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Activation of c-Jun N-Terminal Kinase (JNK) by Lysophosphatidic Acid in Swiss 3T3 Fibroblasts[1]

Takehiko Sasaki, Tomohiko Maehama, Toshiyoshi Yamamoto, Shunsuke Takasuga, Shin-ichi Hoshino, Hiroshi Nishina, Osamu Hazeki, and Toshiaki Katada[2]

Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

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Lysophosphatidic acid (LPA) induced activation of c-Jun N-terminal kinase (JNK) in Swiss 3T3 fibroblasts. This activation reached the maximum at 20 min and required a high concentration of LPA with an EC_{50} value of approximately 3 μg/ml. LPA-induced activation of JNK was not suppressed by prior treatment of the cells with pertussis toxin, whereas it was completely blocked by suramin, a non-selective inhibitor of ligand-receptor interactions. The kinetics and concentration-dependency of LPA-induced JNK activation were in sharp contrast with those of LPA-induced extracellular signal-regulated kinase (ERK) activation, which reached the maximum within 3 min and occurred with an EC_{50} of 0.1 μg/ml. The ERK activation was susceptible to pertussis toxin, whereas it was not inhibited by suramin. These results indicate that the signal transduction pathways of LPA-induced JNK and ERK activations are distinct. Thus, this is the first report showing that LPA induces not only ERK activation but also JNK activation, which may be responsible for the induction of DNA synthesis in LPA-stimulated Swiss 3T3 fibroblasts.

Key words: c-Jun N-terminal kinase (JNK), DNA synthesis, extracellular signal-regulated kinase (ERK), lysophosphatidic acid (LPA).

Phospholipids and a number of their metabolites exhibit a variety of biological activities. Among them, lysophosphatidic acid (LPA), the smallest and structurally simplest phospholipid, evokes various biological responses such as fibroblast proliferation (*1*), platelet aggregation (*2*), smooth muscle contraction (*3*), and cell shape changes (*4*). LPA is generated and released from activated platelets (*5*), injured fibroblasts (*6*), and ovarian tumors (*7*), suggesting both physiological and pathological roles for the lipid (*8-10*). For these reasons, much attention has been paid to the biochemical signal transduction pathways utilized by LPA to elicit cellular responses, especially proliferation in fibroblasts.

Like many G protein-coupled receptor agonists, LPA stimulates a variety of early biochemical responses, including rapid hydrolysis of phosphatidylinositol bisphosphate, mobilization of intracellular Ca^{2+}, and activation of protein kinase C, which may be important signals in the early cell cycle (*1*). Other actions of LPA include the inhibition of adenylate cyclase (*1*), the induction of c-*fos* gene (*11*), and the activation of ERK (*12*), focal adhesion kinase (*13*), Ras (*14*), phospholipase A_2 (*1*), and phospholipase D (*15*). However, reconstitution of these pathways in fibroblasts is insufficient to stimulate DNA synthesis (*7*). The ability of LPA to stimulate DNA synthesis is inhibited by pertussis toxin (PTX), demonstrating a role of the G_i family of G-proteins. There is strong evidence indicating that the Ras-ERK pathway is required for LPA-stimulated DNA synthesis. First, the Ras-ERK pathway is regulated in a PTX-sensitive manner similar to proliferation (*14*). Second, injection of anti-Ras antibodies blocks the DNA synthesis (*16*). Last, dominant-negative mutants of Ras inhibit stimulation of the mitogenesis (*17, 18*). However, LPA is about 100-fold less potent in inducing DNA synthesis than in evoking Ras and ERK activation and the other early responses, and this has been the subject of argument (*7, 19*). Some functional studies have suggested that multiple subtypes of LPA receptors with distinctive signaling properties mediate diverse cellular effects of LPA. Recently, several groups have just reported the isolation of cDNAs encoding two structurally different seven transmembrane-spanning receptors for LPA (*20, 21*).

