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Characterization of Epidermal Growth Factor Receptor Function in Lysophosphatidic Acid Signaling in PC12 Cells

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Abstract Lysophosphatidic acid (LPA) is a lipid metabolite that induces the activation of mitogen-activated protein kinase (MAPK) through binding to the G protein-coupled receptor in a number of cell lines and cultures. Recent studies have revealed that LPA is able to rapidly induce the phosphorylation of MAPK through an epidermal growth factor (EGF) receptor-dependent pathway. We investigated the role of the EGF receptor in the signaling pathway initiated by LPA stimulation in nerve growth factor (NGF)-responsive PC12 cells well known to transiently retract their own neurites upon LPA stimulation. LPA-stimulated MAPK signaling was suppressed by the selective EGF receptor inhibitor and in the dominant negative mutant EGF receptor cell line. As in the EGF signaling pathway, the complex of EGF receptor with adapter proteins Shc and Sos was formed in response to LPA stimulation, suggesting there is an intracellular mechanism for transactivation. A neurite retraction assay was also performed to examine the role of the EGF receptor in PC12 cell differentiation, which related to the involvement of LPA-induced neurite retraction. These results suggest that the receptor tyrosine kinase can be activated in a ligand-independent manner through intracellular crosstalk between the signaling pathways. J. Cell. Biochem. 76:386–393, 2000.

Key words: EGF receptor; LPA-signaling; G-protein coupled receptor; ERK activation; neurite retraction; PC12 cells

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol-3-phosphate) is the smallest and structurally simplest of all phospholipids, and its functions as intracellular signaling molecules are recently being appreciated [Jalink et al., 1994; Moolenaar et al., 1997]. It has become clear that LPA is a normal constituent of serum in the lower micromolar range [Zhang et al., 1994], produced and released by activated platelets during blood clotting. LPA binds strongly to serum albumin, while retaining its biological activity, and LPA becomes protected against hydrolysis by serum-phospholipases [Checovich et al., 1993]. Exogenous LPA largely formed through deacylation of phosphatidic acid can exert quite diverse effects, and the investigations on its other roles are continuing. LPA especially induces neurite retraction and neurotransmitter release in PC12 cells [Checovich et al., 1993; Barry and Critchley, 1994]. There is compelling evidence that LPA exerts its multiple actions via its own G protein-coupled receptor (GPCR) present in numerous cell types. It seems likely that LPA serves various functions in vivo both in normal physiological and pathological conditions, such as a possible growth factor-like role not only in wound-healing and inflammation but probably also in oncogenesis and metastasis.

Abbreviations used: LPA, Lysophosphatidic acid; GPCR, G protein-coupled receptor; NGF, nerve growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; BK, bradykinin; PLC, phospholipase C; EGF, epidermal growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffered saline; PTX, pertussis toxin; MBP, myelin basic protein.

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PC12 cells established from a rat adrenal pheochromocytoma are differentiated into sympathetic neurons upon treatment with a nerve growth factor (NGF) [Marshall, 1995]. This action of NGF in these cells requires activation of extracellular signal-regulated kinases (ERKs), a family of mitogen-activated protein kinase (MAPK), and induction of gene expression.

Biological activities of other external stimuli, such as growth factors, hormones, and neurotransmitters, are generally mediated by another family of receptors possessing seven transmembrane domains and coupled to heterotrimeric G protein [Strosberg, 1991]. The mechanisms by which mitogenic GPCR activates the MAPK-signaling pathway are poorly understood. The candidate protein tyrosine kinases that link GPCR with MAPK include Src family kinase [Sadoshima and Izumo, 1996], the epidermal growth factor (EGF) receptor [Daub et al., 1996], and Lyn [Wan et al., 1996]. Many results indicate that the LPA receptor couples to at least three distinct G proteins which includes G_i, G_q, and $G_{12/13}$. G_i triggers Ras·GTP accumulation and inhibition of adenyl cyclase [van Corven et al., 1993], G_q links the receptor to phospholipase C (PLC), and $G_{12/13}$ mediates Rho-activation [Buhl et al., 1995]. In G_i-mediated Ras·MAPK pathway initiated by LPA, the activation involves the sequential interaction of one or more protein tyrosine kinases with the signaling proteins Shc, Grb2, and the GDP/GTP exchanger Sos [van Biesen et al., 1995; Touhara et al., 1995; Luttrell et al., 1996]. Moreover, it was reported that activation of MAPK by a G_i-coupled receptor, including LPA receptor, is proceeded by the $G\beta\gamma$-mediated tyrosine phosphorylation, leading to an association between Shc, Grb2, and Sos in COS7 cells [van Biesen et al., 1995]. Neuropeptide bradykinin (BK), as well as LPA, elicits a wide range of biological responses in various cell types and tissues [Dikic et al., 1996]. In PC12 cells, BK activates a GPCR that stimulates the production of diacyl glycerol and inositol-1, 4,5-triphosphate by activation of PLC [Lev et al., 1995]. In PC12 cells, PYK2 was phosphorylated on the tyrosine residue and found to form a complex with activated Src when stimulated with either LPA or BK [Proud and Kaplan, 1988]. Activated PYK2 led to initiate MAPK signaling pathway [Fasolato et al., 1988], which occurred through the action of Grb2 and Sos.

