

# Phosphatidic Acid Is a Potent And Selective Inhibitor of Protein Phosphatase 1 and an Inhibitor of Ceramide-mediated Responses\*

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**In the present study, we report that phosphatidic acid (PA) functions as a novel, potent, and selective inhibitor of protein phosphatase 1 (PP1). The catalytic subunit of PP1 $\alpha$  was inhibited by PA dose-dependently in a non-competitive manner with a  $K_i$  value of 80 nM. The inhibition by PA was specific to PP1 as PA failed to inhibit protein phosphatase 2A (PP2A) or PP2B. Furthermore, PA was the most effective and potent inhibitor of PP1 compared with other phospholipids. Because we recently showed that ceramides activated PP1, we next examined the effects of PA on ceramide stimulation of PP1. PA inhibited both basal and ceramide-stimulated PP1 activities, and ceramide showed potent and stereoselective activation of PP1 in the presence of PA. Next, the effects of PA on ceramide-induced responses were examined. Molt-4 cells took up PA dose- and time-dependently such that by 1 and 3 h, uptake of PA was 0.37 and 0.65% of total PA added, respectively. PA at 30  $\mu$ M and calyculin A at 10 nM (an inhibitor of PP1 and PP2A at low concentrations), but not okadaic acid at 10 nM (a PP2A inhibitor at low concentrations) prevented poly(ADP-ribose) polymerase proteolysis induced by C<sub>6</sub>-ceramide. Moreover, the combination of PA with okadaic acid prevented retinoblastoma gene product dephosphorylation induced by C<sub>6</sub>-ceramide. These data suggest that PA functions as a specific regulator of PP1 and may reverse or counteract those effects of ceramide that are mediated by PP1, such as apoptosis and retinoblastoma gene product dephosphorylation.**

Protein phosphatase 1 (PP1)<sup>1</sup> is a serine/threonine phosphatase that regulates diverse processes in cellular functions, such as cell division, muscle contraction, gene expression, glycogen metabolism, and neurotransmission (1–3). Two endogenous proteins, inhibitor 1 and inhibitor 2, as well as Mn<sup>2+</sup> have been reported as factors that regulate PP1 specifically, but not other protein phosphatases, such as PP2A, PP2B, PP2C, or protein-tyrosine phosphatases (4, 5). Recently, we found that ceramide, a novel lipid second messenger, also regulates PP1 activity,

and PP1 may function downstream of ceramide in cells,<sup>2</sup> especially in causing dephosphorylation of the retinoblastoma gene product (Rb).

Phosphatidic acid (PA) is another proposed second messenger in cellular responses. In different cell types, PA can mimic physiological agonists leading to various cellular responses, such as neurotransmission, hormone release, cell proliferation, expression of several proto-oncogenes, and calcium influx in cells. PA has also been shown to modulate several enzyme activities, such as phospholipase C, protein kinases, cyclic AMP-phosphodiesterase, Ras GTPase-activating protein and protein-tyrosine phosphatase (6–8). PA is produced by 1) phospholipase D (PLD), which causes the hydrolysis of phospholipids (such as phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine), 2) diacylglycerol (DAG) kinase, which causes the phosphorylation of DAG, or 3) lysophosphatidic acid (LPA)-acyltransferase, which acylates LPA (9–12). Of these, the PLD pathway has been the best studied in signal transduction, and the generated PA has been proposed as a key mediator of PLD action.

Multiple studies suggest an important role for ceramide in regulating diverse cell responses, such as apoptosis, cell cycle arrest, and cell senescence (13). Our recent finding that ceramide activated PP1 stereoselectively and that PP1 may mediate the effects of ceramide on Rb phosphorylation raised the possibility that PP1 is a lipid-regulated phosphatase.<sup>2</sup> During the investigation of lipid regulation of PP1 and the role of PP1 in ceramide-induced signaling, we found that PA functions as a potent inhibitor of PP1.

In the present study, we report on this inhibition of PP1 by PA. We also provide evidence that there is a role for PP1 in mediating the effects of ceramide on caspases and that PA is capable of inhibiting PP1-mediated effects of ceramide, *i.e.* specifically on activation of caspases and Rb dephosphorylation. The implications of these findings on the lipid regulation of PP1 and on the role of PP1 as a target for PA and ceramide are discussed.

## EXPERIMENTAL PROCEDURES

**Materials**—Recombinant PP1 ( $\alpha$ -isoform from rabbit muscle) was purchased from Calbiochem. The trimeric form (AB'C) of PP2A (purified from bovine cardiac tissue) was kindly provided by Dr. Craig Kamibayashi, Dept. of Pharmacology, University of Texas Health Science Center (Dallas, TX). PAs and other phospholipids were purchased from Avanti Polar Lipids Inc. Monoclonal mouse anti-human Rb antibody was purchased from PharMingen. Mouse anti-PP1 antibody and a polyclonal antibody to an epitope in the automodification domain of PARP were purchased from Transduction Laboratories. Horseradish peroxidase conjugates of a goat anti-rabbit for PARP and of a goat anti-mouse antibody for Rb or PP1 were from Bio-Rad. Sephacryl S300

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<sup>1</sup> The abbreviations used are: PP1, protein phosphatase 1; Rb, retinoblastoma gene product; PA, phosphatidic acid; PLD, phospholipase D; DAG, diacylglycerol; LPA, lysophosphatidic acid; MBP, myelin basic protein; FBS, fetal bovine serum; DiC<sub>8</sub>, dioctanoylglycerol; PARP, poly ADP-ribose polymerase; DiC<sub>18:1</sub>, dioleoylglycerol.