The proto-oncogene c-*jun* acts as an immediate early response gene, coupling short-term signals received at the cell membrane to long-term adaptive cellular responses by altering patterns of gene expression. c-Jun associates with Fos and ATF families, yielding different heterodimeric transcription factors that exhibit differences in DNA-binding specificity (*22, 23*). The transcriptional response of the c-*jun* promoter is mediated by two promoter elements

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[2] To whom correspondence should be addressed: Tel: +81-3-3812-2111 (Ext. 4750), Fax: +81-3-3815-9604, E-mail: katada@mol.f.u-tokyo.ac.jp

Abbreviations: LPA, 1-oleoyl-lyso-phosphatidic acid; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; DTT, dithiothreitol.

which are constantly occupied by pre-existing c-Jun itself dimerized to ATF2. This suggests that the response to external signaling is due to post-translational modification of the pre-bound factors, rather than to an increase in the factor binding. c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases, belong to the mitogen-activated family of protein kinases and phosphorylate c-Jun (at Ser63 and Ser73) and ATF2 (at Thr69 and Thr71) *in vitro*, thereby increasing the transcriptional activity of these proteins (*24, 25*). The same sites in these transcription factors also become phosphorylated when cells are exposed to the cellular stresses that activate JNK, suggesting that they may be physiological substrates for the kinase. Recently, JNKs have been found to be activated by some mitogens and oncogenic proteins such as v-Src tyrosine kinase and v-Ras (*26*). Thus, it is intriguing to know whether LPA, a growth factor-like phospholipid, also causes activation of JNKs.

In the present study, we show that JNK is activated in LPA-stimulated Swiss 3T3 fibroblasts. We also found that LPA stimulates the induction of c-*jun* mRNA. Both of these responses were of the same concentration dependence as was observed for the stimulation of DNA synthesis. Based on temporal and pharmacological criteria, the mechanism by which LPA activates JNK appeared to be different from that of ERK.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP, $^{32}P_i$, and [^{3}H]thymidine were purchased from DuPont NEN. JNK1-specific antibody, antisera to JNK isoforms, and antibody to ERK2 which cross-reacts with ERK1 were from Santa Cruz. Protein A-Sepharose was purchased from Pharmacia. Lysophosphatidic acid (oleoyl) and sphingomyelinase (*Streptomyces*) were from Sigma. All other reagents from commercial sources were of analytical grade.

Cell Culture and Assay of DNA Synthesis—Swiss mouse 3T3 fibroblasts were cultured as described previously (*27*). For JNK and ERK kinase assays and Northern blot analysis, subconfluent cultures of the cells (about 1.2×10^6 cells in 100-mm dish) were arrested in G_0 phase by incubation for 48 h in Dulbecco's modified Eagle's medium (D-MEM) containing 0.2% (v/v) fetal calf serum. If the cells that had been rendered quiescent by contact inhibition were used, JNK activation by LPA was slightly attenuated. DNA synthesis was measured according to the methods described previously (*28*).

Preparation of GST-Fusion Proteins—A DNA fragment encoding the amino-terminal 79 amino acids of c-Jun was obtained by PCR using murine cDNA as a template and the following oligonucleotides:

5′-GATCGGATCCATGACTGCAAAGATGGAAACG-3′
and
5′-GATCGAATTCCAGGCGCTCCAGATACGG-3′.

A DNA fragment encoding ATF-2 (residues 19 to 96) was also obtained by PCR using human cDNA as a template and the following oligonucleotides:

5′-GATCGGATCCATGAGTGATGACAAACCC-3′ and
5′-GATCGAATTCATTCTCAAATGGACTCGC-3′.

The cDNAs were prepared by reverse transcription of total RNA from Swiss 3T3 cells and HeLa cells, respectively. The fragments were cloned between the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia), in frame with the GST gene. The resulting fusion proteins were purified from bacterial lysates with the aid of glutathione-Sepharose 4B beads (Pharmacia) and used as substrates.

