Published in final edited form as:
Biochemistry. 2009 April 7; 48(13): 2997–3004. doi:10.1021/bi8020636.

Activation of Polyisoprenyl Diphosphate Phosphatase 1 Remodels Cellular Presqualene Diphosphate

Troy Carlo[1], **Nicos A. Petasis**[2], and **Bruce D. Levy**[1,*]

[1]Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115

[2]Department of Chemistry, University of Southern California, 837 West 37th Street, Los Angeles, CA 90089

Abstract

Polyisoprenyl diphosphates play diverse and vital roles in cell function in health and disease. The counter-regulatory lipid signaling molecule, presqualene diphosphate (PSDP), is rapidly converted to its monophosphate form (PSMP) upon cell activation (Levy, B. D., Petasis, N. A., and Serhan, C. N. (1997) *Nature* 389, 985–990). The first PSDP phosphatase was recently identified and named polyisoprenyl diphosphate phosphatase 1 (PDP1) (Fukunaga, K., et al (2006) *JBC* 281, 9490-9497). Here, we present evidence that PDP1 displays properties of lipid phosphate phosphatase/ phosphotransferase with distinct substrate preference for PSDP. Cell activation with PMA increased PSDP phosphatase activity in a concentration dependent manner and western analysis suggest that PDP1 is directly phosphorylated by protein kinase C. Cellular PSDP phosphatase activity was also induced by the receptor-mediated agonists insulin and TNF-α. To address PDP1's contribution to cellular PSDP phosphatase activity, HEK293 cells were established that stably expressed PDP1 siRNA, leading to a 60% decrease in PDP1 RNA, and concomitant decreases in PDP1 protein and PMA-initiated PSDP phosphatase activity. HEK293 cells harboring PDP1 siRNA construct also displayed a marked decrease in PMA-initiated conversion of cellular PSDP to PSMP. Together, these findings are the first to indicate that PDP1 is activated during cell responses to soluble stimuli to convert PSDP to PSMP. Moreover, they provide evidence that PDP1 can serve as a new checkpoint for polyisoprenyl phosphate remodeling during cell activation.

Lipid mediators regulate cell responses to inflammatory stimuli (1-3). Lipid phosphate phosphatase/phosphotransferase (LPT) proteins, such as lipid phosphate phosphatases (LPPs), sphingosine-1-phosphate phosphatases (SPPs) and sphingomyelin synthases, comprise a family of membrane proteins that play critical roles in lipid metabolism and signaling in organisms ranging from yeast to humans (4-8). The newest member of this family to be characterized is polyisoprenyl diphosphate phosphatase 1 (PDP1) (aka PPAPDC2, GenBank accession number AK027568.1) (9). Originally identified as a type 2 candidate sphingomyelin synthase (CSS2) that lacked sphingomyelin synthase activity, this protein has a consensus lipid phosphate phosphatase domain (10), and was recently found to convert polyisoprenyl diphosphates to their monophosphate forms (9). Some members of the LPT family can utilize short isoprenyl diphosphates, such as farnesyl diphosphate (FDP), as substrates (11), but PDP1 is the first phosphatase identified that can convert presqualene diphosphate (PSDP) to presqualene monophosphate (PSMP) (9).

Address correspondence to: Bruce D. Levy, Pulmonary and Critical Care Medicine, Brigham and Women's Hospital Boston, Massachusetts 02115, USA. Tel: (617) 525-8362; FAX: (617) 264-5133, E-mail: Blevy@partners.org.

