Tight Binding Inhibition of Protein Phosphatase-1 by Phosphatic Acid

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Phosphatidic acid (PA) has been identified as a bioactive lipid second messenger, yet despite extensive investigation, no cellular target has emerged as a mediator of its described biological effects. In this study, we identify the γ isofrom of the human protein phosphatase-1 catalytic subunit (PP1γ) as a high affinity in vitro target of PA. PA inhibited the enzyme dose-dependently with an IC₅₀ of 15 nM. Mechanistically, PA inhibited the enzyme noncompetitively with the kinetics of a tight binding inhibitor and a Kᵣ value of 0.97 ± 0.24 nM. Together, these data describe one of the most potent in vitro effects of PA. To further elucidate the interaction between PA and PP1γ, structure/function analysis of the lipid was carried out using commercially available and synthetically generated analogs of PA. These studies disclosed that the lipid-protein interaction is dependent on the presence of the lipid phosphate as well as the presence of the fatty acid side chains, because lipids lacking either of these substituents resulted in complete loss of inhibition. However, the specific composition of the fatty acid side chains was not important for inhibition. Using 1-O-hexadecyl,2-oleoyl-PA, it was also shown that the carbonyl group of the sn-1 acyl linkage is not required for the lipid-protein interaction. Finally, using a lipid-protein overlay assay, it was demonstrated that PP1γ specifically and directly interacts with phosphatic acid while not significantly binding other phospholipids. These results identify PA as a tight binding and specific inhibitor of PP1, and they raise the hypothesis that PP1γ may function as a mediator of PA action in cells. They also argue for the existence of a specific high affinity PA-binding domain on the enzyme.

Changes in cellular phosphatidic acid (PA) levels have been correlated to specific cellular responses, but few of these responses have been shown to occur at physiologically relevant concentrations of PA. Cellular PA levels are regulated through three major pathways; 1) the acylation of lysophosphatidic acid (LPA) by lysophosphatidic acid acyltransferase, 2) the phospho-rylation of diacylglycerol (DAG) by diacylglycerol kinase, and 3) the hydrolysis of choline from phosphatidylcholine (PC) by phospholipase D (PLD). The tight regulation of these pathways results in low basal cellular concentrations of PA that can be rapidly induced upon stimulation of cells with cellular agonists. This paradigm forms the basis of the hypothesis that PA is a lipid second messenger and a key mediator of these pathways. However, PA still remains without a clearly defined cellular target.

The most extensively characterized target of PA is the Raf-1 kinase. Ghosh et al. (1–3) have demonstrated that PA binds specifically within a 35-amino acid putative binding domain containing two smaller subdomains, consisting of a small basic region and a small hydrophobic region. Furthermore, they demonstrated in cells that PA plays a critical role in the docking of Raf-1 at the plasma membrane upon translocation by treatment with phorbol ester. In addition to Raf-1, other putative targets have been identified including SHP-1, phospholipase Cy1, p47phox, and an undefined PA-dependent protein kinase (4–7). Yet of these potential targets, few have exhibited direct interaction at physiologically relevant concentrations of PA.

Protein phosphatase-1 (PP1) is a serine/threonine phosphatase expressed in all eukaryotic cells and has been found to regulate a diverse number of cellular functions including cell cycle regulation, muscle contraction, glycogen metabolism, gene expression, and neurotransmission (8–10). PP1 in cells is found as a holoenzyme consisting of a catalytic subunit (PP1c) and single targeting/regulatory subunit. There are three major isoforms of PP1c: α, β, and γ, having greater than 90% homology on the protein level. Regulation of phosphatase activity is thought to occur principally through the action of endogenous peptide inhibitors and association with regulatory/targeting subunits (11, 12). It has been suggested that association with specific targeting subunits can regulate PP1 activity by directing its subcellular localization and substrate specificity, thus allowing it to carry out unique functions in the cell. In addition to the regulation by association with a regulatory subunit, PP1 has also been shown to be modulated by lipid mediators as well as direct phosphorylation of the catalytic subunit (13–17).