To analyze the specific LPA signaling pathway in neuronal PC12 cells, we investigated the involvement of possible proteins, such as EGF receptor and protein kinase C (PKC) on LPA-induced MAPK pathway, and we also performed an acute neurite retraction assay to assess LPA-induced neurite retracting action. We found that the EGF receptor has a specific role in LPA-induced MAPK activation, as well as neurite retracting action in PC12 cells.

MATERIALS AND METHODS

Materials

LPA (Sigma) was dissolved in phosphate-buffered saline (PBS) containing 4% bovine serum albumin at 5 mM final concentration. Anti-EGF receptor pAb, anti-phosphotyrosine monoclonal antibody (mAb) 4G10, anti-Grb2 mAb, anti-Shc pAb, anti-Sos pAb, and anti-sheep IgG pAb were purchased from Upstate Biotechnology, and anti-phosphorylated ERK pAb was from New England Biolab. BK, PTX, GF109203X, AG1478, and PMA were purchased from Calbiochem. Protein G Sepharose was purchased from Pharmacia Biotech. The Western blotting detection reagent ECL™ and anti-mouse IgG pAb were obtained from Amersham. RPMI1640 media, fetal bovine serum, and horse serum were purchased from GIBCO. The other materials were purchased from Sigma.

Cell Cultures

PC12 cells obtained from the American Type Culture Collection were cultured in RPMI1640 supplemented with 10% fetal bovine serum and 5% horse serum (growth medium). Cells were grown on a collagen (Type I; 1 mg/ml)-coated dish. The PC12/HER-CD533/Tet cell line which expresses dominant negative mutant form of EGF receptor HER-CD533 under the control of a tetracycline-sensitive promoter system was kindly provided by Axel Ullrich [Zwick et al., 1997].

ERK Phosphorylation and Immunoprecipitation With Anti-EGF Receptor Antibody

Cells were grown to 90% confluency in a growth medium on 60 mm plates, washed with PBS, and starved for 3 h in a serum-free RPMI1640 medium. The cells were stimulated with appropriate ligands for the indicated times at 37°C, aspirated, and lysed in 0.5 ml lysis buffer, as described [Seedorf et al., 1994; Park

et al., 1998]. The lysates were centrifuged for 15 min at 4°C. For ERK phosphorylation, The supernatants were analyzed by gel electrophoresis and transferred to the nitrocellulose membrane, which was immunoblotted by anti-phosphoERK pAb. For immunoprecipitation, the supernatants were incubated with an excess of anti-EGF receptor pAb overnight, and then with protein G-Sepharose for 2 h at 4°C. Immunoprecipitates were washed with HNTG buffer [Redemann et al., 1992]. The pellets were then resuspended in Laemmli buffer, analyzed, and then detected.

MAPK Assay

A MAPK assay was performed as described [Zwick et al., 1997]. Endogenous ERK2 was immunoprecipitated, washed three times with HNTG buffer, and washed once with kinase buffer (20 mM HEPES, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 uM sodium orthovanadate). Kinase reactions were performed in 30 ul of kinase buffer supplemented with 0.5 mg/ml myelin basic protein, 50 uM ATP, 1 uCi of [γ-^{32}P] ATP for 10 min at room temperature. Laemmli buffer was added to stop the reaction, and the mixture was subjected to the gel electrophoresis.