<sup>2</sup> K. Kishikawa, J. Y. Lee, A. Bielawska, S. H. Galadari, L. M. Obeid, and Y. A. Hannun, manuscript in preparation.

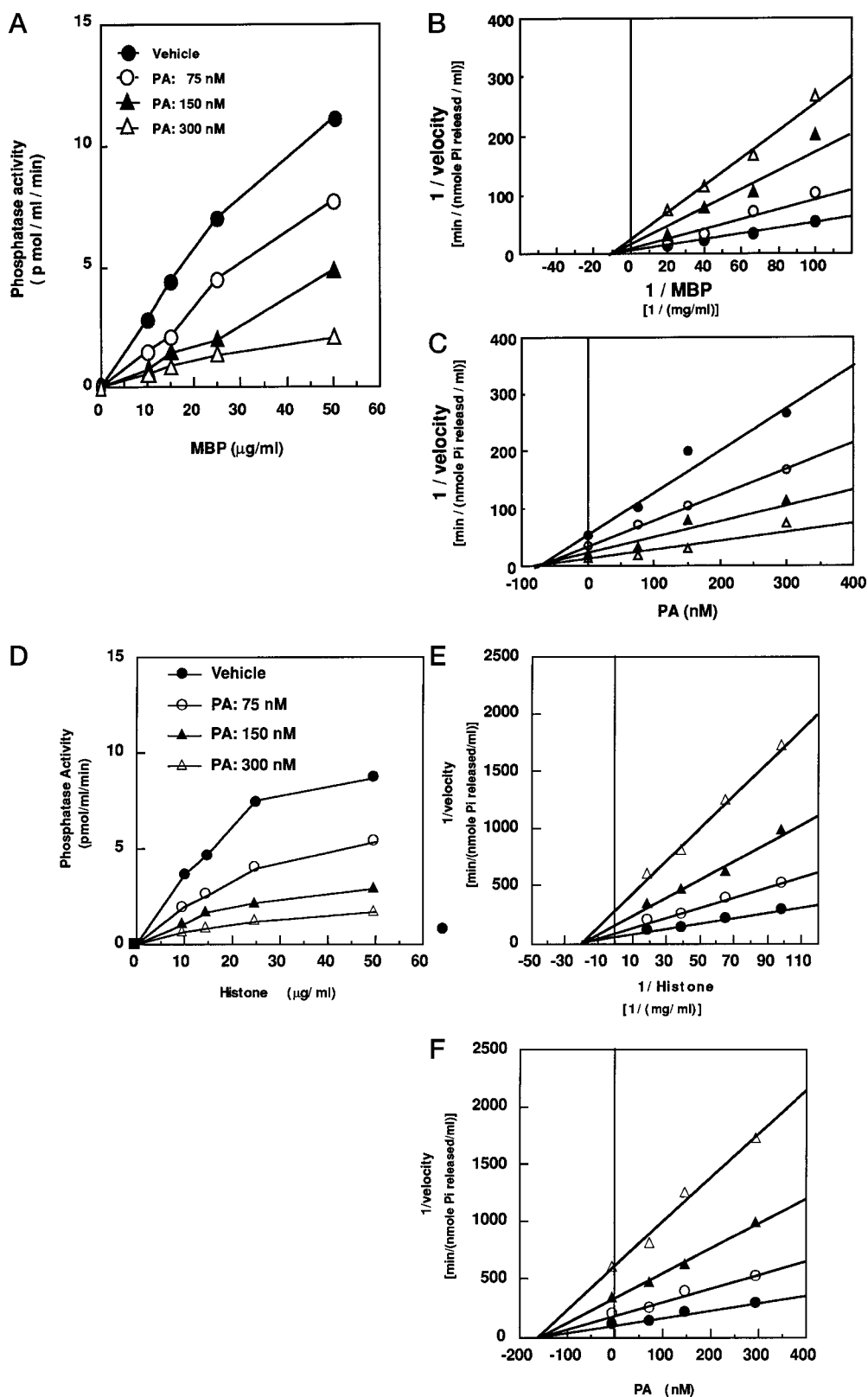


FIG. 1. Inhibition of recombinant PP1 catalytic subunit by PA. *A*, phosphatase activity of PP1 was assayed by the standard method (10–50  $\mu\text{g/ml}$  [ $^{32}\text{P}$ ]MBP, for 15 min at 30 °C) in the absence or presence of the indicated concentrations of PA. *B*, Lineweaver-Burk double-reciprocal plot of the same data. *C*, Dixon plot of the same data. This experiment is representative of two similar experiments. *D*, phosphatase activity of PP1 was assayed by the standard method (10–50  $\mu\text{g/ml}$  [ $^{32}\text{P}$ ]histone, for 15 min at 30 °C) in the absence or presence of the indicated concentrations of PA. *E*, Lineweaver-Burk double-reciprocal plot of the same data. *F*, Dixon plot of the same data. This experiment is representative of two similar experiments.

was from Amersham Pharmacia Biotech. Other materials were obtained from Sigma.