When cells are exposed to provocative stimuli, signaling pathways are activated to initiate functional responses. To regulate these responses, cells employ both “go” and “stop” signals (12). For example, the pro-inflammatory agonists leukotriene B_4 and formyl-methionyl-leucyl-phenylalanine trigger neutrophil NADPH oxidase assembly and reactive oxygen species generation that is activated within seconds but limited so that superoxide anion generation usually stops after several minutes (13). The membrane remodeling that accompanies cell activation and deactivation provides a rich source for bioactive lipid mediators (14). Presqualene diphosphate is present in cell membranes in nanomolar quantities (15). In response to stimuli, PSDP levels in PMN decrease by approximately 30% within seconds and return to baseline within a few minutes (15). There is an equally rapid and reciprocal increase in PSMP. Of interest, counter-regulatory autacoids, such as lipoxins, that block cell activation, also block agonist-triggered PSDP remodeling (16). PSDP and PSDP structural mimetics inhibit two pivotal signaling molecules in cell activation, namely phosphatidlyinositol 3-kinase and phospholipase D, a property not shared by PSMP (13,16,17). Taken together, PSDP's ability to inhibit important enzymes for cell activation and its rapid remodeling to PSMP upon cell exposure to provocative stimuli suggests the presence of a regulated PSDP phosphatase that controls cellular PSDP and PSMP levels. The results from the present experiments are the first to demonstrate agonist-initiated PDP1 activation for PSDP remodeling.

Experimental Procedures

Materials

PSDP was isolated from human neutrophils as in (15). PMA, glucose 6-phosphate (G6P), and FDP were purchased from Sigma Chemical Co. (Saint Louis, MO, USA), pSilencer 3.1 neo system from Ambion, Inc. (Austin, TX, USA), Biomol green reagent from BIOMOL International, L.P. (Plymouth Meeting, PA, USA), Phospho-(Ser) protein kinase C (PKC) Substrate Antibody from Cell Signalling Technology (Danvers, MA, USA), and sphingosine-1-phosphate (S1P), lysophosphatidic acid (LPA C18:1) and phosphatidic acid (PA, C10:1) from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The PSDP structural mimetic, a PSDP amino bis-phosphonate analog, and the (Z)-PSDP isomer were prepared by the organic synthesis core of NIH grant P50-DE016191 (13,17). All synthetic compounds were characterized by NMR spectroscopy. Rabbit Anti-Human Phosphatidic Acid Phosphatase Domain Containing 2 (PPAPDC2) Polyclonal Antibody was from Exalpha Biologicals, Inc. (Maynard, MA, USA).

In Vitro Phosphatase Assay

For recombinant human PDP1 (rhPDP1) experiments, 2μg of recombinant protein, isolated as in (9) was exposed to substrate (0-60μM), and subjected to rotational mixing (20min, RT). The reaction (total volume of 50μl) was allowed to proceed for 30 minutes at 37°C. The amount of free phosphate was determined using malachite green as in (9).

For insect cell extract experiments, Sf21 cells were infected (5:1 MOI) with hPDP1 cDNA expressing baculovirus. 48hours post-infection, infected and mock-infected cells were harvested by scraping and cells pelleted by centrifugation (100×G, 10min, 4°C). After centrifugation, cells were washed to remove residual media in calcium and magnesium free HBSS and then resuspended in lysis buffer (50mM Hepes pH 7.4, 80mM KCl, 3mM EDTA, 4mM DTT and 1X Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA)). Cells were disrupted by sonication (three pulses of 10sec) with a Fisher model 100 Sonic Dismembrator (Fisher Scientific). Remaining intact cells and debris were pelleted by centrifugation (100×G, 10min, 4°C). The resulting supernatant was centrifuged at 10,000×G (60min, 4°C). The pellet was resuspended in lysis buffer (lacking protease inhibitors)

supplemented with 0.1% Triton-X 100. 1μg of extract was combined with lipid and phosphatase activity (30min, 37°C) was measured as above.

For HEK293 cell extracts (see below), 2μg of the extract and 0-60μM of the test compound (PSDP, LPA, PA, FDP, G6P, or S1P) were incubated in a reaction buffer consisting of 50mM Hepes pH 7.4, 80mM KCl, 3mM EDTA, 4mM DTT and 0.1% Triton-X 100. After gentle mixing (20min, RT), the phosphatase reaction was allowed to proceed for 30 minutes at 37° C.