Previously our group has established that PP1cα is inhibited in vitro by acidic phospholipids at relatively high concentrations (13). This inhibition was especially pronounced for PA, which caused complete inhibition at concentrations 10-fold lower than the other phospholipids. In this report, we demonstrate that the γ-isofrom of the human PP1 catalytic subunit (PP1γ) is inhibited by PA with an IC₅₀ of 15 nM, demonstrating high potency. To date, this is one of the most potent in vitro effects of PA observed and may link these two mediators of
cellular signal transduction. We therefore set out to describe the mechanism of this inhibition through studying the structure/function relationship mediating the lipid-protein interaction, the biochemical kinetics of PA inhibition, and the direct interaction of PA with PP1c. The results from these studies lay the foundation for future investigation of the physiological interaction between PA and PP1c.

**EXPERIMENTAL PROCEDURES**

**Materials**—The γ-isform of the Escherichia coli recombinant human catalytic subunit of PP1 (PP1cγ) was purchased from Stratagene (La Jolla, CA). Native PP1c was purified from rabbit skeletal muscle and contains a mixture of PP1c isoforms. This preparation was provided to us as a generous gift from Dr. Mathieu Bollen. All lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Anti-His6 mouse monoclonal antibody was purchased from CLONTECH Laboratories (Palo Alto, CA). Anti-PP1c (E-9) mouse monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-mouse peroxidase was acquired from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The pBAD/His-B E. coli expression vector was purchased from Invitrogen. Restriction enzymes XhoI and KpnI were purchased from Promega (Madison, WI). All other reagents, unless otherwise noted, were purchased from Sigma.

**Preparation of [32P]-Myelin Basic Protein (MBP) and [32P]Phosphorylase a**—[32P]-MBP was labeled as previously described by Kishikawa et al. (13). [32P]Phosphorylase a was prepared according to the manufacturer’s instructions, using the protein phosphorylation assay system from Invitrogen.

**Phosphatase Assay**—Dephosphorylation of [32P]-MBP or [32P]Phosphorylase a was carried out in 50 mM Tris-HCl, 20% glycerol (v/v), pH 7.4. Duplicate reactions containing 10.6 micromolar of PP1cγ (EC 3.1.3.16) (or an equivalent amount of activity for native PP1c) were preincubated in the absence of or absence of lipid vesicles for 10 min at 30 °C. The reactions were then initiated with the addition of [32P]-labeled substrate and were allowed to proceed for 20 min. The reactions were stopped with the addition of 60% trichloroacetic acid to achieve a final concentration of 30% in the reaction mix. To facilitate precipitation of the substrate and enzyme, 250 µg of bovine serum albumin were added as a carrier, and the samples were allowed to incubate on ice for 10 min. Samples were then centrifuged for 5 min at 12,000 g, and 200 µl of the resulting supernatants were scintillation counted. Scintillation counts were expressed as percentages of the vehicle-treated control. 

**Lipid-Protein Overlay Assays**—To determine the kinetic mechanism of PA inhibition, phosphatase assays were carried out as above but included the addition of 100 µM MnCl2 to the reaction buffer to stabilize the basal activity and reduce experiment to experiment variation. The activities of substrate dose-response curves were expressed as pmol of P/min/ml released. The data were analyzed using the enzyme kinetics module of the Sigma Plot 2001 software package. This module was specially equipped to handle tight binding inhibitor kinetics.

**Cabbage PLD-mediated Conversion of PC to PA**—1-Oleoyl-2-stearoyl-PC, dielaidoyl-PC, di-octadecoyl-PC, and 1-O-hexadecyl,2-oleoyl-PC were converted in vitro to their respective PA analogs for use as inhibitors in the phosphatase assay. Briefly, 5 mg of lipid was dried from a chloroform stock under a stream of nitrogen. It was then resuspended in 2 ml of PLD reaction buffer (200 mM sodium acetate, 80 mM calcium chloride, pH 5.6) by sonication with a probe tip sonicator. One hundred units of cabbage PLD (EC 3.1.4.4) (Sigma) solubilized in PLD reaction buffer was added to the lipid suspension, and the mixture was overlaid with 2 ml of methanol. The reaction was allowed to proceed at room temperature and was supplemented with additional enzyme at 2 h increments for 6 h. The reaction was then allowed to proceed overnight at room temperature. The following day, the phase separation was induced under a stream of nitrogen, and the aqueous lipid suspension was extracted using the method of Bligh and Dyer (18). The converted lipid was separated from its PC precursor by thin layer chromatography using chloroform:methanol:acetic acid:water (80:15:8:0.5) as the mobile phase. Precursor and product lipids were visualized by staining with iodine and identified based on standards run on adjacent lanes. The converted lipid was scraped and extracted from the silica resin using chloroform:methanol:water (5:5:1). The extracted lipid was then dried down, resuspended in chloroform, and quantitated by measuring lipid phosphate content as previously described (19). The converted, quantitated lipid was then used in phosphatase assays as indicated above.