Immunoprecipitation—The cell monolayers were rinsed once, D-MEM containing 10 mM Hepes-NaOH (pH 7.4) and 0.1% (w/v) fatty acid-free BSA was added, and the cells were incubated for 4 h. The cells were then treated with LPA or reagents for various times in the medium at 37°C. The incubation was terminated by placing the dishes on ice and quickly aspirating the medium. The cells were rinsed twice with ice-cold Tris-HCl-buffered saline, then lysed on ice with 800 μl of a lysis buffer [10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% (v/v) Triton X-100, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were centrifuged at $10,000 \times g$ for 20 min at 4°C and precleared by incubation with protein A-Sepharose beads at 4°C for 1 h. After brief centrifugation, the supernatants were transferred to a new tube and incubated at 4°C for 1 h with anti-JNK or anti-ERK antibody, then for 1 h with the further addition of protein A-Sepharose beads. The immuno-complex was subjected to the sequential washing with the lysis buffer and a kinase assay buffer consisting of 50 mM Hepes-NaOH (pH 7.5), 0.1 mM EDTA, 75 mM NaCl, 25 mM β-glycerophosphate, 0.5 mM vanadate, 1 mM DTT, and 0.03% (v/v) Brij35. Each washing was repeated three times.

Protein Kinase Assay—For determining JNK activity, immunoprecipitates prepared as described above were resuspended in 30 μl of the kinase buffer containing 3 μg of GST-c-Jun (the amino acid sequence of 1-79) or GST-ATF-2 (19-96) as the substrates. Reactions were initiated by the addition of an ATP solution (10 μl) consisting of 200 μM [γ-^{32}P]ATP (10 μCi) and 40 mM $MgCl_2$. After incubation at 30°C for 20 min, reactions were stopped by addition of 20 μl of sample buffer consisting of 1% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromophenol blue, and 62.5 mM Tris-HCl (pH 6.8). The samples were heated at 100°C for 5 min, then solubilized peptides were electrophoresed through a SDS-polyacrylamide (12%) gel. The radioactivity incorporated into the substrates was analyzed by use of a Fuji BAS2000 bioimaging analyzer.

For ERK activity, immunoprecipitates were resuspended in 30 μl of the kinase buffer containing a synthetic peptide with a sequence based on the threonine phosphorylation site of EGF receptor (Amersham). Reactions were initiated by the addition of 10 μl of the ATP solution. After incubation at 30°C for 20 min, reactions were stopped by cooling on ice. Immunoprecipitates were collected by centrifugation, and the supernatants were applied onto a P-81 ion exchange chromatography paper, which was washed intensively with 1% (v/v) acetic acid before being dried and counted in a scintillation counter.

Measurement of Ras Activation—The cell monolayers cultured in D-MEM containing 0.2% (v/v) fetal calf serum were further incubated with $^{32}P_i$ (200 μCi/ml) for 3 h, followed by stimulation and lysis as described above. Ras was immunoprecipitated with a monoclonal antibody Y13-259 (Oncogene Science) and analyzed for bound guanine

nucleotides by means of thin layer chromatography on PEI-cellulose plates. The radioactivity on GTP and GDP spots was analyzed by use of the BAS2000 bioimaging analyzer.

Northern Blot Analysis—Total RNA was isolated from 3T3 cells that had been treated with LPA for various times by homogenization in guanidinium thiocyanate as described (*29*). Samples containing 20 μg of total RNA were fractionated on 2% formaldehyde-agarose gels, transferred to nitrocellulose membranes, then hybridized with ^{32}P-labeled oligonucleotide probe of c-*jun*. Accuracy in gel-loading and transfer was confirmed by fluorescence under UV light upon ethidium bromide staining and by hybridization with a radiolabeled actin probe. Quantitation of relative strength of induction of c-*jun* mRNA was performed by use of the BAS2000 bioimaging analyzer.

