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Lysophosphatidic acid possesses dual action in cell proliferation

(lipid/cAMP/signal transduction/reverse transformation)

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ABSTRACT **Lysophosphatidic acid (LPA) induces mitogenic responses in cultured fibroblasts through a pertussis toxin-sensitive signaling pathway. In contrast, we have shown that LPA inhibits the proliferation of Sp2/0-Ag14 myeloma cells. To resolve this apparent controversy, LPA-elicited responses in cell proliferation and the underlying second messenger mechanisms were compared in Sp2/0-Ag14 myeloma and NIH 3T3 fibroblast cells. The antimitogenic response was not elicited by micromolar concentrations of phosphatidic acid, phosphatidylglycerol, or diacylglycerol. In NIH 3T3 and Sp2 cells, LPA elicited an increase in inositol trisphosphate and a subsequent transient increase in free cytoplasmic Ca^{2+}. Unlike the mitogenic response in NIH 3T3 cells, the antimitogenic effect was not affected by pertussis toxin; on the contrary, it was accompanied by an increase in cAMP. In Sp2 cells, cAMP analogs, forskolin, and isobutylmethylxanthine inhibited cell proliferation and enhanced LPA action in an additive manner, suggesting that an LPA-elicited increase in cAMP-mediated signaling was responsible for the antimitogenic response. In addition to the mitogenic response in fibroblasts and the antimitogenic response in tumor cell lines, there are some cell types (Jurkat T-cell lymphoma and primary astrocytes) in which LPA is ineffective in altering cell proliferation. The cell-type-specific dual action of LPA suggests that this endogenous lipid mediator when released from activated cells might play an important role as a regulator, rather than a ubiquitous inducer, of cell proliferation.**

There is growing interest in the physiological function and mechanism of action of lysophosphatidic acid (LPA), as this naturally occurring lipid elicits diverse cellular responses in a variety of cell types (1–4). An intriguing effect caused by LPA is its growth factor-like action in fibroblasts (5–10). Since LPA is produced in large amounts in activated platelets during blood clotting (11–13) and growth factor-stimulated fibroblasts (14), its potential growth factor-like role might be important in wound healing and regeneration (4).

Surprisingly, most experiments unveiling the growth factor-like action of LPA were performed only on embryonic and primary fibroblast cell lines (5–10). Earlier experiments carried out in our laboratory with the albumin-bound endogenous form of serum LPA (designated as active serum albumin; ASA) and Sp2/0-Ag14 (Sp2) myeloma cells cultured in serum-free chemically defined medium suggested that ASA did not enhance the growth rate of these cells but, on the contrary, markedly reduced their proliferation, without altering cell viability (4, 15). The apparent contradiction between our initial results with myeloma cells and those of others with fibroblasts initiated the present study on the action of natural (ASA) and synthetic LPA on the proliferation of cells other than fibroblasts. Experiments presented here demonstrate the growth-inhibitory effect of blood-clotting-generated endogenous LPA-like phospholipids in a variety of transformed cell lines and relate their mechanism of action to the cAMP and cAMP-activated protein kinase A signaling pathway.

MATERIALS AND METHODS

Human serum albumin (HSA, Cohn fraction V), fatty acid-free HSA, and all other chemicals were from Sigma unless stated otherwise. Human plasma albumin (HPA, Cohn fraction V) was from Miles–Pentex Biochemicals (Kankakee, IL). L-α-Phosphatidic acids (PA), LPA, 1,2-phosphatidylglycerol, and dioleoylglycerol were from Avanti Polar Lipids. For tissue culture application, lipids were first dissolved in methanol (5 mM stock solution) and then solubilized further by a 2 M excess of fatty acid-free HSA or HPA dissolved in the culture medium. Fura-2 was purchased from Molecular Probes. [^{3}H]Thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was from DuPont/New England Nuclear, whereas [^{3}H]inositol trisphosphate (IP_3; 34 Ci/mmol) and radioimmunoassay kits for cAMP were obtained from Amersham.

Most experiments described in this paper used Sp2 myeloma cells previously adapted to grow in HB101 chemically defined medium (Irvine Scientific; doubling time = 16–18 h, see ref. 16). For cell proliferation assays, 1-ml cultures containing 2×10^4 cells per ml were seeded on 24-well culture plates and allowed to adapt for 24 h before addition of test materials dissolved in 100 μl.