**Preparation and Purification of Recombinant PP1cγ**—The human PP1cγ sequence was cloned by polymerase chain reaction using the following primers: 5’-CCG CTC GAG CAT GGC GGA TTT AGA TAA ACT C-3’, containing an XhoI restriction site and 5’-GGC GTA CCC TAT TTC TTT GCT TGT GTG-3’ containing a KpnI restriction site. The resulting product was restriction digested then ligated into the inducible bacterial expression vector pBAD/His-B, which had been linearized with the same enzymes. The resulting plasmid sequence was verified prior to expression and purification of the recombinant protein. 

**RESULTS**

**In Vitro Inhibition of PP1cγ by PA**—Because PA has been previously shown to inhibit PP1c, we pursued the mechanism of this inhibition. The **E. coli** recombinant catalytic subunit of human PP1γ was incubated in the presence of increasing concentrations of PA. Dose-dependent inhibition of
phosphatase activity was observed with an IC$_{50}$ of 15 nM (Fig. 1A). The control PP1cy phosphatase activity, in this experiment (similar for all subsequent experiments), was 4.9 ± 1.6 pmol P/min/ml released. The inhibition was independent of the substrate used because identical inhibition was observed when using an artificial substrate, $^{32}$P-MBP, versus a physiological substrate, $^{32}$P-phosphorylase a.

Because of reported differences between recombinant and purified preparations of PP1c, we assessed whether PA could inhibit native PP1c (21, 22). As demonstrated in Fig. 1B, native PP1c was inhibited by PA with an IC$_{50}$ of 135 nM. This suggests that PA specifically interacts with a subset of PP1c isoforms contained in the purified preparation. The high degree of potency and specificity of PA for PP1c led us to investigate the mechanism of inhibition.

Biochemical Kinetics of Phosphatidic Acid Inhibition of PP1cy—To define the biochemical parameters of PA inhibition of PP1cy, kinetic analysis was performed on substrate dose-response data carried out in the presence and absence of PA. As shown in Fig. 2A, PA was able to inhibit PP1cy activity to the same degree over a wide range of substrate concentrations. However, when the substrate and inhibitor concentrations were maintained in the presence of varying concentrations of enzyme, inhibition of enzyme activity by PA was lost as the enzyme concentration was increased (Fig. 2B). These data suggest that the inhibitor concentration is not in excess of the enzyme concentration, indicating that PA functions as a tight binding inhibitor of PP1cy. We next performed kinetics analysis using nonlinear least squares regression to determine the kinetics parameters (Fig. 2, C and D). The following equation was used to account for the amount of inhibitor effectively removed from free solution as it bound to the enzyme.

$$v = (v_0/(2\cdot E)) \cdot (E - I - K_i + \sqrt{(E - I - K_i)^2 + 4 \cdot E \cdot K_i})$$  \hspace{1cm} (Eq. 1)

where the velocity was calculated in terms of $v_0$, the initial velocity; $E$, the initial total enzyme concentration; $I$, the free inhibitor concentration; and $K_i$, the inhibition constant. $K_m$ and $V_{max}$ for MBP were determined to be 7.69 ± 1.22 μM and 37.11 ± 4.37 pmol P/min/ml released, respectively. PA inhibited the enzyme noncompetitively (see Fig. 2D), and the $K_i$ of PA was determined to be 0.97 ± 0.24 nm.

Structure/Function Analysis of the Lipid Requirements for PA/PP1cy Interaction—In an attempt to gain a greater understanding of the interaction between PA and PP1cy, a detailed series of experiments was carried out to identify the critical structural components of PA required for inhibition of PP1cy. As seen in Fig. 3, there are three major structural features of PA; the phosphate head group (panel I), the lipid side chains (panel II), and the lipid acyl-linkage (panel III). By using commercially available PA or PC analogs (subsequently converted to PA analogs in vitro; indicated by asterisks in Fig. 3), we tested the requirement of these structural features for their contribution toward PP1cy inhibition.

