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The mitogenic activities of phosphatidate are acyl-chain-length dependent and calcium independent in $C_3H/10T_{1/2}$ cells

Michael J. Krabak and Sek-Wen Hui
Membrane Biophysics Laboratory
Roswell Park Memorial Institute
Buffalo, New York 14263

Phosphatidates (PA or phosphatidic acid) were shown to have mitogenic properties, including the stimulation of DNA synthesis and calcium mobilization in $C_3H/10T_{1/2}$ cells. Their continuous presence for a minimum of 7 h induced DNA synthesis with kinetics similar to that observed when 10% fetal bovine serum was used as a mitogen. PAs with long chain saturated fatty acid moieties were more mitogenic, in a dose-dependent fashion, than PAs with short saturated or unsaturated fatty acid moieties. When compared with lysostearoyl-PA (LSPA), distearoyl-PA (DSPA) was as potent with respect to the induction of DNA synthesis. Lysooleoyl-PA (LOPA) was slightly more potent than dioleoyl-PA (DOPA), but much weaker than DSPA and LSPA. Preincubation with dilauroyl-PA (DLPA) reduces the mitogenic effect of DSPA by 85%. The pattern of mitogenic inhibition suggests that a chain-length-independent, yet PA-specific, mechanism is involved. Both DSPA and DLPA are equally taken up by the cells after 30 min. LOPA, but not LSPA, produced a large calcium transient (1.3 μM), which we found to be derived from intracellular sources. DSPA, the most mitogenic PA tested, produced a weaker transient (0.6 μM). Interestingly, LSPA did not produce any detectable calcium transient. These results suggest that the chain-length-specific step in the signaling mechanism of PA occurs after the initial chain-length-independent partitioning and/or binding to the membrane and that the induction of DNA synthesis is not related to the observed calcium transients.

Introduction

Membrane phospholipids play a major role in the known signal transduction processes in cells. The most well-known example is the phosphatidylinositol cycle (PI cycle), which generates diacylglycerol (DAG) and inositol phosphates. However, recent evidence indicates that, in many cells, phospholipids other than PI are also involved in the signal transduction process. DAG increases resulting from the treatment of myocytes with insulin, or from the transformation of NIH 3T3 cells by H-*ras,* are derived mainly from phosphatidate (PA) rather than from PI (Lacal, 1990; Farese *et al.,* 1987). G-protein–mediated stimulation of human polymorphonuclear leukocytes leads to the hydrolysis of the alkyl analogues of phosphatidylcholine (PC) into PA and DAG, with little involvement of PI (Agwu *et al.,* 1989). DAG is found in large amounts in stimulated cells in excess of that expected from PI turnover. In a recent comment, Pelech and Vance (1989) postulated that an initial burst of PI breakdown is sufficient to trigger the PC cycle, which sustains the activation of protein kinase C (PKC) even after the depletion of inositol phospholipids. This model explains the PI-independent, prolonged phase of stimulation of many cells (Exton, 1988). A key link to both the PC and PI cycles is PA.

PAs have recently been found to possess growth factor–like properties, i.e., at micromole per milliliter quantities, they can induce increases of intracellular calcium, promote the expression of c-*fos* and c-*myc* proto-oncogenes, and activate DNA synthesis. Moolenaar *et al.* (1986) reported that PA, but not PI or phosphatidylserine (PS), elicited a transient calcium release from intracellular storage, raised cytoplasmic pH, induced expressions of c-*fos* and c-*myc* proto-oncogenes, and stimulated DNA synthesis. These workers also found that natural PA and dipalmitoyl-PA (DPPA), but not dimyristoyl-PA (DMPA) or dilauroyl-PA (DLPA), were effective in inducing calcium transients. To date, the chain-length-specific responses elicited by different PAs in various systems have been inconsistent. Yu *et al.* (1988) demonstrated that, in NIH 3T3 cells, DNA synthesis, induced by saturated PAs, could be inhibited by microinjection of a *ras*-specific antibody. In vitro, Tsai *et al.* (1989) demonstrated that unsaturated PAs

could inhibit GTPase activating protein (GAP) activity on purified c-Ha-*ras*, whereas saturated PA's were unable to alter GAP activity. Imagawa *et al.* (1989) found that only unsaturated PAs are mitogenic in primary cultures of normal mouse epithelial cells. Murayama and Ui (1987) demonstrated that unsaturated PAs could cause a greater inhibition of adenylate cyclase than saturated PAs. In addition, a recent article by van Corven *et al.* (1989) demonstrated that lysopalmitoyl-PA (LPPA; 16:0) or lysooleoyl-PA (LOPA; 18:1) is a more potent inducer of DNA synthesis than dioleoyl-PA (DOPA; 18:1) in Rat-1 cells. The mechanism for the growth factor–like action of PA, especially the chain-length dependency of the PA activities, remains uncertain.

Because PA occupies a key position in the PC and PI cycles, and is found to have mitogenic properties, we undertook a comprehensive study of the role of PA as a possible second messenger utilized in signal transduction. In this paper, we report our detailed measurements of the kinetics and chain-length dependency of PA-activated calcium transients and DNA synthesis. A possible mechanism for the chain-length dependence of PA-induced mitogenesis is discussed based on these findings.