Lipids

All lipids were maintained dissolved in $CHCl_3$:MeOH (2:1) at -20°C. For use, the required amount of lipid was brought to dryness under a gentle stream of nitrogen gas. Material was resuspended in Triton-X 100 containing buffer by sonication (1min, RT). The molar ratio of detergent to lipid was kept at a constant 12.5:1 for the Sf21 extract experiments. For all other experiments, the detergent to lipid ratio is described elsewhere.

Constructs

DNA oligonucleotides were cloned into the pSilencer 3.1 neo vector to create transfectable plasmids. The sequence of the oligonucleotides used were: GATCCAATATTA and AGCTTAATATTGG (control), GATCCAGCCAACTTGCTTAAAGAGTTCAAGAGACTCTTTAAGCAAGTTGGCTTTTTTTGGAAA and AGCTTTTCCAAAAAAAGCCAACTTGCTTAAAGAGTCTCTTGAACTCTTTAAGCAAGTTGGCTG (hPDP1 siRNA). After DNA sequencing confirmed each construct, the DNA was transfected into HEK293 cells using SuperFect Transfection Reagent (Qiagen, Valencia, CA). Transfectants were selected by culture in media containing 800 μg/ml G418 from (Sigma Chemical Co., Saint Louis, MO) for four weeks and the resultant polyclonal cell lines were maintained in media containing 100 μg/ml G418.

RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and residual DNA was removed by DNAse I (Invitrogen Life Technologies, Carlsbad, CA, USA). Ready-To-Go Beads (Amersham Biosciences, Piscataway, NJ, USA) were used to create cDNA from total RNA (2 μg/reaction). PCR (35 cycles) using primers specific to human PDP1 (9), two related genes, (phosphatidic acid phosphatase 2a (PAP2A) (9) and CSS2α (Forward: ATGGAGTGATGAACTCGGAAAT, Reverse: ACAAAGGAATCTTGCCAGTGAT)), or β-actin (9) was performed using cDNA (1μg/reaction).

Activation of Cellular PSDP Phosphatase

HEK293 cell lines were grown to approximately 80% confluence (5×10^6 cells) in DMEM plus 10% FCS and then were exposed to PMA (10^{-8}-10^{-6} M) or vehicle (0.1% EtOH) for 10min at 37°C. For experiments with TNF-α and insulin, cells were serum-starved overnight, and then exposed to TNF-α, insulin, or vehicle (PBS) for 10min at 37°C. After incubation, cells were collected by scraping and extracts prepared as described above for Sf21 cell extracts.

Monitoring of Cellular Polyisoprenyl Phosphates

HEK293 cells (5×10^5) (37°C, 5% CO_2) were plated in 6 well dishes in media containing 10% fetal bovine serum and allowed to attach to the plate for 2hrs. After attachment, the cells were washed and incubated overnight in serum-free media. The following day cells were incubated (37°C, 5% CO_2) for two hours with [α-^{32}P]-ATP (4×10^7μCi/mL) (PerkinElmer, Waltham,

MA, USA) in HBSS with calcium and magnesium. Cells were then exposed (0-60s, 37°C) to PMA (2×10^{-6}M). Reactions were terminated by addition of an equal volume (500μl) of 10% KOH in methanol. Cellular materials were saponified (37°C, 30min) as in (15). After saponification, 0.25 vol (125μl) of acetone and 4 vol (2ml) of $CHCl_3$:MeOH (2:1, v/v) were added and the mixture was placed at -20°C overnight. Non-saponifiable lipids were extracted as in (15) and separated by TLC with a mobile phase of $CHCl_3$:MeOH:H_2O (65:25:4, v/v) (45min, RT) in a glass chamber with curtains (Schleicher & Schuell, Keene, New Hampshire). PSDP and PSMP were identified by Rf values. Amounts of radiolabeled materials were determined by phosphoimaging using a Molecular Dynamics Storm 840 phosphoimager and ImageQuant software.

Statistical analysis

Results are expressed as the mean ± SEM. $P < 0.05$ was set as the level of significance as assessed by Student's *t*-test. One-way ANOVA analysis was used to determine differences between groups of three or more.