RESULTS

LPA Stimulates JNK and ERK with Different Time Courses—Quiescent Swiss 3T3 cells were incubated with LPA, and lysate from the cells was treated with an anti-JNK antibody. The kinase activity in the immunoprecipitated JNK was measured using purified GST-c-Jun as a substrate. As shown in Fig. 1A, LPA stimulated JNK activity in a time-dependent manner. Activation of JNK was detected within 5 min after exposure to LPA, reached a peak at 20 min, and persisted for at least 40 min. The maximum increase in JNK activity, 3.2±0.47-fold increase (mean±SD, n=17 experiments), was much smaller than sorbitol-induced activation (300 mM, 20 min; 44±2.7-fold, n=3) and comparable with the effect of platelet-derived growth factor (PDGF)-BB (100 ng/ml, 20 min; 3.5±0.28-fold, n=4). LPA-stimulated JNK activation was also detected when GST-ATF-2 was used as a substrate instead of GST-c-Jun (data not shown), indicating that LPA stimulates catalytic activity of JNK.

Receptor agonists such as EGF and carbachol have been reported to cause rapid ERK activation and gradual JNK activation in NIH 3T3 cells (*29*). Thus, we compared the kinetics of LPA-induced activations of JNK and ERK under the same conditions (Fig. 1B). ERK was activated more rapidly and transiently than JNK. The LPA-stimulated increase in ERK activity was detectable as early as 1 min, peaked at 3 min, and returned to the near-baseline level by 40 min. We have never observed biphasic, sustained activation of ERK as previously reported in LPA-stimulated Rat-1 fibroblasts (*30*). Recent findings have demonstrated that mitogens acting on a large variety of cell surface receptors converge at the level of Ras to induce a cascade of serine-threonine kinases, leading to the activation of ERKs. In addition, it has been shown that expression of oncogenically activated Ras efficiently stimulated JNK (*26*). However, we found that LPA failed to activate Ras, while PDGF could activate it (Fig. 2). Thus, LPA appears not to utilize Ras to activate JNK and ERK cascades in Swiss 3T3 fibroblasts. This may account for the finding that LPA stimulates only transient activation of ERK in the cells (Fig. 1B), since Ras is likely to be important for sustained ERK activation (*18*).

Effects of PTX, Suramin, and PD98059 on LPA-Induced Activation of JNK—To examine the signaling pathways leading to the activation of JNK and ERK, we applied several inhibitors to the intact cells. JNK activation in response to LPA was not suppressed by the prior treatment of the cells with PTX (Fig. 3A), indicating that trimmeric G-proteins of the G_i family may not be involved in the activation. In contrast, the PTX pretreatment caused greater than 80% inhibition of LPA-stimulated ERK activation (Fig. 3B), in agreement with previous reports (*30, 31*). It has been reported that pretreatment of cells with a polysulfonated compound, suramin, can inhibit some effects of LPA (*14, 32*). One of the sites of its action is thought to be a membrane receptor(s) for LPA (*33*). Thus, we examined effects of suramin and found that JNK activation by LPA was markedly attenuated in the presence of this compound (Fig. 3A). The ability of sphingomyelinase or sorbitol to activate JNK was totally unaffected by suramin (data not shown), demonstrating the specific effect of the compound on the LPA action. In contrast, suramin had little effect on LPA-dependent activation of ERK (Fig. 3B). These data demonstrated that suramin is an effective

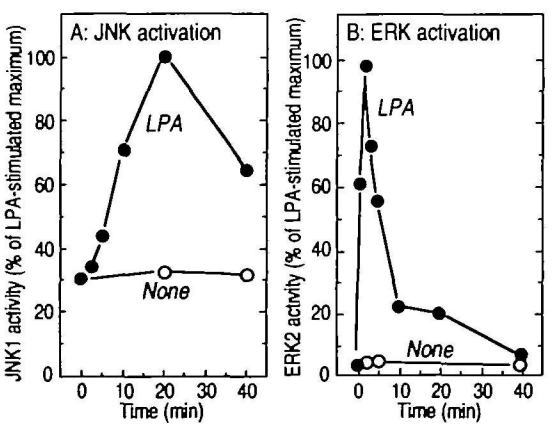

Fig. 1. **Time courses of LPA-induced activation of JNK and ERK.** Quiescent Swiss 3T3 cells were incubated with (closed circle) or without (open circle) 30 μg/ml of LPA for the indicated times. JNK (A) and ERK (B) were immunoprecipitated from the cell lysate, and their kinase activities were measured as described in "MATERIALS AND METHODS." Values shown are the mean of duplicate determinations expressed as percentages of the maximum effects of LPA. Each result is representative of experiments performed at least three times.