Results

To establish the relative mitogenicity of PA, we tested the ability of various lipids and agents to stimulate DNA synthesis in the mouse cell line $C_3H/10T_{1/2}$. These cells exhibit density-dependent inhibition of cell growth and thus can be readily synchronized, making them useful for studying cell-cycle-dependent responses (Reznikoff *et al.*, 1973). Fluorescence-activated cell sorter (FACS) analysis, using propidium iodide, demonstrated that >82% of the cells utilized were proliferatively quiescent in the G_1 phase of the cell cycle. Table 1 shows that distearoyl-PA (DSPA) and lysostearoyl-PA (LSPA) were as potent as 10% fetal bovine serum (FBS) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in inducing DNA synthesis. It should be noted that the technique we used to stage the cells, serum depletion, yields cells that are proliferatively quiescent but not as dramatically responsive to mitogens as cells that have been serum-starved (Cutry *et al.*, 1989). This may be due to the presence of growth-inhibiting factors in the spent media. The assaying condition is, therefore, not the same as in other reported works that used serum-starved cells. Because serum starvation causes marginal survival of $10T_{1/2}$ cells, we prefer to assay under the less-traumatic serum depletion condition. Compared with the positive controls with 10% FBS, DSPA and LSPA are potent mitogens in our system. DLPA, distearoyl-PC (DSPC), phosphatidylethanolamine (PE; egg), DAG (1,2-distearoyl), DAG (dioleoyl), and 1-oleoyl-2-acetoyl glycerol were not mitogenic. Interestingly, DOPA and LOPA were very poor mitogens. These results differ from those obtained in normal mouse mammary epithelial cells (Imagawa *et al.*, 1989) in which unsaturated PAs were potent mitogens. In addition, van Corven *et al.* (1989) suggested that lysoPAs are more mitogenic than their diacyl counterparts, and that some of the PA effects observed might be due to contaminating levels of lysoPA in commercially obtained PA. To eliminate any doubt, we synthesized DSPA and LSPA from DSPC. Our PA and those purchased from Avanti Polar Lipids (DSPA, DPPA, DMPA, DLPA, DOPA, and LOPA) were shown to be chromatographically pure by the Skipsky *et al.* (1964) and Rouser *et al.* (1969) solvent systems. As shown in Table 1, at equimolar concentrations, the DSPA was as potent as LSPA with respect to the induction of DNA synthesis. In addition, DSPA was more potent than LOPA. Because we had a greater variety of PAs with different chain length and saturation available to us, we

Table 1. Ability of various lipids and other agents to stimulate DNA synthesis in quiescent $C_2H/10T_{1/2}$ cells

Stimulus	[^{3}H]thymidine incorporation (fold stimulation)
Control	1.0
10% FBS	4.5
TPA (100 ng/ml)	4.5
*Phosphatidic acid (1,2-distearoyl)	4.5
†Phosphatidic acid (1,2-distearoyl)	4.5
†Phosphatidic acid (1,2-dilauroyl)	1.3
†Phosphatidic acid (1,2-dioleoyl)	1.2
*Lysophosphatidic acid (1-stearoyl)	4.5
†Lysophosphatidic acid (1-oleoyl)	1.5
†Phosphatidylcholine (1,2-distearoyl)	1.0
†Phosphatidylethanolamine (egg)	0.9
†1-oleoyl-2-acetoyl glycerol	1.0
‡DAG (stearoyl)	1.1
‡DAG (oleoyl)	1.1

Stimulation of [^{3}H]thymidine incorporation was assayed as described in Materials and methods. Unless otherwise indicated, concentrations were 15 μM. Values are the mean of three experiments, each performed in triplicate (SE < 7%). Control incorporation of [^{3}H]thymidine was 27 481 ± 720 cpm per 10^6 cells.

* Lipids synthesized in our lab. †Lipids purchased from Avanti Polar Lipids. ‡Lipids purchased from Sigma.