Results

Substrate Preference of PDP1

Recently, PDP1 was shown, *in vitro*, to favor the polyisoprenyl phosphates PSDP and FDP over PA (9). To further characterize PDP1's substrate preference, we first prepared rhPDP1 and exposed PSDP (20μM) or the LPT substrate sphingosine-1-phosphate (S1P) (60μM) in 0.1% Triton-X 100 to rhPDP1 (2μg) for 30 min (37°C). Phosphatase activity was monitored by phosphate release. Despite three-fold higher concentrations of S1P, rhPDP1 released significant amounts of phosphate from only PSDP (Figure 1A). The enzyme was next exposed, in parallel, to equimolar amounts (20μM) to several additional substrates, including LPA and G6P. Only PSDP led to significant phosphate release from rhPDP1 (32.8 nmol phosphate/mg protein) (n=3, P<0.04) with a structure activity relationship for PDP1 that gave a rank order preference of PSDP>S1P≈G6P≈LPA.

There are at least three classes of LPTs with phosphatase activity; namely the LPPs, SPPs, and CSS2s (8). Because PSDP is a poor substrate for the LPPs (9) and CSS2α lacks the amino acid residues predicted for phosphatase catalytic activity (8), we next determined if a representative SPP could utilize PSDP as a substrate. HEK293 cells were transiently transfected with an expression vector containing hSPP1 cDNA or an empty vector as a transfection control. SPP1 released substantial amounts of phosphate from S1P, but hSPP1 transfected cell extracts did not carry significant phosphatase activity for PSDP (Figure 1B). Together, published work (9) and these new results support a preferential relationship amongst the LPTs for PDP1 and PSDP.

To determine PDP1 activity in cell extracts, we next expressed hPDP1 in Sf21 cells and exposed membrane extracts to increasing concentrations (0-50μM) of candidate phosphorylated lipid substrates prepared in Triton-X 100 mixed micelles (see Methods). Because PDP1 converts PSDP to PSMP and not presqualene alcohol (9), we directly compared the amount of phosphate released from PSDP, S1P, FDP, LPA, and PA. The molar ratio (12.5:1) of detergent (Triton-X 100) to lipid was maintained constant for each lipid concentration. At concentrations greater than 6.25μM, PSDP gave the highest rate of phosphatase activity (Figure 1C). At a lipid concentration of 50μM, PDP1's rank order of substrate preference was PSDP>FDP≈S1P>LPA≈PA with rates of 6.9, 1.6, 1.4, 0.8, and 0.7 nmol/mg/min, respectively. A rate of 6.9 nmol/mg/min (50μM) equates to 8.3% conversion of PSDP to PMSP during the 30min experimental time frame. More complete enzyme kinetics were not performed with this

phosphatase assay secondary to the limited availability of PSDP and the presence of contaminating phosphate in Triton-X 100 obtained from multiple suppliers.

Agonist-initiated PDP1 Activity

Because cell activation leads to PSDP remodeling (15), we next investigated the regulation of PDP1 in cells exposed to soluble stimuli. HEK293 cells were chosen as a model system because they express PDP1 (9), are readily available, and are more tractable to molecular manipulation than human neutrophil and neutrophil-like cell lines. HEK293 cells exposed to PMA (10^{-8}-10^{-6}M) gave a concentration dependent increase in phosphate release from PSDP (20μM) (Figure 2A). The hPDP1 amino acid sequence was analyzed at NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) for phosphorylation sites and determined to contain several serine residues in sequence motifs that could serve as potential targets for direct PKC phosphorylation (Fig. 2B). In addition to concentration dependent increases in PSDP phosphatase activity, PMA also led to concentration dependent increases in PDP1 phosphorylation by PKC, as determined by western analysis (See Methods) with a Phospho-(Ser) PKC Substrate antibody (Cell Signaling Technology) that detects phosphorylated serine residues within the context of a PKC phosphorylation motif (Figure 2C). In addition to PMA, extracts of HEK293 cells exposed to either the pro-inflammatory cytokine TNF-α (10ng/ml) or insulin (25μM) also led to significant increases in phosphate release from PSDP (20μM) (Figure 2D). These data indicate that PDP1 phosphorylation and PSDP phosphatase activity in HEK293 cells is stimulated by soluble stimuli and are consistent with a putative role for PDP1 in the conversion of PSDP to PSMP by activated cells.