Acute Neurite Retraction Assay

PC12 cells were seeded on the plate in the growth medium with a density of 5,000 cells/cm^2 and allowed to grow for 24 h. The cells were incubated with a chemically defined medium (RPMI1640 medium containing 10 µg/ml insulin, 6.7 mg/ml sodium selenite, 5.5 µg/ml sodium transferrin, 110 µg/ml pyruvate, 0.5% (w/v) glucose, 1 mM glutamine, 20 nM progesterone, and 0.1 mM putrescine) for 24 h. Then, the cells were incubated with the defined medium containing 50 ng/ml NGF in order to differentiate the cells. After 3 days of differentiation, the differentiated cells were treated with LPA and various inhibitors. To pretreat cells with AG1478 and/or PTX, usually an equal volume of these agents was added to the dishes without removing the medium present. The controls received 0.1% DMSO, which by itself had no effect. After pretreatment, the cells were fed again with LPA and incubated for 1 h at 37°C. After 1 h, the cells were washed with PBS, and then fixed with 4% formaldehyde in PBS for the neurite outgrowth measurement. All the data were counted by two persons and assessed. Three random photographs were made per dish. Each scored microscopic field was considered as one observation, so that, in each experiment, the mean score for each treatment group was the average of nine observations. For the quantification of neurites, fixed cells bearing neurites longer than twice the length of the cell body were considered as positive. A total of over 400 cells were counted, and each data point corresponds to the counts obtained from at least three independent dishes.

RESULTS

The present study provides insights into signaling events that link GPCR to activation of the MAPK pathway.

Transient Phosphorylation of ERK by GPCR Agonists

The analysis of endogenous GPCR signaling events in PC12 cells led to the rapid and transient identification of phosphorylated ERK-1, two with molecular masses of 42,44 kDa in response to the stimulation of LPA and BK. As that shown in Figure 1, comparison of the ERK time course experiments with BK, LPA, and EGF revealed that LPA more rapidly induced ERK phosphorylation, only slightly lagging behind that induced by EGF. LPA-induced phosphorylation of the ERKs reached a maximum within 2 min after stimulation and persisted for about 10 min. Suggesting that both LPA and EGF use EGF receptor to transduce their signals, an additional LPA-triggered signal exists to synergize with the EGF receptor-mediated signaling, leading to a more rapid ERK phosphorylation than that triggered by EGF during the early stage of LPA-stimulated ERK activation. BK-induced ERK phosphorylation was also transient and rapid. In comparison with GPCR-

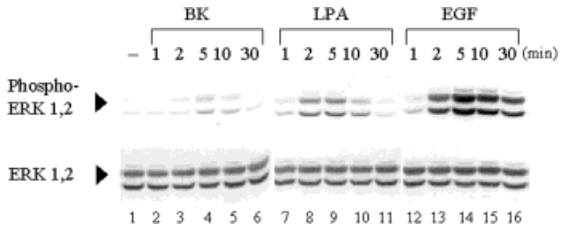

Fig. 1. Time-dependent phosphorylation of ERK by various agonists in PC12 cells. Induction of ERK phosphorylation was analyzed upon stimulation of serum-starved PC12 cells for 1, 2, 5, 10, and 30 min with 1 µM BK, 25 µM LPA and 5 ng/ml EGF, each. Western blotting was performed with anti-phosphoERK antibody and anti-ERK antibody.

mediated stimulations, ERK phosphorylation induced by only 5 ng/ml EGF was more pronounced and was sustained more than 30 min.