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (GIBCO/BRL). To reach quiescence, the medium was replaced by serum-free DMEM for 24 h before test materials were added. DNA synthesis in all cell lines was measured after a 21-h incubation in the presence of the test materials by adding 1 mCi of [^{3}H]thymidine to the medium. Three hours later, cells were harvested and the acid-precipitable radioactivity was determined as described (4).

Flow cytometric analysis of the cell cycle distribution was performed with propidium iodide staining (17) of DNA using an EPICS V (Coulter) flow cytofluorometer. DNA cell cycle analysis was done using the MODFIT program by Verity Software House (Topsam, ME). Populations of 2×10^4 cells were analyzed from each sample, yielding χ^2 values <2 for the statistical fit of the experimental data.

IP_3 extraction and quantitative determinations were performed using a radioreceptor assay as described (18). Levels of cAMP were measured by a scintillation proximity RIA kit (RPA 538) from Amersham. Samples of 2×10^6 cells pretreated with 100 μM isobutylmethylxanthine (IBMX) were

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Abbreviations: LPA, lysophosphatidic acid; PA, phosphatidic acid; HSA, human serum albumin; ASA, active serum albumin; HPA, human plasma albumin; IP_3, inositol trisphosphate; PTX, pertussis toxin; Bt_2-cAMP, dibutyryl cAMP; cAMP[S], adenosine 3',5'-cyclic phosphorothioate; IBMX, isobutylmethylxanthine.

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used for each time point, and total cAMP (intracellular plus released to the medium) was extracted by established procedures (18). Intracellular [Ca^{2+}] measurements were performed using the Ca^{2+} indicator dye fura-2. Description of the apparatus (19), fluorescence monitoring, calibration procedures, and data analysis were similar to those described elsewhere (20).

RESULTS

Effects of LPA-Containing ASA on the Proliferation of NIH 3T3 and Sp2 Cells. We have demonstrated (3, 4) that the serum factor responsible for the antiproliferative action was a set of albumin-bound phospholipids whose most prominent component was LPA. Therefore, HPA, HSA [purified by the Cohn procedure (21)], and synthetic LPA were tested in parallel on the DNA synthesis of Sp2 and NIH 3T3 cultures. As shown in Fig. 1, plasma albumin did not alter [^{3}H]thymidine incorporation in either cell line compared to the appropriate controls. In contrast, HSA caused a 7-fold increase in the incorporation of [^{3}H]thymidine into NIH 3T3 cells (Fig. 1*A*), whereas the same batch of HSA strongly inhibited [^{3}H]thymidine incorporation into Sp2 cells (Fig. 1*B*). Synthetic LPA similarily to HSA induced a mitogenic response in NIH 3T3 fibroblasts, whereas it inhibited DNA synthesis in Sp2 myeloma cells. Cell counts confirmed (data not shown) that reduced [^{3}H]thymidine incorporation was indicative of the inhibition of cell proliferation, whereas an increase in [^{3}H]thymidine incorporation was accompanied by an increase in cell count.

As judged by cell counts (data not shown), in HB101 medium, Sp2 cultures showed vigorous growth in the presence of HPA, PA 18:0, or the solvent with doubling times between 16 and 18 h. In contrast, when exposed to 100 μM HSA or 10 μM LPA 18:0, cells grew at a decreased rate with doubling times >30 h. This reduced proliferation rate was not due to cell death because cell viability in the HSA-treated cultures was virtually identical to that of cultures grown in HB101 medium alone (86 ± 7% vs. 86 ± 4% in control).

Effect of LPA on the Sp2 Cell Cycle. To analyze the mechanism of the antiproliferative effect of LPA, we determined its action on the DNA cell cycle of Sp2 cells (Fig. 2*A*). LPA induced an early drop in the number of M-phase cells at 14 h. A decrease in the number of S-phase cells also became detectable at 14 h, followed by a slow increase that failed to return to the control level even after 48 h. In contrast, the cell cycle distribution of cells cultured in the presence of the solvent control showed only minor variations (<2%) during the 48-h period (data not shown). Analysis of the cell cycle after a 24-h LPA treatment indicated that increasing concentrations of LPA 18:0 elicited a gradual decrease in the S-phase population that was accompanied by an increase of similar size in the G_0–G_1 cells (Fig. 2*B*). A slightly higher decrease in the number of S-phase cells (23.9%) was seen with 10 μM LPA 18:1 compared to equimolar amounts of LPA 18:0 (26.8%), whereas the number of S-phase cells in the 10 μM PA 18:0-treated cultures (35.5%) was virtually identical to that of the solvent control (34.2%).