Preparation of  $^{32}\text{P}$ -Phosphorylated Myelin Basic Protein (MBP)—

[ $^{32}\text{P}$ ]MBP and [ $^{32}\text{P}$ ]histone were prepared in a 0.5-ml reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM  $\text{MgCl}_2$ , 100 mM [ $\gamma$ - $^{32}\text{P}$ ]ATP (20  $\mu\text{Ci}$ ), 5 mM dithiothreitol, 10 mM  $\beta$ -mercaptoethanol, and 1 mg of

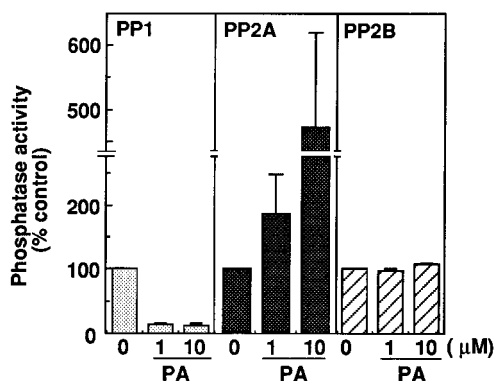


FIG. 2. Effects of PA on various protein phosphatases. Phosphatases were assayed as under "Experimental Procedures" in the absence or presence of the indicated concentrations of DiC<sub>18:1</sub>-PA. Phosphatase activity is presented as the activity relative to control in the absence of PA. Data are averages  $\pm$  S.E. of three separate experiments.

MBP (purified from bovine heart, Sigma) or histone Type IIIss (purified from calf thymus, Sigma). Purified catalytic subunit of protein kinase A (250 units in 100  $\mu$ l of 6 mg/ml dithiothreitol, purified from bovine heart, Sigma) was added, and the reaction proceeded for 2 h at 37  $^{\circ}$ C. Proteins were precipitated with 0.17 ml of cold 100% trichloroacetic acid for 30 min at 0  $^{\circ}$ C, sedimented at 12,000 rpm for 10 min at 4  $^{\circ}$ C, and washed twice with 1 ml of -20  $^{\circ}$ C acetone. The pellet was dissolved in 1 ml of 50 mM Tris-HCl, pH 7.4.

**Phosphatase Assay**—Dephosphorylation reactions contained 50 mM Tris-HCl, pH 7.4, enzyme (PP1 (10 milliunits) or PP2A (10 milliunits)), and the indicated amount of radiolabeled substrate in a final volume of 0.1 ml. PP2B (40 nM) was assayed in the same buffer containing calmodulin (80 nM), MgCl<sub>2</sub> (6 mM), and CaCl<sub>2</sub> (0.1 mM) (14). Ceramide or other lipids were delivered in 1  $\mu$ l of absolute ethanol to give the indicated concentrations. Control assays received a similar volume of ethanol. Egg yolk PA or other phospholipids in chloroform were dried under a stream of nitrogen and suspended in 50 mM Tris-HCl, pH 7.4, by ultrasonication with three 30-s pulses, and 10  $\mu$ l were added to give the indicated concentrations. Reactions were initiated with the addition of substrate and incubated for 15 min at 30  $^{\circ}$ C. Assays were terminated with 0.1 ml of 1 mM KH<sub>2</sub>PO<sub>4</sub> in 1 N H<sub>2</sub>SO<sub>4</sub> and 0.3 ml of 2% ammonium molybdate. After standing for at least 10 min, free <sup>32</sup>P<sub>i</sub> was determined as organic extractable counts after the addition of toluene:isobutyl alcohol (1:1). The amount of organic extractable <sup>32</sup>P<sub>i</sub> in blank assays was typically <0.1% of total radioactivity added. [<sup>32</sup>P]MBP and [<sup>32</sup>P]histone hydrolysis was linear with respect to time and protein and did not exceed 15% of total MBP or histone added.

**Rb Dephosphorylation**—Bcl-2-transfected Molt-4 cells (15) were grown in RPMI 1640 medium and 10% fetal bovine serum (FBS) containing 150  $\mu$ g/ml Hygromycin B1. For experiments, cells were diluted to  $2 \times 10^5$ /ml in RPMI 1640 medium with 2% FBS and treated with C<sub>6</sub>-ceramide, delivered in ethanol. Phospholipids in chloroform were dried under a stream of nitrogen and suspended in 50 mM Tris-HCl, pH 7.4, by ultrasonication with three 30-s pulses. Dioctanoylglycerol PA (DiC<sub>8</sub>PA) was delivered in ethanol. Molt-4 cells were pretreated with the designated phospholipid for 1 h prior to addition of EtOH vehicle or C<sub>6</sub>-ceramide. Rb dephosphorylation was evaluated by Western blot analysis of Rb migration, with the faster migrating forms indicating progressive dephosphorylation of Rb as described (15), using a monoclonal mouse anti-human antibody.

**PARP Proteolysis**—Jurkat cells were grown in RPMI 1640 medium and 10% fetal bovine serum. For experiments, cells were diluted to  $2 \times 10^5$ /ml in RPMI 1640 medium with 2% FBS and treated with C<sub>6</sub>-ceramide, delivered in ethanol. Phospholipids in chloroform were dried under a stream of nitrogen and suspended in 50 mM Tris-HCl, pH 7.4, by ultrasonication with three 30-s pulses. DiC<sub>8</sub> was delivered in ethanol. Molt-4 cells were pretreated with the designated phospholipid for 1 h prior to addition of EtOH vehicle or C<sub>6</sub>-ceramide. PARP proteolysis was evaluated by Western blot analysis as described (16) using a polyclonal rabbit anti-human antibody.