First, the requirement for the phosphate head group was investigated. Inhibition of phosphatase activity was assessed using DAG and monooleoyl-glycerol, both of which lack the phosphate head group. Fig. 4A illustrates that neither DAG nor monooleoyl-glycerol was capable of inhibiting PP1cy activity, suggesting that the phosphate head group may be required for interaction between the protein and lipid. This was further explored by assessing the effects of the dioleoyl-phosphatidyl-alcohols: phosphatidylethanol, phosphatidylethanol, and phosphatidylethanol, on phosphatase activity. We hypothesized that if the phosphate was needed to make a critical contact with amino acids of the enzyme, by placing a methyl group on the phosphate we may be able to inhibit the lipid-protein interaction. Moreover, phosphatidyl-alcohols are formed in cells when phospholipase D is activated in the presence of primary alcohols. Thus, by using a series of phosphatidyl-alcohols, the resulting effects of substituting a hydroxyl group from the phosphomonoester of PA with various primary alcohols allowed us to assess the significance of the phosphate contact with the enzyme. As can be seen in Fig. 4B, when the phosphate of PA has an alkyl substituent, it loses its ability to inhibit PP1cy. These data imply not only that the presence of the phosphate head group is a requirement but that inhibition of the enzyme is dependent on its direct interaction with critical amino acid residues of PP1cy.

Second, the requirement for the presence of an aliphatic side chain for inhibition of PP1cy was determined. To this end, analogs that lacked both lipid side chains were evaluated. As seen in Fig. 5A, the phosphorylated glycerols, $\alpha$-glycerophosphate and $\beta$-glycerophosphate, were not able to inhibit enzyme activity. This argues that there is a requirement for at least one side chain. To determine whether one side chain is sufficient, we next tested LPA, which mimics PA except that it lacks the
Increasing concentrations of dioleoyl-PA: activity was assessed in the presence of increasing concentrations of 32P-MBP substrate. A, 0.25 μM; ○, 0.5 μM; ■, 1.0 μM; □, 2.0 μM; ▲, 2.5 μM; and △, 3.5 μM. A representative experiment is shown. B, inhibition of phosphatase activity by PA was measured in the presence of increasing concentrations of enzyme: ○, 0.214 nM; ●, 0.428 nM; □, 0.856 nM; ■, 1.712 nM; and △, 3.424 nM. A representative experiment is shown. C, phosphatase activity was assessed in the presence of increasing concentrations of dioleoyl-PA: ●, control; ○, 2.5 nM; □, 5.0 nM; and △, 10.0 nM. The enzyme velocities are expressed as functions of substrate concentration. The data represent the means ± S.D. of four replicate experiments. D, Lineweaver-Burk double-reciprocal plot of 1/velocity versus 1/substrate concentration in the presence of increasing concentrations of PA: ●, control; ○, 2.5 nM; □, 5.0 nM; and △, 10.0 nM. The data represent the means ± S.D. of four replicate experiments.

Specific and Tight Binding of PA to PP1

*sn-2* lipid side chain. LPA was not able to inhibit PP1cγ (Fig. 5B), thus suggesting that both side chains are required for PA to inhibit enzyme activity.

Next, the contribution of the length and composition of the lipid side chains toward the inhibition of PP1cγ was explored. Initially, the degree of saturation of the lipid side chains was evaluated to determine the effect on enzyme inhibition. Using the fully saturated PA analogs distearoyl-PA and dioctanoyl-PA, it was observed that neither of the saturated compounds were capable of inhibiting phosphatase activity (Fig. 6A). Interestingly, eliminating the double bond on the lipid side chains abolished inhibitory activity of the lipid, even when the lipid chains were truncated prior to the cis9–10 double bond of PA. These results may indicate that there is a requirement for at least one double bond in the lipid side chains for PA to interact with PP1cγ. However, because these results were somewhat unexpected, we further explored the significance of side chain composition by testing mixed saturated/unsaturated PA analogs. As can be seen in Fig. 6B, 1-steaoyl,2-oleoyl-PA was fully capable of inhibiting PP1cγ phosphatase activity. Furthermore, the use of 1-oleoyl,2-steaoyl-PA was also able to inhibit enzyme activity to the same degree (data not shown). Together these data suggest that there may be a requirement for a single unsaturated side chain; however, there is no specificity for the *sn-1* versus the *sn-2* position in the structure.