Effect of Different Lipids on Sp2 Cell Proliferation. LPA action shows a high degree of structural specificity, suggesting that LPA combines with a receptor-like binding site in the plasma membrane (2–4, 18, 20, 22). Because LPA and PA are both mitogenic in fibroblasts (5, 6, 8, 9, 23–25), we examined the structure–activity relationship for a number of lipids in the Sp2 proliferation assay.

Longer acyl chain lengths and higher degrees of unsaturation in the fatty acid substituents of LPA tended to increase the antiproliferative effect, as was observed from the cell

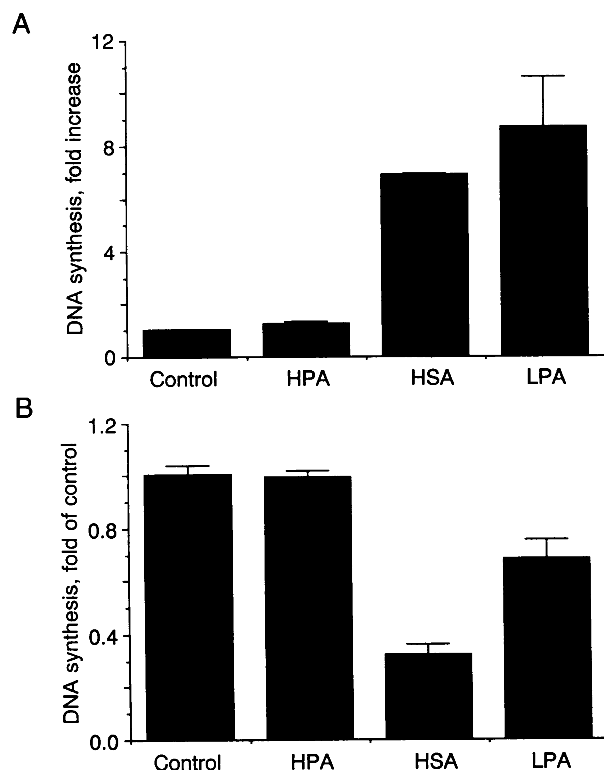

FIG. 1. In quiescent NIH 3T3 fibroblasts, HSA (100 μM) and LPA 18:1 (1 μM) induced DNA synthesis (*A*), or, in Sp2 myeloma cells, HSA and LPA inhibited DNA synthesis (*B*) as measured by [^{3}H]thymidine incorporation. HPA (100 μM) was ineffective. Bars represent the mean ± SEM in [^{3}H]thymidine incorporation over that of control cultures in four experiments. Medium controls consisted of DMEM with the lipid solvent for NIH 3T3 cells and HB101 for Sp2.

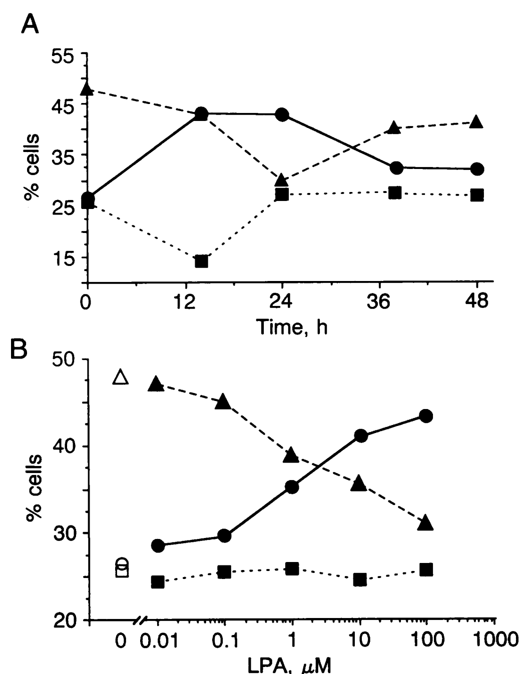

FIG. 2. (*A*) Cell cycle distribution of Sp2 cells cultured in the presence of 20 μM LPA 18:0. Points represent the percentage of cells in M phase (squares), S phase (triangles), and G_0/G_1 phase (circles) for a population of 2×10^4 cells. (*B*) Effect of increasing doses of LPA (solid symbols) on the cell cycle of Sp2 cells after a 24-h culture. Open symbols are cell cycle distribution in cells cultured with the solvent only.

cycle analysis. PA with various saturated acyl chain lengths, as well as PA 18:1, were ineffective up to 20 μM (proliferation indexes, 0.98–1.07), which is higher than the IC_{50} of LPA 18:1 in the antimitogenic response. When applied to the culture medium, dioleoylglycerol (18:0, proliferation index = 1.0), 1,2-phosphatidylglycerol (proliferation index = 0.99), and lysophosphatidylserine (proliferation index = 0.97) up to a concentration of 10 μM were all ineffective in inhibiting the DNA synthesis of Sp2 cells.