EGF Receptor Is Implicated in the LPA-Induced MAPK Pathway

The EGF receptor and erbB2 have been implicated in MAPK activation induced by LPA and other agonists of GPCRs [Daub et al., 1996]. The role of the EGF receptor in GPCR signaling was, therefore, analyzed using a highly specific inhibition of an EGF receptor signal. Myeline basic protein (MBP) phosphorylation, following stimulation with LPA, was inhibited by pretreatment of PC12 cells with tyrphostin AG1478, a selective EGF receptor antagonist with a nanomolar range [Levitzki and Gazit, 1995]. The ERK activation to phosphorylate MBP by NGF stimulation was not affected, whereas that by EGF stimulation was totally blocked. This means that AG1478 specifically inhibits the kinase activity of the EGF receptor (Fig. 2A, upper panel). MBP phosphorylation induced by BK, which transduces its signal by a G_q protein, was also decreased. This decrease in ERK activation may be due to the calcium influx caused by BK upon the basis of the findings that calcium is critical for EGF receptor-transactivation in PC12 cells [Zwick et al., 1997]. BK-induced calcium effects are shown in Figure 4. It has also been suggested that G_q-coupled receptors can mediate stimulation of EGF receptor tyrosine phosphorylation and, moreover, that for both G_i- and G_q-coupled receptor subfamilies, this event is essential for the stimulation of Shc tyrosine phosphorylation and MAPK activation [Daub et al., 1997]. The same effect was achieved in PC12/HER-CD533/Tet (Fig. 2A, lower panel). This cell line expresses the dominant negative EGF receptor mutant HER-CD533 under the control of tetracycline-sensitive promoter system. This mutant lacks the cytoplasmic domain and disrupts EGF receptor downstream signaling. In this cell line, expression of HER-CD533 is suppressed in the presence of tetracycline, whereas the removal of tetracycline results in an approximately 20-fold induction within 48 h [Zwick et al., 1997]. Expression of HER-CD533 strongly interfered with EGF receptor tyrosine phosphorylation upon the treatment with EGF (Fig. 2B). As a result, it appears that both LPA receptor-coupled G_i protein and BK receptor-coupled G_q protein are essential in order to activate the

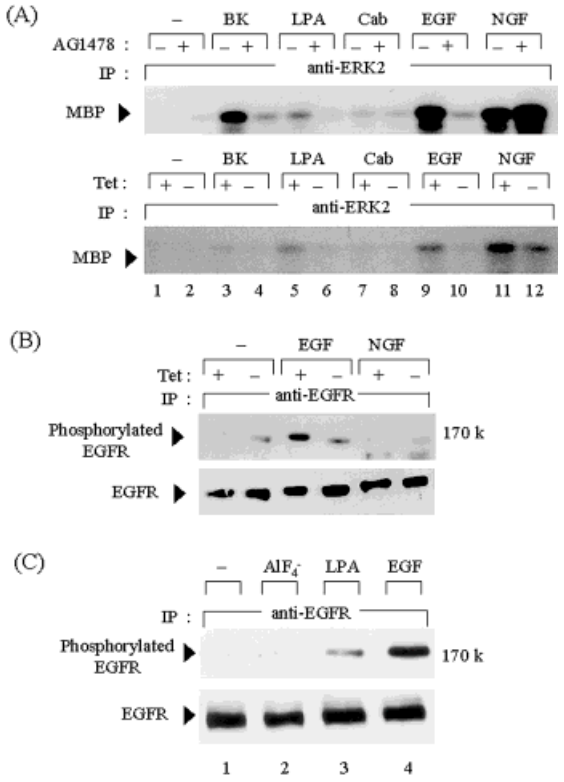

Fig. 2. EGFR is implicated in LPA-induced MAPK pathway. **A**: PC12 cells **(upper panel)** and PC12/HER-CD533/Tet **(lower panel)** were pretreated with or without AG1478 (500 nM) for 5 min (upper panel) or tetracycline for 48 h (lower panel) to inhibit EGF receptor kinase activity. The cells were stimulated with indicated ligands (1 μM BK, 25 μM LPA, 1 μM Carbachol (Cab), 10 ng/ml EGF and 20 ng/ml NGF) and lysed, and endogenous ERK2 activity was determined using MBP. Phosphorylated MBP was detected by autoradiography after gel electrophoresis. **B**: In PC12/HER-CD533/Tet, the EGF receptor was immunoprecipitated using anti-EGF receptor antibody. The tyrosine-phosphorylated EGF receptor was detected by anti-phosphotyrosine antibody **(upper panel)**, followed by reprobing of the same filter with anti-EGFR antibody **(lower panel)**. **C**: Serum-starved PC12 cells were treated with described ligands (LPA, AlF^{4-} (30 mM NaF and 10 μM $AlCl_3$) and EGF) and lysed. Immunoprecipitation with anti-EGF receptor antibody was performed, and then it was immunoblotted with appropriate antibodies on the same filter. IP, immunoprecipitation.