To further address the issue of lipid side chain composition, we examined the ability of 1-steaoyl,2-arachidonoyl-PA to inhibit PP1cγ. This compound contains a fully saturated *sn-1* linked side chain and a polyunsaturated (cis5, 8, 11, 14) *sn-2* linked side chain. As can be seen in Fig. 6C, this analog was fully capable of inhibiting PP1cγ to the same extent as PA. Similarly, dielaidoyl-PA (diC18:1), containing a [trans9–10] double bond in place of the cis9–10 double bond, also inhibited phosphatase activity to the same extent as dioleoyl-PA (Fig. 6D). Together these data support the suggestion that the composition of the lipid side chains is not a major determinant of PP1cγ inhibition. This requirement for at least one double bond in one of the hydrocarbon chains may result from physical conditions such as the solubility of PA and/or the need for vesicle formation and not from differences in interaction with the enzyme.

To further evaluate this possibility, we tested a few key compounds for their ability to inhibit PP1cγ when delivered in mixed detergent/lipid micelles. Mixed micelle delivery systems have been used extensively for delivering lipids, which do not easily form vesicles, to soluble enzymes. To explore the possibility that saturated PAs were not forming suitable vesicles, we employed Triton X-100 micelles to deliver dioleoyl-PA, distearoyl-PA, and dioleoyl-DAG to PP1cγ *in vitro*. As shown in Fig. 7, both dioleoyl-PA, and distearoyl-PA were able to inhibit PP1cγ activity, whereas dioleoyl-DAG was not. These data substantiate the notion that lipid side chain composition is not

\[ K_m = 7.69 \pm 1.22 \, \mu M \, MBP \]
\[ V_{\text{max}} = 37.11 \pm 1.37 \, \text{pmol} \, P_i \, \text{released/min/ml} \]
\[ K_i = 0.97 \pm 0.24 \, nM \]
a major determinant of enzyme inhibition by these analogs, while further supporting the evidence that the presence of the phosphate group is crucial for PP1c inhibition. Moreover, the mode of lipid delivery to PP1c is of critical importance and may indicate a requirement for the presence of a lipid surface to coordinate the lipid-protein interaction.

It should be noted that Triton X-100 detergent micelles alone were capable of activating PP1c 2-fold when delivered as a 0.3% solution. Although the observed activation was dose-dependent, it is believed to be a result of a general sensitivity of the enzyme to hydrophobic compounds, because we observed a similar response with other detergent micelle preparations (data not shown).

The last major feature of the PA structure to be investigated was the requirement of the acyl-linkage of the lipid side chains for PP1c inhibition. To gain insight into the significance of the carbonyl group, a mixed alkyl/acyl-PC-derivative was converted to the respective PA analog using cabbage PLD. The converted compound was purified by thin layer chromatography, quantitated, and used to inhibit PP1c. As can be seen in Fig. 8, 1-O-hexadecyl-2-oleoyl-PA lacking the carbonyl at the sn-1 position was still capable of potent inhibition of PP1c. Unfortunately, the dialkyl-derivative did not serve as a suitable substrate for cabbage PLD and thus could not be converted to its corresponding PA analog. In light of this, these data demonstrate that the sn-1 carbonyl group is not required for

**Fig. 3.** Scheme showing the structures of the compounds used in this study. The compounds are grouped according to the structural feature of PA. The compounds marked with asterisks indicate those that were purchased as PC-derivatives and converted to PA-derivatives in vitro.
Given the unavailability of the dialkyl-derivative, we could not determine the contribution of the sn-2 carbonyl, if any, in respect to inhibition of PP1c/H9253.

Binding of PP1c/H9253 to PA—To demonstrate a direct physical interaction between PP1c/H9253 and PA, binding studies were carried out using the lipid-protein overlay method developed by Boss and co-workers (23). By this method, PA and other phospholipids were immobilized on a nitrocellulose membrane, and binding was assessed by incubation with commercially available E. coli recombinant human PP1c/H9253 (PP1c/H9253), followed by immunostaining with anti-PP1c (pan-isoform-specific) antibody. Fig. 9A demonstrates that PP1c/H9253 physically interacted with dioleoyl-PA. Because of low sensitivity of the antibody used and a nonspecific interaction resulting in a rim of DAG binding, we chose to repeat this study using purified His6-tagged PP1c/H9253 (His6-PP1c/H9253) and a higher sensitivity anti-His6 antibody (Fig. 9B). It was observed that His6-PP1c/H9253 bound specifically to PA, with modest binding to 1,2,3,4-tetraoleoyl-cardiolipin, whereas no binding was seen to the other phospholipids immobilized. These data reinforce the in vitro biochemical results, suggesting that the phosphate contact with the enzyme is critical, because no binding was seen to either dioleoyl-DAG or the phosphatidyl-alcohols. Because of the qualitative nature of these experiments, it was not possible to accurately calculate the binding constants.