Effects of HSA and LPA on Cell Shape. Sp2 cells cultured in HB101 serum-free chemically defined medium show a highly monomorphic circular shape (Fig. 3*A*). This typical morphology remains unchanged when the medium is supplemented with 100 μM HPA, similar to the concentration of albumin found in 10% (vol/vol) serum-supplemented medium formulations. In contrast, supplementation of the medium with 100 μM HSA or 10 μM LPA 18:1 (Fig. 3*B*) brings about a marked change in cell morphology of Sp2 cells, characterized by the occurrence of club-shape cells. These club-shape cells become noticeable after a 1-h incubation, continue increasing during the first 48 h when >85% of the cells show this altered shape, and then slowly decline after 72 h of culture.

Signal Transduction Events Accompanying the Antiproliferative Action of LPA in Sp2 Cells. In Sp2 cells, ASA and LPA elicited a transient increase in cellular IP_3 content that peaked within 5 min [with 100 μM ASA, ≈8.4 (±1.3)-fold, n = 3] and decayed to near resting levels (1 ± 0.1 pmol per 10^6 cells, n = 3) after 15 min. In contrast, neither fatty acid-free HSA nor HPA elicited an appreciable and lasting change in IP_3 concentration.

IP_3 liberates Ca^{2+} from intracellular stores, leading to a typically transient increase in free intracellular Ca^{2+} concentration (5, 20). To assess such consequences of LPA action, fura-2 ratio imaging technique was applied (20) to measure the magnitude and time course of the Ca^{2+} transients. LPA at 10 μM elicited a rapid (within 60 sec after exposure) and transient ≈3-fold increase over the 45 nM resting level of free intracellular Ca^{2+}, whereas the solvent was ineffective. It is interesting that similar changes were detected in IP_3 and Ca^{2+} transients when other cell lines were studied, regardless of whether the cellular response to LPA was antiproliferative (e.g., Sp2 cells) or proliferative (e.g., NIH 3T3 cells).

Among the different second messenger pathways activated by LPA, the G_i/G_o protein-coupled branch of the cAMP system was implicated in mediating the mitogenic effect (8). We examined whether pertussis toxin (PTX), which ADP-ribosylates and blocks G_i/G_o proteins regulating adenylyl cyclase activity, would also inhibit the antiproliferative effect of LPA. The results indicated that addition of PTX to the cultures, up to 1 μg/ml did not alter the decrease in DNA synthesis elicited by LPA (Fig. 4). In NIH 3T3 fibroblasts (Fig. 4*A*), in contrast, PTX at as little as 1 ng/ml completely abolished the mitogenic response elicited by 20 μM LPA. The lack of effect on the inhibition of Sp2 cell proliferation in the continued presence of PTX (Fig. 4*A*) at a 10^3 higher concentration than the IC_{50} value reported for the mitogenic response in fibroblasts indicates that the PTX-sensitive branches of cAMP signaling pathways are not involved in the LPA-elicited antiproliferative response.

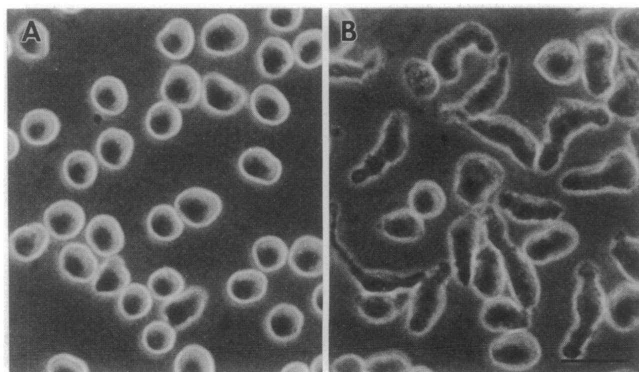

FIG. 3. Morphological changes in Sp2 myelomas cultured in HB101 chemically defined medium (*A*) or exposed to 10 μM LPA 18:1 (*B*). Phase-contrast microscopy. Pictures were taken after 4 h in the presence of LPA. (Bar = 50 μm.)