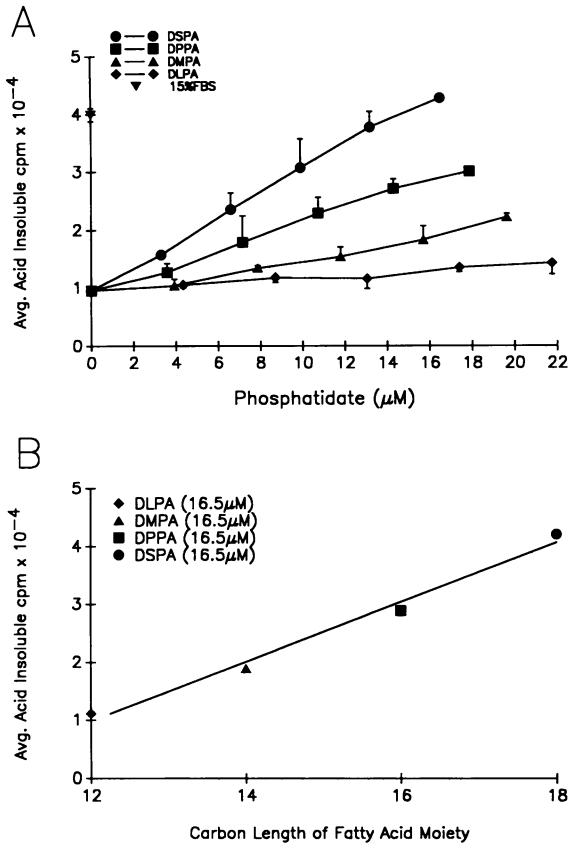

Figure 1. **The relative mitogenic activity of PA.** (A) Concentration dependence of PA-induced [^{3}H]thymidine incorporation. (B) Fatty acid chain length dependence of PA-induced [^{3}H]thymidine incorporation. The cultures were last subjected to a medium change with 10% FBS in BME 96 h before T = 0. At T = 0 the cultures were stimulated with either 10% FBS or 16.5 μM of DSPA, DPPA, DMPA, or DLPA. Simultaneously, cultures were pulsed with [^{3}H]-thymidine and at T = 24 h the [^{3}H]thymidine incorporated into the acid-insoluble material was measured as described in Materials and methods. Each data point represents the combined result of four repeating experiments (n = 4).

chose to concentrate our efforts on studying PA in subsequent experiments.

In an effort to define more clearly the chain length effects of PA-induced DNA synthesis, we tested the concentration and kinetic responses of various saturated PAs. As shown in Figure 1A, all of the PAs tested demonstrated concentration-dependent responses with respect to incorporation of [^{3}H]thymidine into the acid-insoluble material. The dose response appeared to be linear between 0 and 20 μM and did not increase further above 20 μM (data not shown). The most potent response was observed on stimulation with DSPA. Above 16 μM, in the range tested, the response was greater than that observed with 15% FBS. The least potent response was observed on stimulation with DLPA. Its response was relatively close to the control. As determined by incorporation of [^{3}H]thymidine into the acid-insoluble material, the relative mitogenic potentials of the various PAs tested are DSPA > DPPA > DMPA > DLPA. As shown in Figure 1B, when [^{3}H]thymidine incorporation into the acid-insoluble material is plotted as a function of the fatty acid chain length for a given PA, a linear correlation between mitogenic potential and chain length is observed.

To be certain that the results shown in Figure 1 were indicative of a differential mitogenic effect of various PAs and were not due to varied response kinetics, we performed a time course of PA-induced [^{3}H]thymidine incorporation. As shown in Figure 2, all of the PAs tested demonstrated similar kinetics for [^{3}H]thymidine incorporation into the acid-insoluble material. Peak incorporation occurred at ~21 h after stimulation. The data in Figure 2 suggest that, indeed, a differential mitogenic effect of various phosphatidates is observed. In addition, the observed kinetics of S-phase entry and potent mitogenic response relative to 15% FBS are consistent with the data previously published by Yu *et al.* (1988).

The ability of exogenous PA to stimulate DNA synthesis is related to its continuous presence in the culture medium. As shown in Figure 3, the cells require a minimum of 7 h of continuous exposure to elicit a mitogenic response. The type of PA utilized does not alter the required

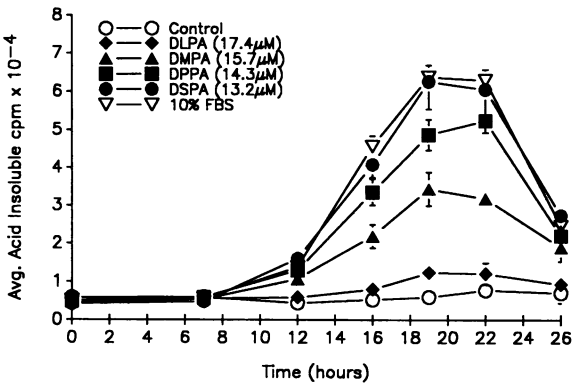

Figure 2. **Time course of PA-induced [^{3}H]thymidine incorporation.** The cell culture procedures were the same as described for Figure 1. At T = 0 the cultures were stimulated with 10 μg/ml of DSPA (13.2 μM), DPPA (14.3 μM), DMPA (15.7 μM), or DLPA (17.4 μM). The cultures were given 1-h pulses with [^{3}H]thymidine as indicated at respective times. [^{3}H]thymidine incorporation into the acid-insoluble material was measured as described in Materials and methods. Each data point has a repeating value of n = 4.

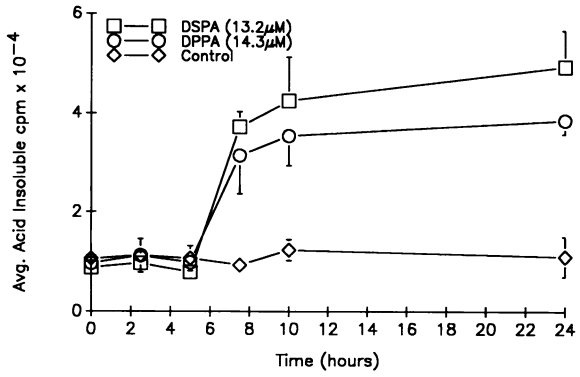

***Figure 3.* Exposure time required for PA-induced [^{3}H]thymidine incorporation.** The cell culture procedures were the same as described for Figure 1. At T = 0 the cultures were stimulated with DSPA (13.2 μM) or DPPA (14.3 μM). At the times indicated, the cultures were washed with BME and placed in agonist-free media for the remainder of the experiment. The cultures were given 24-h pulses with [^{3}H]thymidine beginning at T = 0. Each data point has a value of n = 4.

time of exposure, as illustrated by the comparison of DSPA and DPPA.