**PA Uptake and the Effect of PA on Uptake of C<sub>6</sub>-ceramide in Cells**—For PA uptake, dioleoylglycerol was labeled using diacylglycerol kinase producing [<sup>32</sup>P]DiC<sub>18:1</sub>-PA. Bcl-2-transfected Molt-4 cells ( $2 \times 10^5$ /ml in RPMI 1640 medium with 2% FBS) were incubated at 37  $^{\circ}$ C for the indicated time periods with 30  $\mu$ M [<sup>32</sup>P]DiC<sub>18:1</sub>-PA. Cells were harvested

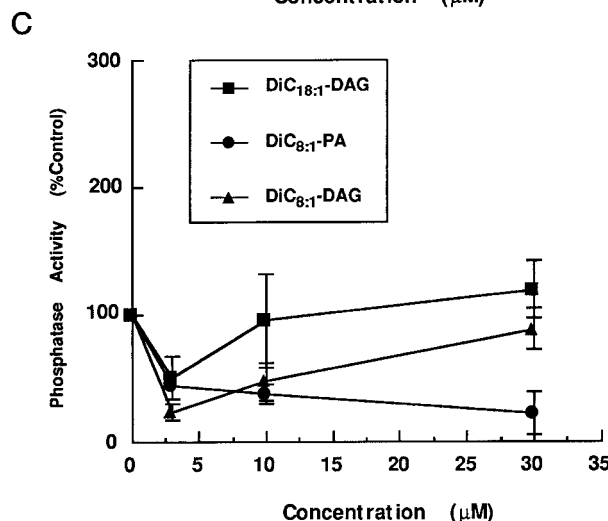
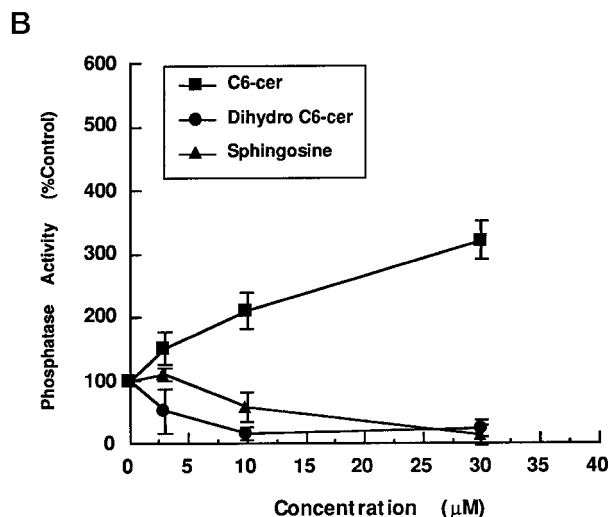
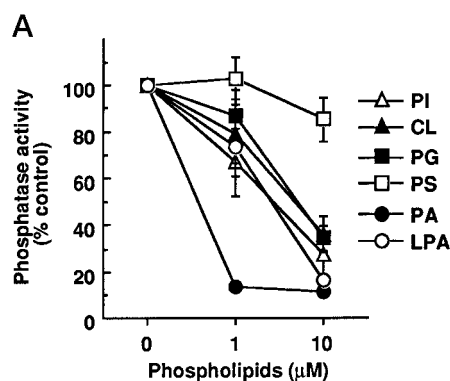


FIG. 3. Effects of lipids on PP1. A, phosphatases were assayed as in Fig. 1 in the absence or presence of the indicated concentrations of lipids. Data are averages  $\pm$  S.E. of five separate experiments. B, effect of other sphingolipids on PP1 activity. PP1 was assayed as in Fig. 1 in the presence of the indicated lipids at concentrations of 0, 3, 10, and 30  $\mu$ M. Phosphatase activity is presented as the activity relative to vehicle control. Data are averages  $\pm$  S.E. of three separate experiments. C, effect of short chain PA and DAG on PP1 activity. PP1 was assayed as in Fig. 1 in the presence of the indicated lipids at concentrations of 0, 3, 10, and 30  $\mu$ M. Phosphatase activity is presented as the activity relative to vehicle control. Data are averages  $\pm$  S.E. of three separate experiments.

by centrifugation and washed twice with cold phosphate-buffered saline, pH 7.4. The cells were suspended in 1 ml of deionized water and lysed by ultrasonication with three 30-s pulses. Radioactivity in the suspension was determined by liquid scintillation counting.

For ceramide uptake experiments, Bcl-2-transfected Molt-4 cells ( $2 \times$

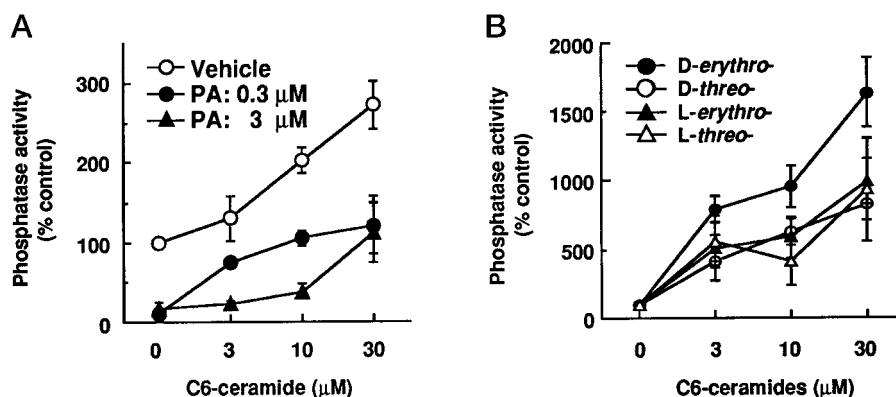


FIG. 4. Effects of PA on C<sub>6</sub>-ceramide-activated PP1. A, PP1 was assayed as in Fig. 1 in the absence or presence of the indicated concentrations of DiC<sub>18:1</sub>-PA and C<sub>6</sub>-ceramide. Phosphatase activity is presented as the activity relative to control in the absence of ceramide. Data are averages ± S.E. of three separate experiments. B, effect of stereoisomers of C<sub>6</sub>-ceramide on PA action. PP1 was assayed as in Fig. 1 in the absence or presence of the indicated concentrations of stereoisomers of C<sub>6</sub>-ceramide in the presence of 0.3 μM DiC<sub>18:1</sub>-PA. Phosphatase activity is presented as the activity relative to control in the presence of PA. Data are averages ± S.E. of three separate experiments.