EGF receptor in MAPK pathway. The contribution of an EGF receptor to LPA-induced MAPK pathway was further investigated by immunoprecipitation with anti-EGF receptor polyclonal antibody. Following treatment with LPA, endogenous EGF receptor was found to be tyrosine-phosphorylated (Fig. 2C) and Shc and Sos-1 were associated with tyrosine-phosphorylated EGF receptor (data not shown). However, when cells were treated with AlF_4^-, that is non-hydrolyzable analog of GTP and continuously activates G proteins, cells did not show the

effects of LPA to induce the tyrosine-phosphorylation of the EGF receptor and further association with Shc, which suggests that there is another specific mechanism that leads to activate the EGF receptor by LPA. This also suggests the analogous signaling pathway between LPA/BK and EGF.

PKC Is Involved in LPA-Induced ERK Phosphorylation

The incubation of PC12 cells with 200 nM phorbol 12-myristate 13-acetate (PMA) for 5 min induced the phosphorylation of ERK, suggesting that the phosphorylation of ERKs could also be mediated by PKC activation. It is possible that GPCRs transduce their signals through PKC to activate ERKs. To test this hypothesis, PMA-sensitive PKC isoenzymes were down regulated by prolonged treatment of PMA. The down-regulated PKC completely abolished the subsequent ERK phosphorylation on the LPA and transient PMA treatment, but had only a minor effect on BK-stimulated phosphorylation of ERKs (Fig. 3). This suggests that the phosphorylation of ERK can be induced by a PKC-dependent mechanism by LPA, but by a PKC-independent mechanism by BK.

LPA-Induced ERK Activation Does Not Involve Calcium Influx

As it was recently reported that LPA and BK activate calcium influx in PC12 cells [Tigyi et al., 1996], it was tested whether the extracellular calcium upon the stimulation with LPA and BK has any role in phosphorylating ERKs. Elimination of extracellular calcium by the addition of 3 mM EGTA to the medium did not affect LPA-induced ERK phosphorylation,

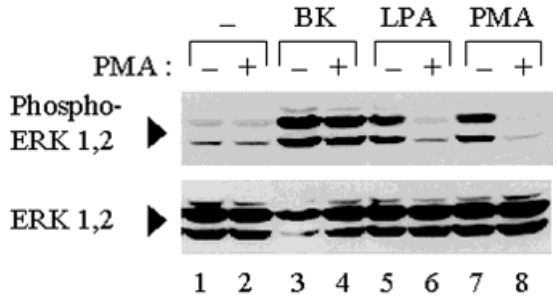

Fig. 3. Effects of specific inhibition of PKC on ERK phosphorylation. Quiescent cells were pretreated with PMA (200 nM) for 20 h to down-regulate PKC, and then treated with indicated agonists (1 μM BK, 25 μM LPA, and 200 nM PMA). Cell lysates were immunoblotted as described.

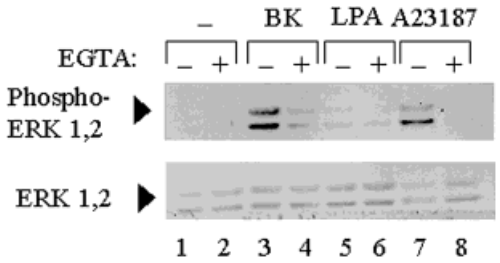

Fig. 4. Effects of EGTA on GPCR agonists-induced ERK phosphorylation. The Ca^{2+} chelator, EGTA (1 mM) was preincubated for 3 min following the incubation with the indicated agonists (1 μM BK, 25 μM LPA, and 2.5 μg/ml A23187).

whereas it did affect BK-induced phosphorylation (Fig. 4). Moreover, when using the calcium ionophore, A23187, to directly elevate intracellular calcium levels, the enhanced ERK phosphorylation was remarkably reduced, as expected, by EGTA. Based on this result, it seems that LPA action to phosphorylate ERK does not essentially involve calcium influx, while BK phosphorylates ERKs through a pathway involving calcium influx in PC12 cells.