In an effort to clarify whether the mitogenic potency reflects a differential membrane affinity of PA, we tested the ability of DLPA to block the mitogenic action of DSPA. As shown in Figure 4A, DLPA demonstrated a concentration-dependent reduction of DSPA-induced incorporation of [^{3}H]thymidine into the acid-insoluble material. Maximal reduction of DSPA (13.2 μM) occurred and saturated at DLPA concentrations of 34.8 μM. An 85% reduction of the DSPA-induced stimulation was observed. The data did not fit a saturable binding formula but followed a model based on the behavior of an ideal solution consisting of two components. If Total Activity = $\sum_i A_i x_i[i]$, where A_i, x_i, and [i] are, respectively, the specific activity coefficient, mole fraction, and molar concentration of the i^{th} component, then, for a given mixture of DLPA and DSPA (assuming $A_{DLPA} = 0$), the expression becomes Total Activity = $A_{DSPA}([DSPA]^2/[DSPA]+[DLPA])$. The approximate linear relationship shown in Figure 4B stems from the hypothesis that, at one stage, DSPA and DLPA behave like an ideal solution in which one component dilutes the other without preferential "binding" or differential "affinity." However, this finding alone cannot resolve whether a non–chain-specific receptor or a non–chain-specific partitioning of PA into the bilayer is the nondiscriminating step. This seems to indicate the existence of a common receiving mechanism for PA in general, be it the binding of PA to a common PA receptor or a partitioning of PA into the bilayer.

Because transient increases in cytoplasmic calcium have been observed to be one of the earliest cellular responses to various growth factors (Moolenaar *et al.* 1986), we decided to measure cytosolic free calcium levels in cells treated with various PAs. As shown in Figure 5, treatment of the cells with LOPA and DSPA resulted in the production of calcium transients. On stimulation with LOPA, an increase in intracellular calcium up to 1.3 μM was observed. This calcium transient was present in the presence or absence of external calcium, as shown by

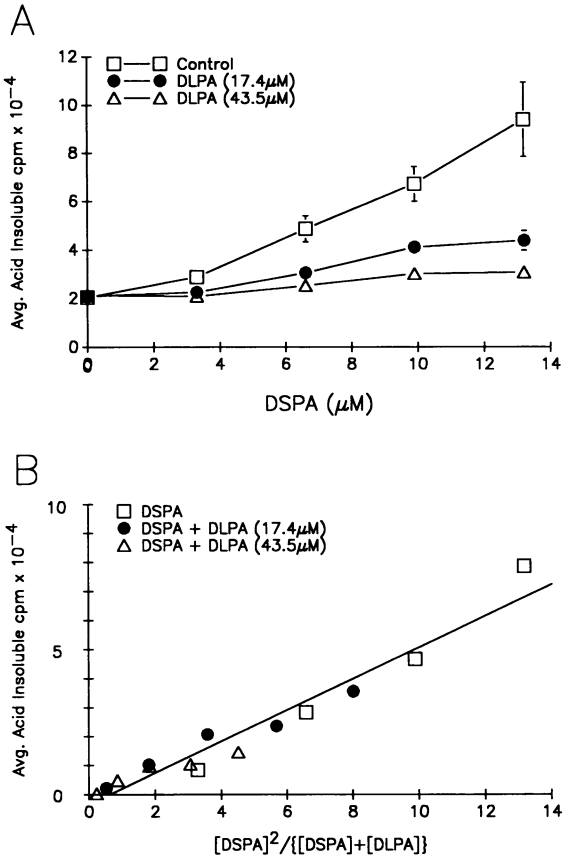

***Figure 4.* DLPA competition of DSPA-induced [^{3}H]thymidine incorporation.** (A) Concentration dependence of DLPA competition. The cell culture procedures were the same as described for Figure 1. Five minutes before T = 0, the cultures were incubated with DLPA at the indicated concentrations. At T = 0 the cells were stimulated with DSPA (13.2 μM). Simultaneously, cultures were pulsed with [^{3}H]thymidine and at T = 24 h the [^{3}H]thymidine incorporated into the acid-insoluble material was measured. All of the values obtained were normalized to the values obtained in the absence of DLPA. (B) Plot of average counts of induced [^{3}H]thymidine incorporation vs. $(DSPA)^2/([DSPA] + [DLPA])$. The line represents the result of linear regression for all data points.

