins, L., Ahern, D. and Clark, M. (1994). Identification and characterization of a lysophosphatidic acid receptor. *Mol. Pharmacol.* **45**, 718-23.

Tokumura, A., Harada, K., Fukuzawa, K. and Tsukatani, H. (1986). Involvement of lysophospholipase D in the production of lysophosphatidic acid in rat plasma. *Biochim. Biophys. Acta.* **875**, 31-38.

van der Bend, R. L., Brunner, J., Jalink, K., van Corven, E. J., Moolenaar, W. H. and van Blitterswijk, W. J. (1992). Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. *EMBO J.* **11**, 2495-2501.

Webster, D. R., Gundersen, G. G., Bulinski, J. C. and Borisy, G. G. (1987). Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Nat. Acad. Sci. USA* **84**, 9040-9044.

Xie, M. and Low, M. G. (1994). Identification and characterization of an ecto-(lyso)phosphatidic acid phosphatase in PAM212 keratinocytes. *Arch. Biochem. Biophys.* **312**, 254-259.

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