EGF Receptor Has Its Own Role in LPA-Induced Neurite Retraction

The effects of AG1478 and PTX on LPA-treated PC12 cells were examined by performing an acute neurite retraction assay. Upon application of LPA to cultures of PC12 cells differentiated with 50 ng/ml NGF for 3 days, there was a change in the morphology (Fig. 5A, upper panel). The most prominent change was the rapid retraction of neurites, which occurred only within the first 1 h. This neurite-retracting activity of PC12 cells was impaired when the EGF receptor was specifically inhibited by tyrphostin AG1478 (Fig. 5A, lower panel). To analyze this loss of neurite-retracting activity, cells bearing neurites were counted and then diagrammatized in the Figure 5B. Percent cells bearing neurites of the LPA-treated group have decreased only by 43% in the presence of AG1478, whereas that of the control group decreased by 66%. On the other hand, LPA-induced neurite-retracting activity was damaged very little by the preincubation of G_i-sensitive PTX. This was an expected result due to the fact that neurite retracting action by LPA in PC12 cells is dependent on PTX-insensitive $G_{12/13}$ α subunit not G_i protein [Tigyi et al., 1996a]. These results demonstrate that the EGF receptor is related to LPA-induced neurite-

retracting action, as well as LPA-induced ERK phosphorylation.

DISCUSSION

Time course experiments in Figure 1 show that LPA-induced phosphorylation of the ERKs persists for about 10 min, which was very transient compared with EGF-stimulated phosphorylation of ERKs, which could be sustained up to 30 min. This means that accelerated receptor down-regulation possibly exists in mediating LPA-specific cellular responses. With NGF stimulation, however, phosphorylation of ERKs is reported to be sustained for more than several hours [Marshall, 1995]. A recent report supports this idea that more sustained ERK phosphorylation by NGF is necessary for differentiating PC12 cells [York et al., 1998]. Therefore, this transient activation of ERK could explain the mitogenic rather than the differentiation action of LPA.

It is well known that many GPCR ligands activate MAPK in various cell types. Endothelin-1, for example, induced MAPK activation, which involved Shc, Grb2, and probably Raf-1 in primary cultures of astrocytes [Cazaubon et al., 1993, 1994]. And stimulation of the thrombin receptor phosphorylated the adaptor protein Shc on tyrosine residue in a pertussis toxin (PTX)-insensitive manner, leading to ERK activation in a CCL39 fibroblast [Chen et al., 1996]. Endothelin-1 (100 nM) and thrombin (2 U/ml) were, therefore, tested in PC12 cells, which resulted in no phosphorylation of ERKs (data not shown).

And we confirmed that LPA-induced MAPK pathway is dependent on the Gi protein in PC12 cells by using Gi protein-sensitive PTX (data not shown), as shown in Cos-1 cells of previous report [Howe and Marshall, 1993].

In this study, we used the quinazoline drug, tyrphostin AG1478 as an inhibitor for a selective EGF receptor. Tyrphostin AG1478 is highly specific for EGFR. The amount of inhibitors required to inhibit the EGFR kinase activity by 50%(IC50) is in the nanomolar range, whereas micromolar concentrations are required to inhibit the HER2-ErbB2 kinase and much higher concentrations are required to inhibit the platelet-derived growth factor receptor (PDGFR) in vitro or in intact cells [Levitzki and Gazit, 1995].

Based on the following findings, we concluded that G protein-mediated EGF receptor activation is involved in both BK- and LPA-induced MAP kinase activation: i) Inhibition of EGF receptor in intact PC12 cells by AG1478 that specifically inhibits the kinase activity of EGF receptor interferes with LPA-induced MAP kinase activation (Fig. 2A); ii) LPA-induced EGF receptor activation, as inferred from tyrosine phosphorylation of EGF receptor (Fig. 2C) and complex formation with Shc and Sos, was sensitive to PTX hence downstream of Gi (data not shown). Other investigators suggested that transactivation of the EGF receptor links GPCR