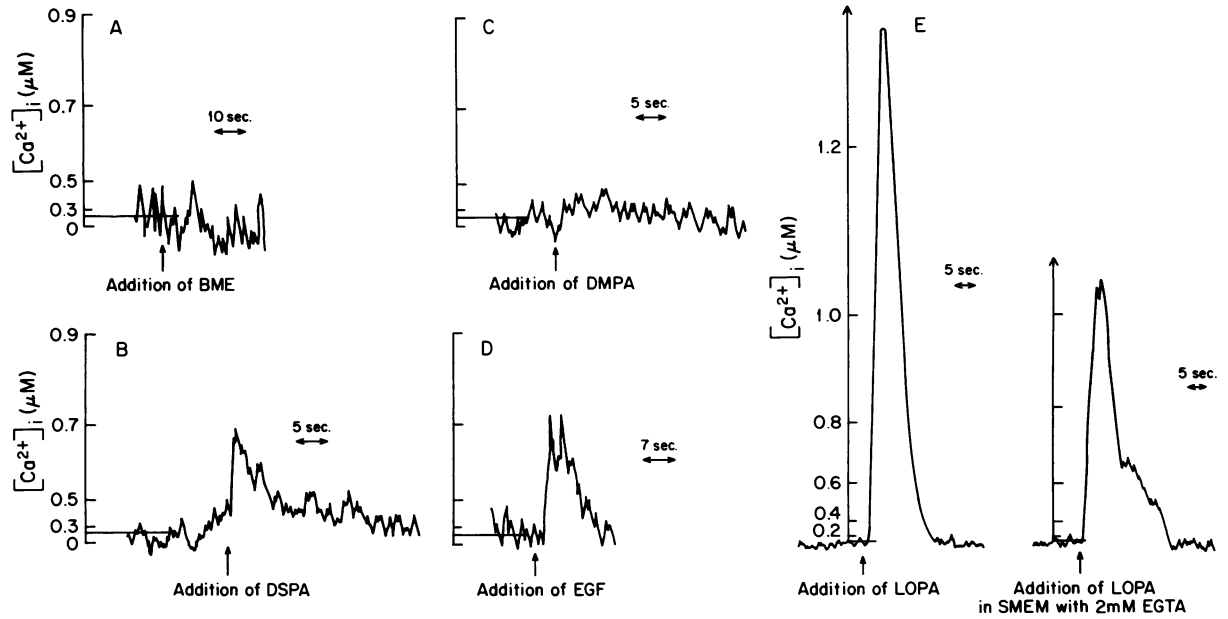

Figure 5. **Effect of DSPA, LOPA, DMPA, and epidermal growth factor (EGF) on $C_3H/10T_{1/2}$ cell intracellular calcium levels.** The calcium indicator photoprotein aequorin was scrape loaded into nearly confluent $C_3H10T_{1/2}$ cells and $[Ca]_i$ levels were calculated as described in Materials and methods. The tracings are the results of representative experiments repeated six times. Spent medium (unless otherwise noted), plus the indicated chemicals, was added at the times indicated by the arrows: (A) none, (B) DSPA (13.2 μM), (C) DMPA (15.7 μM), (D) EGF (20 ng/ml), (E) LOPA (13.2 μM), and LOPA (13.2 μM) in SMEM containing 2 mM EGTA.

the use of supplemented Eagle's minimum essential medium (SMEM), a calcium-free media containing 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and consistent with the result of Jalink *et al.* (1990). DSPA produced an increased intracellular calcium level with maximum at 600 nM. Within the limits of the aequorin technique, LSPA (data not shown) and DMPA did not produce any calcium mobilization. All of the transients occurred within 5 s after the addition of the stimulus and lasted for 15–20 s. The data in Figure 5 suggest that the transient calcium mobilization observed immediately after stimulation is not relevant to the mitogenic action of PA or lysoPA in our cell type.

Discussion

Our present understanding of the mitogenic activity of PA is unclear. PA, but not other phospholipids, has been shown to incorporate rapidly into the cytosol of fibroblasts (Pagano and Sleight, 1985), because of the fact that most of the PA was rapidly converted into DAG on the cell surface (Pagano and Longmuir, 1985). The chain length and/or saturation dependence of this process has not been reported. Initially, PA was implicated to behave like a calcium ionophore in the plasma membrane, eliciting an influx of calcium (Putney *et al.*, 1980; Smaal *et al.*, 1987). Moolenaar *et al.* (1986), as well as Kawase and Suzuki (1988), have demonstrated that the observed PA ionophore effect is actually mediated by inositol trisphosphate (IP_3). PA has also been shown to stimulate Na^+/Ca^{2+} exchange in sarcolemma (Philipson and Nishimoto, 1985). Proll *et al.*, (1985) as well as Murayama and Ui (1987) showed that treatment of fibroblasts with unsaturated PA resulted in a pertussis toxin–sensitive decrease in cyclic AMP levels. These findings led to the proposal that PA has its own receptor and works through the G_i protein. Stacey's group (Yu *et al.*, 1988; Tsai *et al.*, 1989) proposed that PA inhibits GAP, which, in turn, inhibits the *ras* GTPase. The inhibitory effect is lowest for PAs with long saturated chains (most favorable for growth stimulation). van Corven *et al.* (1989) demonstrated that the mitogenic action of lysoPA and possibly PA occurs through G_i or a related pertussis toxin substrate. The mechanism by which extracellular lysoPA activates these G protein–regulated effector systems is unknown, although it appears that the PI cycle is not involved (van Corven *et al.*, 1989). Imagawa *et al.* (1989) ob-

tained further results in normal mouse epithelial cells demonstrating that the minimal esterified fatty acid requirement for optimal mitogenesis is the presence of one polyunsaturated fatty acyl group at position 2 of the glycerol moiety.

Other biologically active lipids also exhibit acyl chain specificity with regard to function. For example, structure activity relations indicate that PKC is specifically activated by the *sn*-1,2-diacylglycerol stereoisomer. Activation also requires the presence of the hydroxyl at the 3 position, the esters at the 1 and 2 positions, and acyl chains of at least six carbons in length (Davis *et al.,* 1985; Ganong *et al.,* 1986). Structure activity studies with sphingosine analogues, specific inhibitors of PKC, showed that the 18-carbon molecule had optimal cellular and in vitro activity and that both shorter and longer chain analogues showed progressively less activity (Hannun and Bell, 1989).