10<sup>5</sup>/ml in RPMI 1640 medium with 2% FBS) were incubated at 37 °C for the indicated time periods with 20 μM [<sup>14</sup>C]C<sub>6</sub>-ceramide in the absence or presence of 30 μM PA. Cells were harvested by centrifugation and washed twice with cold phosphate-buffered saline, pH 7.4. The cells were suspended in 1 ml of deionized water and lysed by ultrasonication with three 30-s pulses. Radioactivity in the suspension was determined by liquid scintillation counting.

## RESULTS

**Effects of PA on PP1 activity for [<sup>32</sup>P]MBP in Vitro**—The recombinant catalytic subunit of PP1α (rabbit muscle) was incubated in the presence of increasing concentrations of DiC<sub>18:1</sub>-PA. The catalytic subunit of PP1α was inhibited by PA dose-dependently when assayed with 10–50 μg/ml [<sup>32</sup>P]MBP (Fig. 1A). Control PP1 activity for MBP showed Michaelis-Menten kinetics, yielding a *K<sub>m</sub>* of 100 μg/ml and a *V<sub>max</sub>* of 100 pmol/ml of P<sub>i</sub> released per min. As seen from Lineweaver-Burk analysis (Fig. 1B), PA caused a decrease in *V<sub>max</sub>* at lipid concentrations up to 300 nM in a noncompetitive manner. From Dixon analysis, DiC<sub>18:1</sub>-PA showed a *K<sub>i</sub>* value of 80 nM (Fig. 1C). Egg yolk PA produced the same results, and using [<sup>32</sup>P]histone as a substrate also produced similar results, yielding a *K<sub>m</sub>* of 58 μg/ml and a *V<sub>max</sub>* of 35 pmol/ml of P<sub>i</sub> released per min (Fig. 1D). Again, PA decreased the *V<sub>max</sub>* in a noncompetitive manner, with DiC<sub>18:1</sub>-PA demonstrating a *K<sub>i</sub>* value of 150 nM (Fig. 1, E and F).

**Specificity of PA Inhibition of PP1**—Previous work indicated that PA modulates protein-tyrosine phosphatase by activating the CD45-tyrosine phosphatase (8). Therefore, the effects of PA on other protein phosphatases were investigated. The inhibitory effect of PA was specific to PP1 compared with PP2A or PP2B. Interestingly, at 10 μM, DiC<sub>18:1</sub>-PA also activated PP2A by 5-fold (Fig. 2).

To evaluate whether other phospholipids are able to inhibit PP1, the effects of acidic phospholipids were examined. At 1 μM, only DiC<sub>18:1</sub>-PA inhibited PP1 completely, and at 10 μM, DiC<sub>18:1</sub>-LPA also inhibited PP1, probably due to structural similarity. Phosphatidylinositol, cardiolipin, and phosphatidylglycerol, but not phosphatidylserine, also showed more than 50% inhibition at 10 μM (Fig. 3). We also examined the effects of several other lipids, short chain PA, long and short chain diacylglycerol, sphingosine, and D-e-dihydro-C<sub>6</sub> ceramide in conjunction with D-e-C<sub>6</sub> ceramide on PP1 activity. With the exception of D-e-C<sub>6</sub> ceramide, each compound demonstrated modest inhibitory effects above 1 μM. Sphingosine (IC<sub>50</sub> = 10 μM), D-e-dihydro-C<sub>6</sub>-ceramide (IC<sub>50</sub> = 3 μM), DiC<sub>8:1</sub>-PA (IC<sub>50</sub> = 2.5 μM), DiC<sub>8:1</sub>-DAG (IC<sub>50</sub> = 2.2 μM), and DiC<sub>18:1</sub>-DAG (IC<sub>50</sub> = 3 μM) were inhibitory to PP1, but, similar to the phospholipids