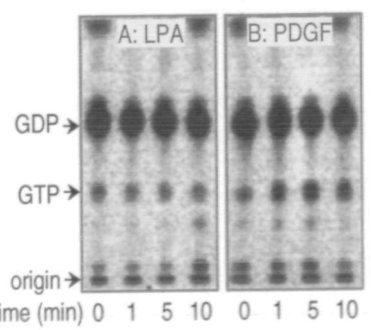

Fig. 2. **Failure of LPA to stimulate Ras.** Quiescent Swiss 3T3 cells were incubated with $^{32}P_i$ and stimulated with LPA (A, 100 μg/ml) or PDGF-BB (B, 30 ng/ml) for the indicated times as described in "MATERIALS AND METHODS." Ras was immunoprecipitated with a monoclonal antibody Y13-259, and the guanine nucleotides bound to Ras were eluted and separated by thin layer chromatography.

inhibitor of LPA-induced JNK activation without significantly affecting the ERK pathway. PD98059, a specific inhibitor of MEK (*34*), suppressed ERK activation to 20% of control, but higher concentrations of PD98059 did not bring about complete inhibition of ERK activation. In contrast to ERK, LPA-induced activation of JNK was unchanged by pretreatment with PD98059 (Fig. 3A).

Concentrations of LPA Required for the Activation of JNK, ERK, the Induction of c-Jun mRNA, and DNA Synthesis—Next, we examined the concentration-dependent effects of LPA on the activation of JNK and ERK. As shown in Fig. 4A, increasing concentrations of LPA resulted in increasing levels of JNK activity. The concentrations of LPA required for the half-maximal and maximal activation of JNK were about 3 μg/ml and 30-100 μg/ml, respectively. The concentration-dependent effect of LPA on ERK activation is also illustrated in Fig. 4A. Significant and maximum increases in ERK activity were observed with 0.01 μg/ml and 3-10 μg/ml of LPA, respectively. The EC_{50} for the activation of ERK was about 0.1 μg/ml, consistent with the value previously reported in Rat-1 fibroblasts (*30*). Together with the kinetics and susceptibility to inhibitors, PTX and suramin, these results indicate that LPA differentially regulates JNK and ERK pathways.

The c-*jun* promoter is regulated by c-Jun-ATF2 heterodimer, which is phosphorylated and activated by JNK (*23*). We therefore examined whether c-*jun* mRNA can be induced after LPA treatment. As shown in Fig. 4B, LPA caused the induction of c-*jun* mRNA in a concentration-dependent manner. The EC_{50} (3 μg/ml) for induction of c-*jun* mRNA was in good agreement with that for JNK activation. Under the same conditions, the effect of LPA on the cell proliferation was also determined by [^{3}H]thymidine incorporation. LPA-stimulated DNA synthesis and JNK activity (and induction of c-*jun* mRNA) were in close agreement, with EC_{50} values of approximately 5 and 3 μg/ml, respectively. These values were more than one order of magnitude higher than that for ERK activation (0.1 μg/ml). Thus, these results suggest that the full mitogenic signaling potential of LPA is only realized when the JNK pathway is activated.

Inhibition of DNA Synthesis by PTX, PD98059, and Suramin—Previous studies have shown that PTX-sensitive activation of ERKs plays an important role in mitogenesis induced by LPA (*14, 30*). Therefore, we examined the