In this paper we found that PAs and lysoPAs with long saturated fatty acid moieties promote DNA synthesis in quiescent postconfluent $C_3H/10T_{1/2}$ cells. The mitogenic potential of a given PA is correlated with the chain length of its fatty acid moieties. The longer the chain length, the more mitogenic it appears to be. These data agree with the limited findings obtained when saturated PAs were utilized as mitogens in NIH 3T3, Rat-1, and A-431 cells (Moolenaar *et al.,* 1986; Yu *et al.,* 1988). Unsaturated PAs were inefficient in our system, contrary to the findings of others (Imagawa *et al.,* 1989). In addition, the mitogenic activity of PA is not due to contamination of PA preparations with lysoPA. We found the mitogenic potency of pure LSPA to be similar to that of pure DSPA. If indeed lysoPAs are more potent mitogens than PAs, as claimed by van Corven *et al.* (1989), our DSPA would have to be mostly LSPA to match the mitogenic activity of the latter. This apparently is not the case, as shown by lipid analysis.

The differential aqueous solubility of PA and lysoPA with different acyl chain length varies considerably, the former being vesicular and the latter being micellar. Both PA and lysoPA are precipitable by calcium in the medium if not protected by serum albumin. We have taken considerable measure to ensure their uniform dispersion in aqueous medium. Certain inconsistencies in the literature could be attributed to the method utilized to disperse PA into aqueous medium.

The ability of DLPA to compete out the mitogenic effect of DSPA by dilution suggests that the initial binding of PA to a putative receptor or partitioning into the plasma membrane occurs in a chain-length-independent fashion. There must be an early stage that involves the absence of differential affinity or binding constants for either PA before a chain-specific step in the mitogenic signaling pathway.

In addition, we found that the mitogenic potential of saturated PAs was correlated with the magnitude of the weak calcium transients they induced. Relative to PAs, the strong calcium transient that LOPA produced and the absence of a transient on stimulation with LSPA did not correspond to their mitogenic potentials. It is unlikely that the observed transients, which were probably produced by PI turnover (Jalink *et al.,* 1990), are an integral part of the signal transduction pathway utilized by PA. A recent article by Hill *et al.* (1990) demonstrates that PI turnover, resulting from the addition of platelet-derived growth factor, is not necessary for its mitogenic action in rat liver cells.

Pagano and Sleight (1985) suggested that, on incorporation into the outer leaflet of the cell membrane, exogenously added PA is dephosphorylated to DAG and transported to the inner leaflet of the membrane. It is then transferred to the endoplasmic reticulum, the mitochondria, and the nuclear membrane. Langmuir and Malnick (1989) have recently demonstrated that at 2°C the transfer of PA from liposomes to Chinese hamster ovary cells occurs in a collision-dependent fashion, resulting in the exclusive formation of 1,2-diacylglycerol. Our preliminary experiment shows that the cellular uptake of ^{14}C-DLPA and ^{14}C-DSPA over a 30-min period was 13.5 ± 0.9% and 14.0 ± 0.7% of the total counts added for ^{14}C-DLPA and ^{14}C-DSPA, respectively. The uptake of each lipid is expressed as the mean of five experiments with the accompanying SD. The results demonstrate that there is no significant difference in the uptake of ^{14}C-DLPA and ^{14}C-DSPA over the time period measured.

In $C_3H/10T_{1/2}$ cells, potency of PA to induce mitogenesis could be related to the rates of metabolism of the exogenous PA. Thus, the chain-length specificity may reside in the ability of a given phosphatidate to resist metabolic degradation. The magnitude of PA-sensitive signal is then determined by the availability of exogenous PA at any given time. This is consistent with our data indicating that the continuous presence of exogenous PA is required for a minimum of 7 h to elicit a mitogenic response. Alternatively, the continued presence of exogenous PA may be necessary for the production of a precursor essential for mitogenesis. The metabolic conversion of exogenous PA into the

precursor may occur in a chain-length-dependent fashion. We are currently investigating both alternatives by the use of ^{14}C-DLPA and ^{14}C-DSPA.

It is likely that PA is operating via more than one mechanism (van Corven *et al.*, 1989). It is of interest to speculate that the continuous presence of exogenous PA may serve to constitutively activate a receptor-mediated transduction pathway. Concomitantly, exogenous PA may serve as a sustained source of DAG. Pelech and Vance (1989), as well as Dunlop and Larkins (1989), have suggested that a sustained increase in DAG is a necessary component of many signaling mechanisms utilized by various growth factors. The interdependence of lipid cycles is complex, involving a multitude of metabolic pathways (Hannun and Bell, 1989; Pelech and Vance, 1989). The unique routes of PA transport and intracellular trafficking, the role of PA in the PC and PI cycles, and the possibility of direct modulation of G-proteins and protein kinases all seem to contribute to the role of PA in the signal transduction process. We are currently investigating the effects of PA in more detail in an effort to clarify the mechanism of PA-induced mitogenesis.