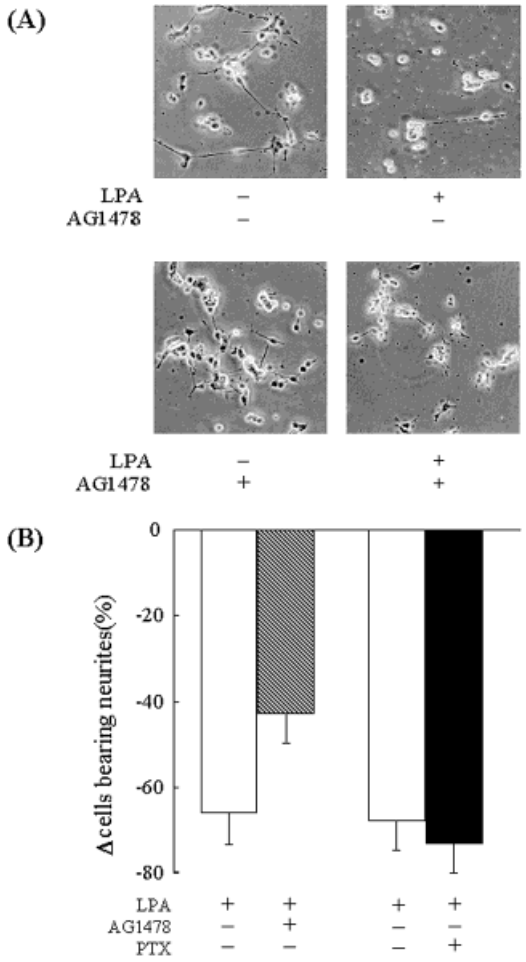

Fig. 5. EGF receptor is implicated in LPA-induced neurite retraction. **A**: PC12 cultures differentiated for 3 days by NGF (50 ng/ml) were preincubated with AG1478 (500 nM, 5 min) and/or PTX (100 ng/ml, 20 h) and then exposed to LPA (25 μM) for 1 h. To exclude the effect of the presence of LPA in the serum, PC12 cells were serum-starved in the chemically defined medium described above. A total of over 400 cells was counted by using three dishes per treatment group. **B**: The percent difference of cells bearing neurites after the treatment of LPA in the presence of AG1478 ▧, was decreased when compared to the one after by only adding LPA □. On the other hand, after adding PTX ■ the difference in cells bearing neurites was changed little. All the data are reported as means ± standard deviation.

to Ras-dependent MAPK activation in HaCaT keratinocytes, primary mouse astrocytes, and COS-7 cells, as well as Rat-1 cells [Daub et al., 1996, 1997]. Others using Rat-1, COS cells [Kranenburg et al., 1997] and even PC12 cells [Proud and Kaplan, 1988] have not been able to detect LPA-induced phosphorylation of EGF receptor. In addition, as a novel mediator, there was a report that Src family kinases may act in concert with the cell type-specific PYK2 tyrosine kinase to activate ERKs in response to LPA or BK in PC12 cells [Proud and Kaplan, 1988].

In our experiment, as shown in Figure 3, PKCs found to be involved in LPA-induced ERK phosphorylation. The involvement of PKC was tested by down-regulating PMA-sensitive PKC isoenzymes. This prolonged treatment with PMA completely abolished the subsequent ERK activation caused by LPA and PMA treatment, but had only a minor effect on BK-stimulated phosphorylation of ERKs. In EAhy926 cells, LPA-induced activation of MAPK is wholly dependent on PKC [Mclees et al., 1995]. Moreover the phosphorylation of PYK2, which was reported to link GPCR-induced ERK activation, was reduced on the treatment of BK by prolonged treatment of PMA [Fasolato et al., 1988], meaning that PKC is involved in the phosphorylation of PYK2. There are still conflicting reports that LPA-induced activation of ERKs occurred mainly via the PKC-independent, PTX-sensitive activation of $p21^{ras}$ in COS7 cells [van Biesen et al., 1995].

We showed that the neurite-retracting activity of LPA decreased by a third, suggesting that EGF receptor have its role in neurite-retracting action. The Ras-related GTPase Rho was reported to mediate cell rounding and neurite retraction in response to LPA in PC12 cells [Tigyi et al., 1996]. Phosphorylation of ERKs in response to LPA was not so high comparing with those in response to BK or other growth factors such as EGF or NGF in PC12 cells. In Figure 4, we could see the diminution of the phosphorylated ERKs to the basal level in response to A23187, the calcium ionophore, whereas the LPA-induced ERKs were still phosphorylated upon the treatment with EGTA.

Using LPA and BK as GPCR agonists, the present report has established that, in response to LPA, the tyrosine-phosphorylated EGF receptor forms a complex with Shc and Sos and meets criteria for linking the G protein to MAPK activation in PC12 cells. Moreover, an EGF receptor is also involved in LPA-induced neurite-retracting action. These findings provide a new basis for the investigation of GPCR-mediated signals and the significance for biological phenomena such as neural cell survival and neurodegenerative diseases. More work is necessary to study the effect of LPA and LPA-like lipid mediators in primary neuronal cells, particularly because the highest amount of specific binding of LPA was found in the brain [Thomson et al., 1996].

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