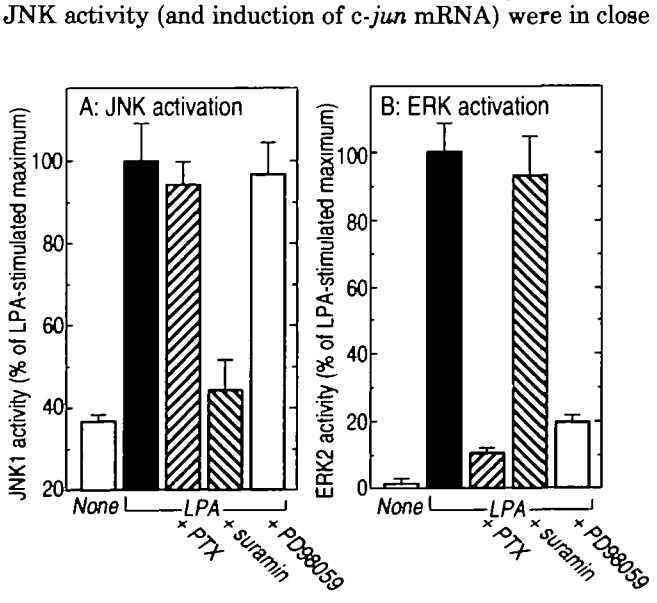

Fig. 3. **Effects of PTX, suramin, and PD98059 on LPA-induced activation of JNK and ERK.** Quiescent 3T3 cells were first incubated in the presence or absence of 100 ng/ml of PTX for 4 h, 1 μg/ml of suramin for 30 min, or 50 μM PD98059 for 30 min, then incubated with or without 30 μg/ml of LPA for 20 min for JNK activity (A) or for 3 min for ERK activity (B). Values shown are the means±SD of triplicate determinations expressed as percentages of the effects of LPA in the absence of inhibitor. Each result is representative of experiments performed at least twice.

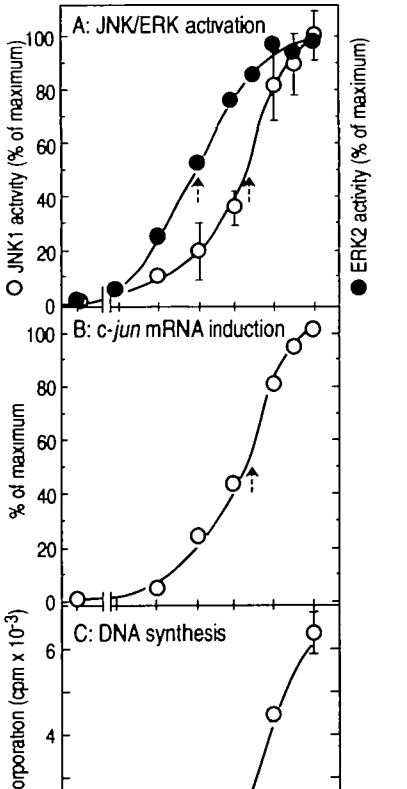

Fig. 4. **Concentration dependence for LPA-stimulated cellular responses.** Quiescent 3T3 cells were incubated with increasing concentrations of LPA for 20 min for JNK activity (A, open circle), 3 min for ERK activity (A, closed circle), 60 min for c-*jun* mRNA induction (B), or 48 h for DNA synthesis (C). Induction of c-*jun* mRNA was analyzed by Northern blotting of total RNA isolated from the cells, and DNA synthesis was measured by [^{3}H]thymidine incorporation in trichloroacetic acid-insoluble material as described in "MATERIALS AND METHODS." [^{3}H]Thymidine incorporation in unstimulated- and LPA-(100 μg/ml) stimulated cells were 100 and 6,200 cpm, respectively. In (C), PTX (closed circle), suramin (closed triangle), or PD98059 (closed square) was also added as shown in Fig. 3. Values in panels (A) and (B) are expressed as percentages of the maximal increases above unstimulated control cells. Values shown in (A, circle) and (C) are the means±SD of triplicate determinations. Values shown in (A, closed circle) and (B) are the mean of duplicate determinations. The arrows in panels show EC_{50} values of LPA. Each result is representative of experiments performed at least three times.