Materials and methods

Cell culture

$C_3H/10T_{1/2}$ fibroblasts (clone 8) were maintained as previously described by Reznikoff *et al.* (1973), using Eagle's Medium (GIBCO, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT) and 0.001% Gentamicin sulfate (GIBCO) as an antimicrobial agent. The cells were inoculated at a density of 1×10^4 cells/ml with subsequent media changes every 4 d unless otherwise indicated. Cultures were grown to confluency and experiments were performed in a 95% air/5% CO_2 atmosphere at 37°C.

Phosphatidate treatment

One micromole of lyophilized PA was added directly to 1 ml of distilled water, transferred into a sterile sonication tube, and sonicated for 5 min in a bath sonicator maintained at a temperature above the gel/fluid phase transition temperature. The single lamellar vesicle (SUV) was diluted 10-fold in spent medium that had been collected from the culture dishes of the cells to be used in the experiment. The use of spent media is to ensure minimal perturbation of the system on addition of PA. The diluted SUV suspension was then added to the culture dishes to give the desired concentration of lipid. Control cultures were treated identically without the inclusion of PA. All PA solutions were optically clear after sonication.

Analysis of DNA synthesis

Measurements of DNA synthesis were made using [^{3}H]thymidine added to cultures at 1.0 μCi/ml of culture media (SA = 85 Ci/mmol) at the designated times. The method used for the detection of [^{3}H]thymidine into the acid-insoluble fraction is as previously described by Van Obberghen-Schilling *et al.* (1983). In brief, after the cells were labeled for a given period of time, the medium containing the label was aspirated and the cells were fixed in 10% trichloroacetic acid for 20 min. At the end of the 20-min time period, the cells were washed twice with phosphate-buffered saline and then solubilized in 0.5N NaOH overnight. The solubilized fractions were placed in scintillation vials and counted using a Beckman LS-20 scintillation counter.

Intracellular calcium measurements

High-density $C_3H/10T_{1/2}$ fibroblasts (7.0×10^7 cells/150-mm dish) were loaded with the calcium indicator photoprotein aequorin (Dr. J. Blinks, Mayo Clinic, Rochester, MN) by the scrape loading technique (McNeil and Taylor, 1985). Briefly, the culture medium was aspirated and the cells were rinsed three times with sterile calcium-free buffered saline at 37°C. The final rinse was removed and 250 μl of the aequorin stock solution was added. A sterile rubber policeman was then used to gently scrape the cell, and 2 min were allowed to elapse before complete culture medium was added. The scraped cells from one 150-mm dish were replated into 8 wells of a 12-well plate (Costar, Cambridge, MA) in basal medium, Eagle's (BME) supplemented with 10% FBS and 0.001% Gentamicin sulfate. Sixteen hours after replating, this medium was replaced with spent medium or SMEM containing 2 mM EGTA. Three hours later, the experiments were performed. Aequorin luminescence measurements were made as previously described (Onuma and Hui, 1988). Precaution was taken to exclude luminescence because of cell rupture during the addition of agents. Resting and stimulated intracellular calcium levels were calculated according to the method of Allen and Blinks (1979).

^{14}C-labeled lipid synthesis and analysis

^{14}C-labeled PCs (Amersham, Arlington Heights, IL) were converted to PA by reaction with phospholipase D isolated from savoy cabbage. The products were purified by thin-layer chromatography and the activity was determined. After labeling with ^{14}C-PA we washed the $C_3H/10T_{1/2}$ fibroblasts three times with ice-cold phosphate-buffered saline and then trypsinized them. The trypsinized cells were solubilized and then counted in a Beckman LS-8 scintillation counter.

Chemicals

Compounds were obtained as noted through Materials and methods. Additionally, $C_3H/10T_{1/2}$ fibroblasts (clone 8) were obtained from ATCC (Rockville, MD). DAG were purchased from Sigma (St. Louis, MO). DSPA and LSPA were synthesized in our laboratory. All other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). [^{3}H]thymidine, ^{14}C-stearate, and ^{14}C-laurate were purchased from Amersham.

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References

Agwu, D.E., McPhail, L.C., Chabot, M.C., Daniel, L.W., Wykle, R.L., and McCall, C.E. (1989). Choline linked phosphoglycerides. J. Biol. Chem. *264,* 1405–1413.

Allen, D.G., and Blinks, J.R. (1979). The interpretation of light signals from aequorin-injected skeletal and cardiac muscle cells: a new method of calibration. In: Detection and Measurement of Free Calcium in Cells, ed. C.C. Ashley, and A.K. Campbell, Amsterdam: Elsevier/North Holland, 159–174.

Cutry, A.F., Kinniburgh, A.J., Krabak, M.J., and Hui, S.W. (1989). Induction of c-*fos* and c-*myc* proto-oncogene expression by epidermal growth factor and transforming growth factor α is calcium-independent. J. Biol. Chem. *264,* 19700–19705.

Davis, R., Ganong, B., Bell, R., and Czech, M. (1985). Structural requirements for diacylglycerols to mimic tumor-promoting phobol diester action on the epidermal growth factor receptor. J. Biol. Chem. *260,* 5315–5322.

Dunlop, M.E., and Larkins, R.G. (1989). Effects of phosphatidic acid on islet cell phosphoinositide hydrolysis, Ca^{2+}, and adenylate cyclase. Diabetes *38,* 1187–1192.

Exton, J.H. (1988). Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme. FASEB J. *2,* 2670–2676.