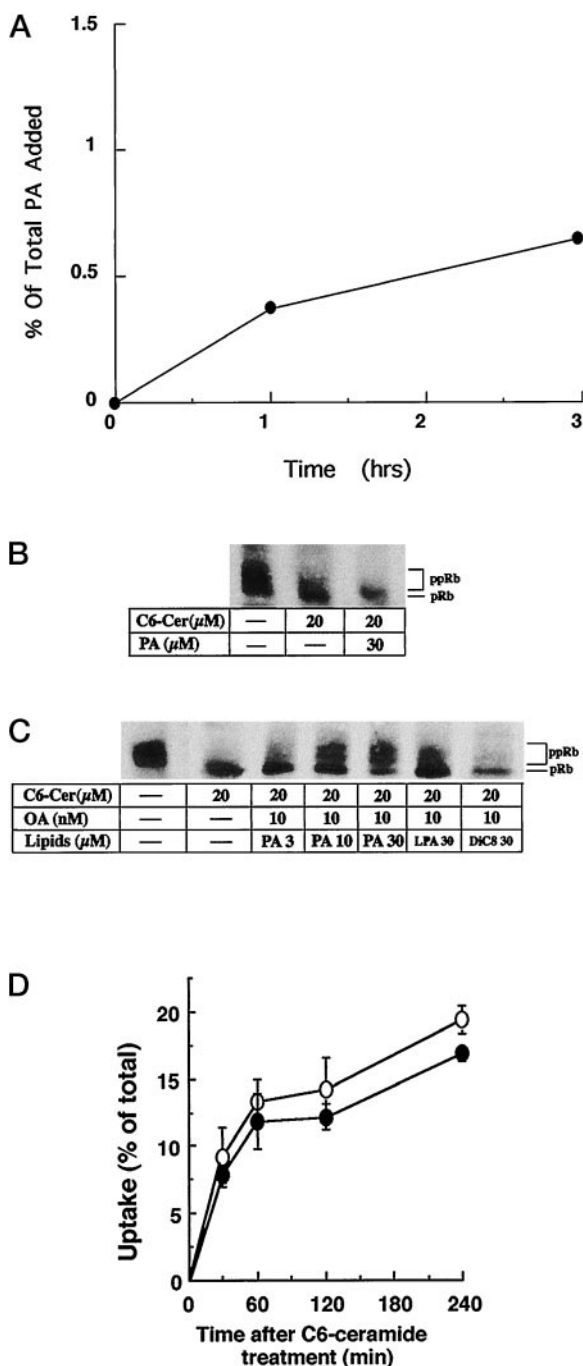
above, not as potently as DiC<sub>18:1</sub>-PA (IC<sub>50</sub> = 80 nM) (Fig. 3, C and D). These data strongly suggest that long chain PA is a potent and selective inhibitor of PP1.

**The Opposing Action of PA and Ceramide on PP1**—Recently, we found that ceramide activated PP1 in vitro and in cells.<sup>2</sup> Therefore, we next examined the effects of PA on ceramide activation of PP1. DiC<sub>18:1</sub>-PA inhibited both basal and ceramide-activated PP1 activities, but ceramide showed potent activation of 12.1- and 6.6-fold in the presence of 300 nM and 3 μM PA, respectively, compared with 2.7-fold in the absence of PA (Fig. 4A). Furthermore, in the presence of PA, D-erythro-C<sub>6</sub>-ceramide (the natural stereoisomer of ceramide) activated PP1 more potently than other stereoisomers of C<sub>6</sub>-ceramide (Fig. 4B).

**Effects of PA on Rb Dephosphorylation Induced by C<sub>6</sub>-ceramide in Cells**—We previously showed that ceramide induces cell cycle arrest (17) through activation of Rb in the Molt-4 T leukemia cells (18). Ceramide also activates caspases in cells resulting in cleavage of many substrates (13), including Rb (data not shown). We therefore performed the following studies in cells overexpressing Bcl-2, which inhibits the effects of ceramide on proteases but not on Rb dephosphorylation (15, 21). We also recently found that the dephosphorylation of Rb induced by ceramide may involve PP1.<sup>2</sup>

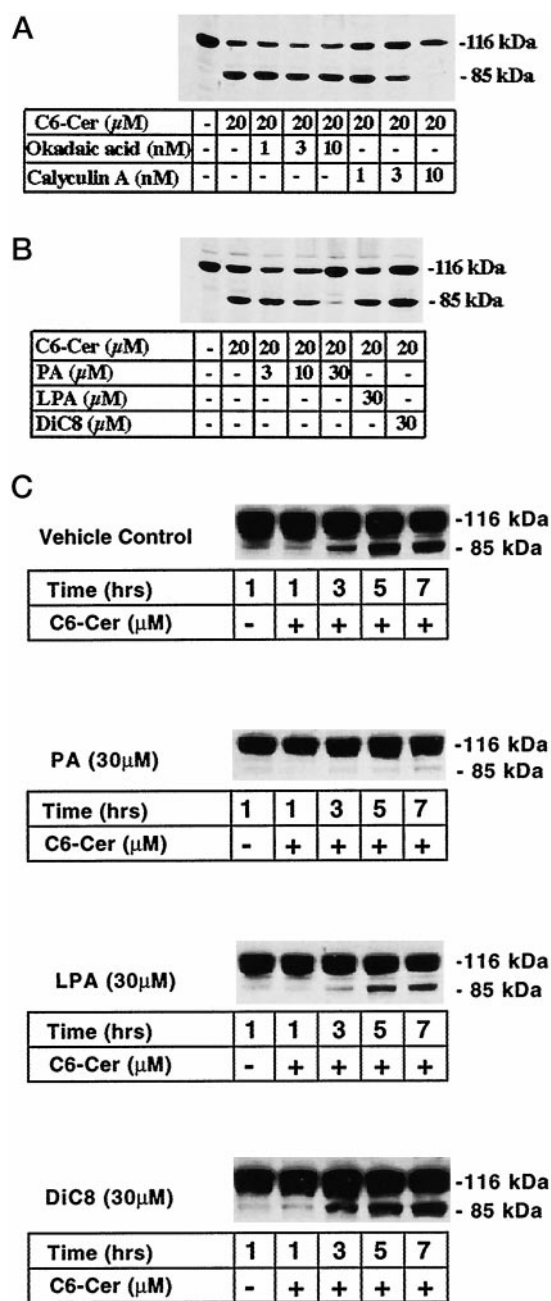
To evaluate the effects of PA, DiC<sub>18:1</sub>-PA was added to cells, and its effects on Rb were examined. First, we examined DiC<sub>18:1</sub>-PA uptake by Molt-4 cells. The uptake of PA was 0.37% after 1 h and 0.65% after 3 h, making the effective dose *in vivo* approximately 200-fold greater than *in vitro* (Fig. 5A). Therefore, using larger doses of PA for treatment of Molt-4 cells, we found that neither DiC<sub>18:1</sub>-PA (3, 10, and 30 μM) alone (Fig. 5B) nor okadaic acid (a specific PP2A inhibitor) prevented C<sub>6</sub>-ceramide-induced Rb dephosphorylation, but the combination of DiC<sub>18:1</sub>-PA with okadaic acid inhibited C<sub>6</sub>-ceramide-induced Rb dephosphorylation (Fig. 5C). Importantly, the effects of PA were not due to effects on ceramide uptake, as 30 μM DiC<sub>18:1</sub>-PA did not affect uptake of C<sub>6</sub>-ceramide (Fig. 5D). Moreover, the direct metabolites of PA, LPA, and DiC<sub>8</sub> had no effects on C<sub>6</sub>-ceramide-induced Rb dephosphorylation (Fig. 5C).