contribution of the ERK pathway to LPA-stimulated reinitiation of DNA synthesis, by using PD98059. The inhibitor reduced LPA-induced ERK activation to 20% (Fig. 3B), and decreased [^{3}H]thymidine incorporation to 29± 0.5% of control value (Fig. 4C, closed square), suggesting that the MEK-dependent ERK activation is also required for the mitogenic response. This is also supported by the results with PTX, which inhibits both ERK activation (Fig. 3B) and mitogenesis (Fig. 4C, closed circle). LPA-stimulated DNA synthesis was almost completely inhibited in the presence of 1 μg/ml of suramin (Fig. 4C, closed triangle), which had little effect (10% inhibition) on fetal calf serum-induced DNA synthesis (data not shown). Suramin had little effect on LPA-dependent activation of ERK at the concentration tested (Fig. 3B). This implied that an effector route other than the ERK pathway, which is blocked by suramin, also plays a role in DNA synthesis induced by LPA. Suramin inhibited LPA-induced DNA synthesis and JNK activation in a similar concentration range (data not shown).

DISCUSSION

JNKs are activated by stimuli that stress cells, including inflammatory cytokines, ultraviolet irradiation, DNA-damaging chemotherapeutic drugs, and heat shock (*25*). Recently, growth factors acting on a large variety of cell surface receptors have been also shown to stimulate JNK (*29, 35*). We found in the present study that LPA, a potent mitogen for fibroblasts, activated JNK. Although the precise mechanism by which LPA activates JNK has not been determined, we showed that it regulated JNK differently from ERK on the basis of several criteria. The polysulfonated drug suramin can form complexes with proteins, thereby exerting profound effects on protein tertiary structure (*32*). The difference in susceptibility of JNK and ERK activation to the drug may indicate that LPA interacts with distinct receptors and thereby activates each kinase independently, because suramin is membrane-impermeant at low concentration and the site of its action seems to be proteins on the outer leaflet of plasma membranes. Although we cannot rule out the possibility of a single receptor coupling to these pathways at different occupancies, there remain marked discrepancies in the sensitivity to suramin and PTX and in the concentrations of LPA required for activating JNK and ERK. JNK activation and expression of c-*jun* gene (and proliferation) required higher concentration of LPA than ERK activation (Fig. 4). We found that LPA caused tyrosine phosphorylation of focal adhesion kinase and paxillin with EC_{50} values of 100 ng/ml (data not shown), which is consistent with ERK activation. Other LPA actions such as activation of phospholipase C, phospholipase D, and Ras have also been reported to be elicited in the same concentration range as activation of ERK (*7*). Recently, an LPA receptor of *Xenopus* oocytes (*36*) and two mammalian LPA receptors, Vzg-1/Edg2 (*21*) and Edg4 (*20*), have been cloned. Further studies will be required for the identification of LPA receptor(s) responsible for these pathways.

Our findings have implications about the mechanisms of signal transduction underlying the mitogenic action of LPA. It has been shown that Ras and ERK are important for the mitogenic effect of LPA (*18, 30*). We found that LPA stimulated ERK activation without stimulating the GDP/GTP exchange reaction of Ras in Swiss 3T3 cells (Fig. 2). At present, we have no idea how ERKs are activated in LPA-stimulated cells. Our data obtained by the use of PD98059 supported the previous findings that ERK activation is required in stimulating proliferation (Fig. 4C). We concluded that activation of ERK is, however, not sufficient for LPA-stimulated DNA synthesis, since LPA is about 100-fold less potent in inducing DNA synthesis than in evoking the stimulation of ERK activity, and since suramin almost completely inhibited DNA synthesis induced by LPA without affecting the activation of ERK largely. A pathway susceptible to suramin is likely to transduce the cell-growth signal of LPA in concert with the ERK pathway. The JNK signaling cascade is a prime candidate for the induction of apoptosis or cell death (*25*). Additionally, recent studies have demonstrated the potential integration of this cascade into cell differentiation, survival, and proliferation (*37-39*). Thus, the LPA-stimulated JNK cascade might play a role in inducing cell proliferation.

In conclusion, the present results reveal a previously unrecognized pathway in the actions of LPA. Identification of the physiological significance of this pathway and elucidation of the precise intracellular mechanisms by which LPA activates JNK warrant further investigation.

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