Farese, R.V., Konda, T.S., Davis, J.S., Stundaert, M.L., Pollet, R.J., and Cooper, D.R. (1987). Insulin rapidly increases diacylglycerol by activating de novo phosphatidic acid synthesis. Science *236,* 586–588.

Ganong, B., Loomis, C., and Hannun, Y. (1986). Specificity and mechanism of protein kinase C activation by sn-1.2-diacylglycerols. Proc. Natl. Acad. Sci. USA *83,* 1184–1188.

Hannun, Y., and Bell, R. (1989). Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. Science *243,* 500–507.

Hill, T.D., Dean, N.M., Mordan, L.J., Lau, A.F., Kanemitsu, M.Y., and Boynton, A.L. (1990). PDGF-induced activation of phospholipase C is not required for induction of DNA synthesis. Science *248,* 1660–1663.

Imagawa, W., Bandyopadhyay, G., Wallace, D., and Nandi, S. (1989). Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture. Proc. Natl. Acad. Sci. USA *86,* 4122–4126.

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. J. Biol. Chem. *265,* 12232-9.

Kawase, T., and Suzuki, A. (1988). Phosphatidic acid–induced calcium mobilization in osteoblasts. J. Biochem. *103,* 581–582.

Lacal, J.C. (1990). Diacylglycerol production in *Xenopus laevis* oocytes after microinjection of $p21^{ras}$ proteins is a consequence of activation of phosphatidylcholine metabolism. Mol. Cell. Biol. *10,* 333–340.

Langmuir, K.J., and Malnick, L.A. (1989). Transfer of phosphatidic acid liposomes to cells is collision dependent. Am. J. Physiol. *256* (Cell Physiol. *25*), C522–C531.

McNeil, P.L., and Taylor, D.L. (1985). Aequorin entrapment in mammalian cells. Cell Calcium *6,* 83–93.

Moolenaar, W.H., Kruijer, W., Tilly, B.C., Verlaan, I., Bierman, A.J., and de Laat, S.W. (1986). Growth factor–like action of phosphatidic acid. Nature *323,* 171–173.

Murayama, T., and Ui, M. (1987). Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. J. Biol. Chem. *262,* 5522–5529.

Onuma, E.K., and Hui, S.W. (1988). Electric field–directed cell shape changes, displacement, and cytoskeletal reorganization are calcium dependent. J. Cell Biol. *106,* 2067–2075.

Pagano, R.E., and Longmuir, K.J. (1985). Phosphorylation, transbilayer movement, and facilitated intracellular transport of diacylglycerol are involved in uptake of a fluorescent analog of phosphatidic acid by cultured fibroblasts. J. Biol. Chem. *260,* 1909–1916.

Pagano, R.E., and Sleight, R.G. (1985). Defining lipid transport pathways in animal cells. Science *229,* 1051–1057.

Pelech, S.L., and Vance, D.E. (1989). Signal transduction via phosphatidylcholine. Trends Pharmacol. Sci. *14,* 28–30.

Philipson, K.D., and Nishimoto, A.Y. (1985). Stimulation of Na^{+}-Ca^{2+} exchange in cardiac sarcolemmal vesicles by phospholipase D. J. Biol. Chem. *259,* 16–19.

Proll, M.A., Clark, R.B., and Butcher, R.W. (1985). Phosphatidate and monooleylphosphatidate inhibition of fibroblast adenylate cyclase is mediated by the inhibitory coupling protein, N_i. Mol. Pharmacol. *28,* 331–337.

Putney, J.W., Jr., Weiss, S.J., Van De Walle, C.M., and Haddas, R.A. (1980). Is phosphatidic acid a calcium ionophore under neurohumoral control? Nature *284,* 345–347.

Reznikoff, C.A., Brankow, D.A., and Heidelberger, C. (1973). Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. *33,* 3231–3238.

Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C., and Bauman, A.J. (1969). Diethylamino and triethylamino cellulose column chromatographic procedures for phospholipids, glycolipids, and pigments. Methods Enzymol. *14,* 272–317.

Smaal, E.B., Nicolay, K., Mandersloot, J.G., de Gier, J., and de Kruijff, B. (1987). ^{2}H-NMR, ^{31}P-NMR and DSC characterization of a novel lipid organization in calcium-dioleoylphosphatidate membranes. Implications for the mechanism of the phosphatidate calcium transmembrane shuttle. Biochim. Biophys. Acta *897,* 453–466.

Skipsky, V.P., Peterson, R.F., and Barclay, M. (1964). Quantitative analysis of phospholipids by thin-layer chromatography. Biochem. J. *90,* 374–378.

Tsai, M.H., Yu, C.L., Wei, F.S., and Stacey, D.W. (1989). The effect of GTPase activating protein upon *ras* is inhibited by mitogenically responsive lipids. Science *243,* 522–526.

van Corven, E.J., Groenink, A., Kees, J., Eichholtz, T., and Moolenaar, W.H. (1989). Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G-proteins. Cell *59,* 45–54.

Van Obberghen-Schilling, E., Perez-Rodriguez, R., Franchi, A., Chambord, J.C., and Pousseygeur, J. (1983). Analysis of growth factor "relaxation" in chinese hamster lung fibroblasts required for tumoral expression. J. Cell. Physiol. *115,* 123–130.

Yu, C.L., Tsai, M.H., and Stacey, D.W. (1988). Cellular *ras* activity and phospholipid metabolism. Cell *52,* 63–71.