To study further the role of cAMP signaling in the LPA-induced antiproliferative effect, we also determined total cAMP levels in LPA-treated Sp2 myeloma cultures (Fig. 4*B*). In contrast to the LPA-induced decrease in cAMP reported in fibroblasts (refs. 7–9 and G.T., unpublished data), LPA application in Sp2 cells was followed by an increase in cAMP that was evident even in forskolin-prestimulated cells. This elevation in cAMP level was not inhibited by PTX pretreatment (200 ng/ml, 1 h) either in forskolin-treated or untreated cells. PTX pretreatment slightly lowered the forskolin-induced increase of cAMP, without attenuating the LPA-induced elevation in cAMP level. On the contrary, LPA induced a steeper increase in cAMP levels in PTX-treated cells (≈12 pmol per min per 10^6 cells) compared to that in cells that were treated with 1 μM forskolin alone (≈8 pmol per min per 10^6 cells). LPA alone (data not shown) and in PTX-pretreated cells (Fig. 4*B*) elicited an increase in cAMP,

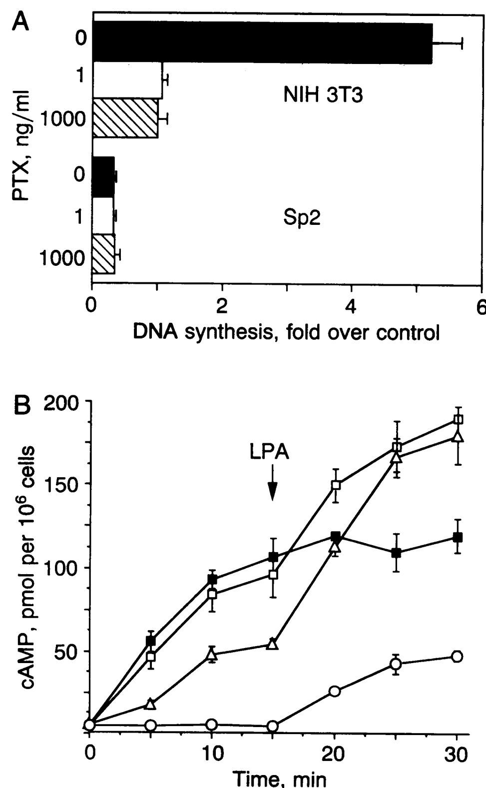

FIG. 4. (*A*) PTX inhibits the mitogenic action of LPA 18:0 (20 μM) in NIH 3T3 fibroblasts (upper bars) but fails to alter the antimitogenic response in Sp2 myeloma cells (lower bars). Bars (PTX in ng/ml) represent the mean ± SEM of three experiments. (*B*) Changes in cAMP content in Sp2 myelomas treated with forskolin (1 μM, solid squares), LPA 18:1 (20 μM) plus forskolin (open squares), PTX (200 ng/ml) followed by forskolin plus LPA (open triangles), and PTX followed by LPA (open circles). LPA was added at 15 min to the cells and elicited an increase in cellular cAMP content even after forskolin or PTX pretreatments. The cAMP content (mean ± SEM) in the cell extracts was plotted for each time point in the different treatment groups. Data shown in this figure are representative of two experiments.

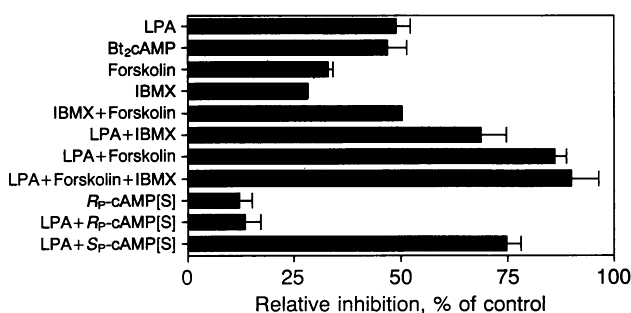

FIG. 5. In Sp2 cells, elevated cAMP levels cause an inhibition proliferation and enhance the action of LPA. Doses of the treatments are as follows: LPA at 10 μM, Bt_2cAMP at 1 mM, forskolin at 10 μM, IBMX at 200 μM, R_P-cAMP[S] at 25 μM, and S_P-cAMP[S] at 25 μM. Bars represent the decrease in cell count (mean $\pm$ SEM) for at least three experiments.

whereas PTX treatment alone did not cause significant changes in cAMP during the experiment.