PDP1 Functions as a PSDP Phosphatase

To determine the quantitative contribution of PDP1 in PMA initiated PSDP phosphatase activity (Figure 2), we established stable polyclonal cell lines harboring either an siRNA construct designed to reduce PDP1 expression (PDP1 siRNA) or a control construct (Mock). Introduction of the siRNA construct reduced PDP1 mRNA levels approximately 60%, as assayed by semi-quantitative RT-PCR (Figure 3A and 3B). To control for off-target effects, expression levels of closely related LPT genes CSS2α and LPP1 were determined. Neither CSS2α, LPP1 nor β-actin was affected by expression of PDP1 siRNA (Figure 3B). To determine protein expression in the PDP1 siRNA cells, blots of extracts from PMA (10^{-7} M) treated Mock and PDP1 siRNA cells probed with either a PDP1 specific antibody (1:800 dilution) or Phospho-(Ser) PKC Substrate antibody (1:1000 dilution). In parallel with the decreased RNA expression, PDP1 siRNA cells also expressed approximately 60% less PDP1 protein as measured by western analysis using both PDP1 specific and Phosph-(Ser) PKC specific antibodies (Figure 3C and 3D).

To determine the impact of PDP1 on agonist-initiated PSDP phosphatase activity, PDP1 siRNA and mock transfected cells were exposed to PMA (10^{-7}M, 37°C, 10min) and baseline activity was determined in non-transfected cells exposed to vehicle (0.1% EtOH). Incubation (37°C, 30min, 0.1% Triton-X 100) of cell extracts (2μg) with PSDP (20μM) led to significant increases in phosphate release by the PMA activated Mock transfected cells (360 ± 83 nmol/mg) compared to non-transfected control cells (203 ± 19 nmol/mg) ($n \geq 3$, $P < 0.01$) (Figure 4A). In the PDP1 siRNA transfected cells, decrements were present in PMA-initiated phosphate release from PSDP (271 ±30 nmol/mg) ($n=4$, $P<0.01$ vs. Mock cells exposed to PMA) (Figure 4A) that paralleled the decrements in PDP1 RNA and protein expression (Figure 3). Despite the capacity of rhPDP1 to release phosphate *in vitro* from S1P, FDP, LPA, and PA (Figure 1), there was no substantial effect of PMA or PDP1 siRNA on agonist-initiated phosphate release from these potential lipid substrates for cellular PDP1 (Figures 4B-E). Of note, basal levels of phosphatase activity were seen for FDP and PA in these cell extracts, yet in contrast to PSDP

phosphatase activity, PMA did not increase phosphate release from these phosphorylated lipids.

PDP1 Regulates Cellular PSDP Remodeling

To determine if PDP1 remodels polyisoprenyl phosphates in intact cells, endogenous PSDP and PSMP levels were monitored before and after cell activation. HEK Mock and PDP1 siRNA stably-transfected cells were exposed (37°C, 0-60s) to PMA (2μM). Immediately after cells were stimulated, lipids were extracted and analyzed by TLC (see Methods). The amount of materials with the Rf values of PSDP and PSMP was calculated by phosphoimaging (Figure 5). Both PSDP and PSMP were present in cells at baseline (time 0). PMA triggered decrements in PSDP and a significant increase in PSMP in Mock cells at 60s (32.4±13.4 percent increase compared to baseline (n=3, $P<0.05$)). In the PDP1 siRNA cells, both PSDP and PSMP were still detected, but in sharp contrast to Mock cells, PMA-initiated PSDP remodeling was completely blocked in the PDP1 siRNA line (Figures 5A-B). These results demonstrate a critical role for PDP1 in the agonist-initiated conversion of cellular PSDP to PSMP and that PSDP remodeling occurs in a time frame and amount consistent with a role in signal transduction.