DISCUSSION

Direct cellular targets for PA have long been elusive yet have remained as an important objective in elucidating pathways of cell signaling regulated by PA. It has been shown that phospholipids have an inhibitory effect toward PP1c activity when delivered at micromolar concentrations and that PA was much more potent than other phospholipids in this regard (13). Therefore, we set out to define the mechanism of this inhibition through analyzing the biochemical kinetics, the structure/function relationship between PA and PP1c/H9253, and the direct binding of PP1c/H9253 to PA. We now report that PP1c/H9253 binds specifically to PA and that PA functions as a noncompetitive tight binding inhibitor of PP1c/H9253 in vitro.

Using classical enzyme kinetic models, Michaelis-Menten and Lineweaver-Burk analyses were performed only to find that the models were not sufficient to accurately describe the data. Because inhibition by PA was observed in the low nanomolar range, we hypothesized that PA may function as a tight binding inhibitor. This was supported by the observation that inhibition by PA did not change with changing substrate concentration but decreased dramatically with increasing enzyme concentration. This confirmed that PA did not interact with the substrate and that the inhibitor concentration was not in great excess as compared with the enzyme concentration. Therefore, to study the kinetics, we chose to use nonlinear least squares.
regression analysis for tight binding kinetic data. These models take into account the amount of inhibitor effectively removed from free solution as it binds the enzyme. The resulting data were consistent with a noncompetitive tight binding inhibitor model, yielding a $K_m$ of 7.69 ± 1.22 nM and a $V_{max}$ of 37.11 ± 4.37 pmol P/min/ml released. Dixon analysis was also performed, yielding a $K_i$ for PA at 0.97 ± 0.24 nM. These data imply that the PA-binding site is distinct from the active site and that upon
interaction with PA, the enzyme velocity is lowered without changing the affinity to substrate. The fact that this inhibition occurs at low nanomolar concentrations suggests that PP1c may have a high affinity binding site for PA.

To better understand the interaction between PA and PP1c, we set out to define the lipid requirements for interaction by structure/function analysis. The most critical determinant of interaction was the phosphate head group and the presence of the sn-1 and sn-2 fatty acid side chains. Interestingly, when fully saturated distearoyl-PA was assayed, no inhibition was observed. This was puzzling and led us to test mixed saturated/unsaturated PA analogs for inhibitory activity. Upon determining that 1-stearoyl,2-oleoyl-PA and 1-oleoyl,2-stearoyl-PA were capable of inhibiting the enzyme equally, we hypothesized that the degree of side chain saturation was not important for inhibition but perhaps was critical for solubility and/or vesicle formation and lipid delivery to the enzyme.

To assess the ability of distearoyl-PA to inhibit PP1c activity, the lipids were delivered in mixed detergent/lipid micelles of Triton X-100. It was observed that dioleoyl-PA was capable of inhibiting the enzyme, whereas dioleoyl-DAG was still incapable of inhibition. Distearoyl-PA, however, was now able to inhibit the enzyme to a similar degree as dioleoyl-PA. Thus, the presence of a lipid surface appears to be necessary for coordinating the interaction between PA and PP1c. This is also supported by studies using dioctanoyl-PA, which is present in solution as a monomer yet did not show any inhibition of the enzyme. Also, biophysical considerations further support this conclusion because the phase transition temperature for saturated lipids is higher than that of unsaturated lipids (e.g., -8 °C for dioleoyl PA versus 75 °C for distearoyl PA) (24, 25).

We also studied the effects of 1-stearoyl-2-arachidonoyl-PA because El Bawab et al. (26) have determined that it is a measurable physiological species of PA in rat thymocytes. 1-Stearoyl-2-arachidonoyl-PA was capable of inhibiting PP1c to the same degree as the dioleoyl species. Thus, physiological species of PA are capable of inhibiting PP1c.

The direct interaction of PP1c with PA was evaluated using the lipid-protein overlay assay (fat blot), which has proven to be a powerful tool for probing lipid-protein interactions. It was used previously to demonstrate the high affinity interactions between pleckstrin homology domain-containing proteins and the various phosphoinositides, as well as other high affinity lipid-protein interactions (20, 23, 27, 28). PP1c bound PA specifically, but it did not interact with other phospholipids including the metabolic precursors of PA: LPA, PC, and DAG. These data demonstrate for the first time that there is a direct and highly specific interaction between PP1c and PA, indicating that PP1c has a high affinity binding domain for PA.