Because LPA application caused an increase in cellular cAMP content, we also tested the effects of drugs that elevate the cAMP level (Fig. 5). In Sp2 cells, exposure to the membrane-permeable dibutyryl-cAMP (Bt_2 cAMP, 1 mM) decreased cell proliferation by 47 $\pm$ 3.6% ($n = 4$) over the control. Stimulation of adenylyl cyclase by forskolin and/or inhibition of cAMP phosphodiesterase by IBMX also inhibited cell proliferation. All these treatments with cAMP-elevating drugs when applied in combination caused enhanced inhibition. LPA application in combination with either of these drugs resulted in higher inhibition of cell proliferation than was detected when the drugs were applied individually. This cooperativity between LPA and cAMP-inducer drugs was additive in the submaximal dose range. The S_P- and R_P-diastereomers of adenosine 3′,5′-cyclic phosphorothioate (cAMP[S]) are membrane-permeable analogs of cAMP that activate and inhibit protein kinase A, respectively (26). When applied in combination with LPA, the S_P-diastereomer enhanced whereas the R_P-diastereomer inhibited the antiproliferative response (Fig. 5).

Dual Effects by LPA on the Proliferation of Other Types of Cells. To determine whether the antiproliferative and mitogenic action elicited by LPA can be generalized to cell types other than Sp2 and NIH 3T3, a variety of transformed and embryonic cell lines was tested, including primary fibroblast and astrocyte cultures. Results indicated that LPA promoted the proliferation of embryonic (L-M, Rat2, and primary foreskin fibroblasts) and transformed (NCTC-2472 sarcoma) fibroblast-type cells, transformed epithelial cells (HeLa), and PC12 pheochromocytoma cells, whereas it inhibited the proliferation of a variety of other types of cells, mostly of myeloid and lymphoid origin (YAC-1 lymphoma, EL4 lymphoma, F0 myeloma, MRC-5 embryonic lung cells, and C6 glioma). Moreover, we found at least two types of cells, Jurkat T-cell lymphoma and rat primary astrocytes, that did not change their rate of proliferation significantly in the presence of a concentration of LPA as high as 10 μM.

DISCUSSION

Synthetic and endogenous albumin-bound LPA-like lipid mediators, in micromolar concentration, evoked an antimitogenic effect in Sp2 myelomas and a mitogenic response in NIH 3T3 fibroblasts. These results reinforce that special care should be taken with the use of serum albumin in tissue culture and cell assay medium because it carries a variety of LPA-like lipid mediators that might either evoke or interfere with cellular responses.

LPA is presumed to act through a putative plasma membrane receptor (2–4, 7–10, 15, 18, 20, 27, 28). The antimitogenic effect showed a structure–activity relationship similar to that described in other cellular systems (cf. refs. 4, 9, 29). However, the antimitogenic effect was not elicited by PA. This is a major difference when compared to the ligand specificity of the mitogenic response, which is readily elicited by PA (23–25). The difference in ligand specificities between the mitogenic and antimitogenic responses may be a reflection of different receptor types for mitogenic lipid mediators such as LPA and PA. Murakami-Murofushi *et al.* (30) reported that a cyclic phosphate- and cyclopropane-containing LPA isolated from *Physarum* inhibited eukaryotic DNA polymerase α *in vitro*. However, non-cyclic-phosphate-containing LPA does not block DNA polymerase (K. Murakami-Murofushi, personal communication). Moreover, PA has also been shown to inhibit the activity of purified DNA polymerases α and γ *in vitro* (31), yet it was ineffective in inhibiting cell proliferation when applied to cultured Sp2 myeloma cells. Thus, in light of the differential response to PA, a direct LPA-induced inhibition of DNA polymerase can hardly account for the effect.

LPA induced rapid changes in cell shape and cell–cell adhesion. LPA has recently been implicated as the major factor in serum causing stress fiber formation in fibroblasts (32) and reported to cause other morphological changes including neurite retraction in PC12 cells (4, 18–20) and cell rounding in neuroblastoma cells (33). Because all these morphological responses to LPA are related to changes in polymerization of the actin cytoskeleton, there is an intriguing possibility that the development of club-shaped Sp2 cells is also due to activation of the small molecular weight GTP-binding protein $p2_1$ rho that was implicated in stress fiber formation (32). However, no club-shape cells were found in Bt_2cAMP-, S_P-cAMP[S]-, forskolin-, and IBMX-treated Sp2 cultures, indicating that the morphological response is probably mediated through an other signaling mechanism. Nonetheless, further experiments are necessary to investigate whether cytoskeletal rearrangements underlying the LPA-induced shape change are in a causative relation with the inhibition of proliferation.