**Effects of PA, Calyculin A or Okadaic acid on PARP Proteolysis Induced by C<sub>6</sub>-ceramide in Cells**—Ceramide has been shown to induce caspase activation as measured by proteolysis of PARP (20, 21). First, and in order to determine whether phosphatases are involved in the induction of the proteolysis of PARP by ceramide, we examined the effects of calyculin A or okadaic acid on ceramide-induced PARP proteolysis in Molt-4



**FIG. 5. Effects of PA and okadaic acid (OA) on  $C_6$ -ceramide ( $C_6$ -Cer)-induced Rb dephosphorylation in cells.** *A*, the uptake of [ $^{32}$ P]DiC $_{18:1}$ -PA was evaluated in Molt-4 cells after 1 and 3 h of treatment. Data are averages of three separate experiments. *B*, Rb dephosphorylation in Bcl-2-transfected Molt-4 cells was evaluated by Western blot analysis using a monoclonal mouse anti-human antibody in the absence or presence of 20  $\mu$ M  $C_6$ -ceramide and 30  $\mu$ M DiC $_{18:1}$ -PA. *C*, effects of PA, LPA, or DiC $_8$  with okadaic acid on  $C_6$ -ceramide-induced Rb dephosphorylation. Rb dephosphorylation was evaluated as in *A* in the absence or presence of 20  $\mu$ M  $C_6$ -ceramide, 3 nM okadaic acid, or the indicated concentrations of DiC $_{18:1}$ -PA, LPA, or DiC $_8$ -PA. These experiments are representative of three separate experiments. *D*, effects of PA on  $C_6$ -ceramide uptake by cells. Cells were treated with [ $^{14}$ C] $C_6$ -ceramide in the absence or presence of 30  $\mu$ M DiC $_{18:1}$ -PA, and uptake was determined. Data are averages  $\pm$  S.E. of three separate experiments.

cells. Calyculin A, but not okadaic acid, prevented PARP proteolysis induced by  $C_6$ -ceramide (Fig. 6A). These results strongly suggest that PP1 is the primary phosphatase involved



**FIG. 6. Effects of PA, calyculin A, or okadaic acid on  $C_6$ -ceramide-induced PARP proteolysis in cells.** *A*, PARP proteolysis in Molt-4 cells was evaluated by Western blot analysis using a polyclonal rabbit anti-human antibody in the absence or presence of 20  $\mu$ M  $C_6$ -ceramide ( $C_6$ -Cer) and the indicated concentrations of calyculin A or okadaic acid. *B*, PARP proteolysis in Molt-4 cells was evaluated by Western blot analysis using a polyclonal rabbit anti-human antibody in the absence or presence of 20  $\mu$ M  $C_6$ -ceramide and the indicated concentrations of DiC $_{18:1}$ -PA, LPA, or DiC $_8$ -DAG. *C*, PARP proteolysis was evaluated after the indicated times in the absence or presence of 20  $\mu$ M  $C_6$ -ceramide and the presence of 30  $\mu$ M DiC $_{18:1}$ -PA, LPA, or DiC $_8$ -DAG. These experiments are representative of three separate experiments.

in mediating the effects of exogenous ceramides on caspase activation. Next, we evaluated the effects of DiC $_{18:1}$ -PA on ceramide-induced PARP proteolysis. DiC $_{18:1}$ -PA, but not LPA or DiC $_8$  prevented, at least in part,  $C_6$ -ceramide-induced PARP proteolysis in a dose-dependent manner (Fig. 6B). We also examined whether PA treatment had an effect on the time course of PARP cleavage in response to ceramide. Neither LPA nor DiC $_8$  affected the induction of PARP cleavage by ceramide treatment which began at approximately 3 h post-ceramide

treatment (Fig. 6C). On the other hand, DiC<sub>18:1</sub>-PA-treated cells did not demonstrate PARP cleavage in response to C<sub>6</sub>-ceramide until 7 h (Fig. 6C). These data suggest that ceramide may induce PARP proteolysis through PP1 activation and that PA is able to inhibit activation of PP1 by ceramide in cells.