In this report we describe a novel mechanism for inhibiting PP1c through its interaction with PA. These data are important for several reasons. First, the observed inhibition is one of the most potent effects of PA recorded to date, having an IC_{50} in the low nanomolar range, whereas other reported biochemical effects of this lipid have required micromolar amounts. The potency and specificity of this interaction not only support the hypothesis that this interaction could occur on a physiological level but also suggest the presence of a specific site on PP1c that binds PA. Subsequent studies will focus on identifying and characterizing this binding domain, which may lead to the identification of a consensus PA-binding site.

Second, this observation links two cellular signaling molecules: a bioactive lipid second messenger and a cellular phosphatase. PA constitutes a small proportion of the total cellular phospholipid content, yet stimulation of cells with cellular agonists leads to rapid and transient increases in cellular PA levels (29–34). Over the past several years, the role of PLD in cellular signaling has become a topic of great interest. Its role in signal transduction has been firmly established, yet its functional product, PA, remains without a clearly defined cellular target. The work presented here identifies a new high affinity in vitro target for PA that could play a significant role in cellular signal transduction.

PP1 in cells is found to be a holoenzyme consisting of a catalytic subunit and single targeting/regulatory subunit. There have been a growing number of regulatory subunits of PP1 identified that are believed to direct subcellular localization as well as substrate specificity (11, 12). Interestingly, Ito et al. (35) have reported that PA is capable of binding the myosin targeting subunit of PP1. This could indicate that PA has multiple binding sites on the holoenzyme, all working toward negatively regulating its activity at the site of targeting within the cell. Although the work reported here has focused on inhibition of the catalytic subunit of PP1 alone, subsequent studies will focus on the role of regulation of the holoenzyme. Furthermore, because PA inhibited PP1c to a much greater degree than native PP1c, containing a mixture of isoforms purified

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**FIG. 9. Lipid-protein overlay assay.** A. Human E. coli recombinant PP1c binding to phospholipids immobilized on a nitrocellulose membrane, visualized using anti-PP1c (pan-isoform-specific) antibody. B. His6-PP1c binding to phospholipids immobilized on a nitrocellulose membrane, visualized using anti-His_{6} antibody. The following dioleoyl lipids were immobilized on the membrane as indicated: PA, phosphatidylserine (PS), phosphatidylinositol (PI), PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), tetraoleoyl-cardiolipin (CL), DAG, LPA, phosphatidylmethanol (PMt), phosphatidylethanolamine (PEt), and phosphatidylbutanol (PBt). The results shown are representative experiments of at least three independent replicate experiments.

| Lipid | 500.0 | 37.5 | 25.0 | 12.5 | 6.0 |
|-------|-------|------|------|------|-----|
| PA    | PA    | PA   | PA   | PA   | PA  |
| PS    | PS    | PS   | PS   | PS   | PS  |
| PI    | PI    | PI   | PI   | PI   | PI  |
| PC    | PC    | PC   | PC   | PC   | PC  |
| PE    | PE    | PE   | PE   | PE   | PE  |
| PG    | PG    | PG   | PG   | PG   | PG  |
| CL    | CL    | CL   | CL   | CL   | CL  |

| Lipid | 500.0 | 37.5 | 25.0 | 12.5 | 6.0 |
|-------|-------|------|------|------|-----|
| PA    | PA    | PA   | PA   | PA   | PA  |
| PS    | PS    | PS   | PS   | PS   | PS  |
| PI    | PI    | PI   | PI   | PI   | PI  |
| PC    | PC    | PC   | PC   | PC   | PC  |
| PE    | PE    | PE   | PE   | PE   | PE  |
| PG    | PG    | PG   | PG   | PG   | PG  |
| CL    | CL    | CL   | CL   | CL   | CL  |

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Specific and Tight Binding of PA to PP1

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from rabbit skeletal muscle, we conclude that there may also be isoform-specific differences in the ability of PA to inhibit PP1c. Additional studies will be needed confirm this observation.

In conclusion, these studies define a mechanism of the interaction between PA and PP1c, which results in potent inhibition of PP1c activity. Future studies will focus on defining the physiological significance of this discovery, with the hope of defining PP1c as a direct cellular target of PA, further illustrating its role as a mediator of cellular signaling.

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