Flow cytometric analysis of the DNA cell cycle in LPA-treated cultures of Sp2 cells showed a dose-dependent increase in the S-phase block, accompanied by an increase in the G_o/G_i population. Considering both the early decrease in M-phase population and later decrease in S-phase cells, it seems logical that there are at least two LPA-sensitive periods: the prereplicative G_1 and the premitotic G_2. These two have been shown to be the most sensitive periods of the cell cycle to changes in cAMP (34, 35).

Application of LPA to many cell types leads to the activation of the inositol phospholipid/Ca^{2+} and the inhibition of the cAMP second messenger systems (2–10, 18, 20). The mitogenic effect caused by LPA in fibroblasts is mediated through a PTX-sensitive G_o/G_i-protein-coupled signaling pathway, whereas the phospholipase C/protein kinase C pathway is neither essential nor sufficient for this response (8). The role of cAMP has long been known in the regulation of cell proliferation, and numerous reports in the literature support the dual role of cAMP as a negative and positive regulator of cell growth (for reviews, see refs. 34–38). Similarly, the phospholipase C/protein kinase C/Ca^{2+} signaling pathway has been implicated in the regulation of cell proliferation (see ref. 39). We found that even large doses of PTX did not alter the antimitogenic effect, indicating that G_i/G_o protein-mediated signaling is not necessary to evoke the antimitogenic response in Sp2 cells. In parallel experiments with NIH 3T3 fibroblasts, as little as 0.1 ng of PTX was sufficient to block the mitogenic response elicited by LPA, which confirms earlier reports by van Corven *et al.* (8) in Rat1

cells. In contrast to fibroblasts (8, 10), in Sp2 cells, LPA caused an elevation of cAMP levels that was insensitive to PTX. Thus, we conclude that the signaling pathway mediating the antimitogenic effect is different from that mediating the mitogenic response in fibroblasts. The mechanism underlying the antimitogenic response in Sp2 cells may depend on the LPA-elicited increase in cAMP alone, because elevation of its level by Bt_2cAMP, S_P-cAMP[S], forskolin, and IBMX treatment and their combinations were sufficient to inhibit cell proliferation. Moreover, all these cAMP-inducing agents when applied together with LPA caused enhanced levels of inhibition in an additive manner, whereas they failed to alter the mitogenic response to LPA in NIH 3T3 cells (10). R_P-cAMP[S], a membrane-permeable analog that inhibits cAMP-activated protein kinases inhibited the LPA-induced antimitogenic effect. van Corven *et al.* (10) have shown that cholera toxin and β-adrenergic stimulation do not affect the LPA-elicited mitogenic response in Rat1 and CCL39 fibroblasts. In these two cell lines, LPA induced a PTX-sensitive activation of $p21^{ras}$ that was independent of cAMP levels and was not altered by cholera toxin. However, further experiments will have to be done to establish whether $p21^{ras}$ and similar small molecular weight guanine nucleotide binding proteins play a role in the signal transduction pathway of the antiproliferative response to LPA.

Lastly, LPA-like lipid mediators generated from activated cells emerge as an important class of endogenous growth-regulatory molecules. These phospholipids are likely to act at the site of their production in concert with polypeptide growth factors in regulating cell proliferation with a distinct cell type specificity.