#### DISCUSSION

In this study, we demonstrated that PA may function as a potent and selective inhibitor of PP1 and opposes the effects of ceramide on PP1 *in vitro* and in cells. Notably, the *in vitro* inhibition of PP1 by PA shows relatively high potency with some specificity to PA and high selectivity for PP1. PA had a  $K_i$  value of 80 nM in the inhibition of PP1, whereas other reported biochemical effects of PA have required a micromolar range (7, 9, 23). Other phospholipids were either without effects or with only weak effects seen with other anionic phospholipids, such as phosphatidylinositol, phosphatidylglycerol, and cardiolipin, which inhibited PP1 $\alpha$  by less than 40% at 1  $\mu$ M. Conversely, PA did not inhibit other serine/threonine phosphatases that were tested, thus demonstrating selectivity of action. The inhibition of PP1 by PA carries important implications for a possible physiologic role of PP1 in PA action, and for possible dual regulation of PP1 by PA and ceramide.

The *in vitro* results suggest a direct interaction between PA and the catalytic subunit of PP1 $\alpha$  in the absence of any other regulatory subunits. Recently, however, acidic phospholipids, including PA, were reported to bind the regulatory subunit (130 kDa) of PP1 (muscle myosin phosphatase) and may cause its association with liposomes (23). Thus, PA may have multiple sites of interaction with heteromeric PP1. Indeed, these studies raise the possibility that PP1 may function as a preferred cellular target for the action of PA.

PA has received increased attention as a candidate second messenger/lipid mediator, and PA formation in cells has been shown to be regulated by the action of either PLD or DAG kinase. Agonist-induced activation of PLD has been the better studied and documented of these two pathways, and it appears to involve the action of small G proteins and protein kinase C (6, 22, 24–27). This regulated formation of PA has led to the hypothesis that PA may function as a lipid second messenger; however, further evaluation of this hypothesis has been hampered by the difficulty in delivering PA to cells and by the difficulty in distinguishing effects of PA from those of DAG (with which it is metabolically interconvertible through the action of PA phosphohydrolases and DAG kinases). Despite these difficulties, a number of candidate direct targets for the action of PA have been identified *in vitro* and proposed as mediators of cellular action of PA. These include protein kinases, Ras GTPase-activating protein, phospholipase C, and cyclic AMP phosphodiesterases (6, 22, 28–30). These *in vitro* effects require  $\mu$ M concentrations of PA. In contrast, the effects of PA on PP1 are pronounced in the nM range, and as such PP1 is the most sensitive target for PA *in vitro* identified thus far. Moreover, the specificity of PA effects (even over LPA) provides further support for a potent and specific interaction of PP1 with PA.

The ability of PA, delivered exogenously, to inhibit PP1-dependent activities, such as activation of caspases, shows that cellular PP1 is responsive to changes in PA. Moreover, these effects were specific to PA in that neither DAG nor LPA, the two metabolites usually associated with PA formation, was active in these assays. The effects of exogenous PA were not as potent as the *in vitro* effects due to low cellular uptake of PA (approximately 1/200 of the [<sup>32</sup>P]DiC<sub>18:1</sub>-PA was taken up by the cells). Indeed, it was somewhat surprising that this negatively charged lipid exerted significant cellular effects.

These considerations lead us to propose that PP1 may func-

tion as a direct intracellular target for PA generated from agonist stimulation of PLD (or possibly from the action of DAG kinase). PP1 is reported to be the major protein phosphatase associated with the microsomes of several tissues (5), and Ito *et al.* (23) recently reported that myosin binding subunit of myosin phosphatase (PP1 $\delta$ ) was observed in the cytosol and the plasma membrane throughout the mitotic cycle. Indeed, the inhibition of PP1 by PA is consistent with the proposed mitogenic effects of PLD. Results from this study and from others are beginning to implicate PP1 in activation of caspases and Rb, thus connecting PP1 to apoptosis and cell cycle arrest, respectively.

In previous studies, ceramides were shown to inhibit PLD (31–35). In addition, PA was reported to have opposite actions to ceramide on cellular responses: 1) ceramide causes cell cycle arrest (14), but PA causes cell proliferation and opposes the effects of ceramide on proliferation (6, 22). 2) Ceramide inactivates PKC $\alpha$  (36), but PA may activate PKC (6, 22). 3) Ceramide inhibits superoxide formation and calcium influx (37), but PA activates NADPH oxidase to form superoxide and induces calcium influx (22).

Here, we find that PA has effects opposing those of ceramide on PP1. In cell studies, we show that the combination of PA and okadaic acid potentially prevent Rb dephosphorylation induced by ceramide. Lee *et al.*<sup>3</sup> showed that Rb is a direct substrate for both PP1 and PP2A. This is consistent with the current results and with our previous data showing that okadaic acid alone did not prevent ceramide-induced Rb dephosphorylation in cells.<sup>2</sup> We also show that calyculin A, but not okadaic acid, prevented ceramide-induced PARP proteolysis. This finding strongly suggests that PP1 is involved in the ceramide-induced apoptotic machinery. Importantly, PA also prevented ceramide-induced PARP proteolysis. This result further suggests that PA has an inhibitory activity on PP1 in the apoptotic pathway. Obviously, more studies are required to define the cellular mechanisms by which PA inhibits PP1 to prevent C<sub>6</sub>-ceramide-induced Rb dephosphorylation and PARP proteolysis.

In conclusion, these studies show specific and direct interactions between PP1 and PA. They also show opposite functions between PA and ceramide on PP1 *in vitro* and in cells. Indeed, the activity of PP1 in cells may be subject to a balance of the relative effects of PA and ceramide. The results support an emerging hypothesis that PP1 is a lipid regulated enzyme.

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