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1. Moolenaar, W. H., Jalink, K. & van Corven, E. (1992) *Rev. Physiol. Biochem. Pharmacol.* **119**, 47–65.
2. Tigyi, G., Dyer, D., Matute, C. & Miledi, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1521–1525.
3. Tigyi, G., Henschen, A. & Miledi, R. (1991) *J. Biol. Chem.* **266**, 20602–20609.
4. Tigyi, G. & Miledi, R. (1992) *J. Biol. Chem.* **267**, 21360–21367.
5. Jalink, K., van Corven, E. J. & Moolenaar, W. H. (1990) *J. Biol. Chem.* **265**, 12232–12239.
6. Krabak, M. J. & Hui, S. W. (1991) *Cell. Regul.* **2**, 57–64.
7. Plevin, R., MacNulty, E. E., Palmer, S. & Wakelam, M. J. O. (1991) *Biochem. J.* **280**, 609–615.
8. van Corven, E. J., Groenink, A., Jalink, K., Eicholtz, T. & Moolenaar, W. H. (1989) *Cell* **59**, 45–54.
9. van Corven, E. J., Rijswik, A., Jalink, K., van der Bend, R. L., van Blitterswijk, W. J. & Moolenaar, W. H. (1992) *Biochem. J.* **281**, 163–169.
10. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L. & Moolenaar, W. H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1257–1261.
11. Gerrard, J. M. & Robinson, P. (1989) *Biochim. Biophys. Acta* **1001**, 282–285.
12. Eicholtz, T., Jalink, K., Fahrenfort, I. & Moolenaar, W. H. (1993) *Biochem. J.* **291**, 677–680.
13. Tokomura, A., Harada, K., Fukuzawa, K. & Tsukatani, H. (1986) *Biochim. Biophys. Acta* **875**, 31–38.
14. Fukami, K. & Takenawa, T. (1992) *J. Biol. Chem.* **267**, 10988–10993.
15. Dyer, D. L., Tigyi, G. & Miledi, R. (1989) in *Proceedings of the Miami Bio/Technology Winter Symposium*, eds. Rotundo, R. L., Ahmad, F., Bialy, H., Black, S., Brew, K., Chaitin, M. H., Glaser, L., Magleky, K. K., Neary, J. J., Van Brunt, J. & Whelan, W. J. (IRL, Oxford), p. 322.
16. McHugh, Y. E., Walfhall, B. J. & Steimer, K. S. (1983) *BioTechniques* **1**, 72–77.
17. Taylor, I. W. (1980) *J. Histochem. Cytochem.* **28**, 1021–1024.
18. Dyer, D. L., Tigyi, G. & Miledi, R. (1992) *Mol. Brain Res.* **14**, 293–301.
19. Lewis, R. L. & Cahalan, M. D. (1989) *Cell Regul.* **1**, 99–112.
20. Dyer, D. L., Tigyi, G. & Miledi, R. (1992) *Mol. Brain Res.* **14**, 302–309.
21. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. & Uroma, E. (1950) *J. Am. Chem. Soc.* **72**, 465–474.
22. van der Bend, R. L., Brunner, J., Jalink, K., van Corven, E. J., Moolenaar, W. H. & van Blitterswijk, W. J. (1992) *EMBO J.* **11**, 2495–2501.
23. Moolenaar, W. H., Krujier, W., Tilly, B. C., Verlaan, I., Bierman, A. J. & de Laat, S. W. (1986) *Nature (London)* **323**, 171–173.
24. Yu, C.-L., Tsai, M.-H. & Stacey, D. W. (1988) *Cell* **52**, 63–71.
25. Tsai, M.-H., Yu, C.-H., Wei, F.-S. & Stacey, D. W. (1989) *Science* **243**, 522–526.
26. Parker Bothelo, L. H., Rothermel, J. D., Coombs, R. V. & Jastroff, B. (1988) *Methods Enzymol.* **159**, 159–172.
27. Durieux, M. E., Salafranca, N. M., Lynch, K. R. & Moorman, J. R. (1992) *Am. J. Physiol.* **263**, 896–900.
28. Fernhout, B. J. H., Dijcks, F. A., Moolenaar, W. H. & Ruigt, G. S. F. (1992) *Eur. J. Pharmacol.* **213**, 313–315.
29. Tokomura, A., Fukuzawa, K. & Tsuzatani, H. (1978) *Lipids* **13**, 572–574.
30. Murakami-Murofushi, K., Shioda, M., Kaji, K., Yoshida, S. & Murofushi, H. (1992) *J. Biol. Chem.* **267**, 21512–21517.
31. Yoshida, S., Tamiya-Koizumi, K. & Kojima, K. (1989) *Biochim. Biophys. Acta* **1007**, 61–66.
32. Ridley, A. J. & Hall, A. (1992) *Cell* **70**, 389–399.
33. Jalink, K. & Moolenaar, W. H. (1992) *J. Cell Biol.* **118**, 509–517.
34. Boyton, A. L. & Whitfield, J. F. (1983) *Adv. Cyclic Nucleotide Res.* **15**, 193–294.
35. Pardee, A. B. (1989) *Science* **246**, 603–608.
36. Cho-Chung, Y. S. (1992) *Biochem. Soc. Trans.* **20**, 425–430.
37. Lockwood, A. H., Murphy, S. K., Lazarus, A. & Pendergast, M. (1987) *J. Cell. Biochem.* **33**, 237–255.
38. Seuwen, K. & Poyssegur, J. (1992) *Adv. Cancer Res.* **58**, 75–94.
39. Nishizuka, Y. (1992) *Science* **258**, 607–614.