PDP1 Dephosphorylation of PIPPs and Bioactive PSDP Mimetics

Structural mimetics of PSDP have been prepared and structure-activity relationships indicate that a (Z)-PSDP isomer with a change in double bond geometry and a PSDP amino bis-phosphonate analog with a modified diphosphate unit (Figure 6, inset) display counter-regulatory properties for human neutrophil activation *in vitro* and acute inflammation *in vivo* (13,17). To determine if these bioactive PSDP mimetics are substrates for rhPDP1, each mimetic (10μM) was exposed (37°C, 30min) to rhPDP1 (2μg) and phosphatase activity measured. While rhPDP1 released substantial amounts of phosphate from native (E)-PSDP and FDP, no significant phosphate was released from either the (Z)-PSDP isomer or the PSDP amino bis-phosphonate analog (Figure 6). In addition, neither mimetic inhibited rhPDP1's ability to release phosphate from PSDP (data not shown). These results indicate that the mimetics' protective *in vitro* and *in vivo* actions may result from their capacity to resist PDP1-mediated inactivation to their corresponding monophosphates.

Discussion

PSDP is rapidly and transiently remodeled to PSMP during cell activation (15-17). Here, we provide several lines of evidence that PDP1 serves as a pivotal PSDP phosphatase. This LPT displays a unique preference for PSDP as a substrate, even when compared to closely related phosphorylated lipids that can serve as substrates for other LPTs. Cellular PSDP phosphatase activity is present in resting HEK293 cells and markedly upregulated by both receptor mediated agonists and PMA in a concentration-dependent manner. Decreased expression of PDP1 blocked agonist-initiated PSDP phosphatase activity, and bioactive structural mimetics of PSDP resist metabolic inactivation by PDP1. Together, these findings implicate PDP1 as a regulated PSDP phosphatase and are the first to demonstrate modulation of PDP1 expression can change endogenous PSDP and PSMP in activated cells.

The LPT family of proteins has been segregated by sequence homology into five distinct classes, including the LPPs, SPPs, sphingomyelin synthases, lipid phosphatase-related proteins/plasticity-related genes (LPR/PRGs), and the CSS2s and more distant active site homology with glucose 6-phosphatase (8). LPPs are phosphatases that hydrolyze a large range of phosphorylated lipids, including LPA, PA, and S1P, with similar levels of activity towards each phospholipid (18,19). All three LPPs were recently tested for PSDP phosphatase activity and despite their broad range of substrate utilization, were not able to efficiently utilize PSDP

as substrate(9). In contrast to the LPPs wide spectra of substrates, the SPPs are reported to selectively dephosphorylate one principle substrate, S1P (20), and here extracts from cells transfected with SPP1 released substantial amounts of phosphate from S1P, but not from PSDP. Sphingomyelin synthases serve as lipid phosphotransferases not lipid phosphatases catalyzing the transfer of a phosphocholine group to ceramide to create sphingomyelin (21). LPR/PRGs lack the consensus amino acids required for phosphatase enzymatic activity and do not appear to function as either lipid phosphatases or sphingomyelin synthases (reviewed in (8)). To date, two CSS2s have been identified, CSS2α and PDP1 (formerly CSS2β). While these proteins lack sphingomyelin synthase activity (21), PDP1 carries a lipid phosphate phosphatase domain and activity for PIPPs (9). Recently, cell lysates with increased expression of PDP1 were reported to have phosphatase activity for PA (C16:0) (22). Of note, CSS2α, similar to the LPR/PRGs, lacks consensus sequences predicted to be critical for lipid phosphatase activity. PDP1 is predicted to have a core protein structure consisting of six transmembrane α helices with a defined active site split between two extramembranous loops (8). Here, we provide further evidence that, *in vitro*, rhPDP1 was able to utilize a wide variety of substrates, yet displayed distinct substrate preference for PSDP confirming that PDP1 is a member of the LPT family as a PSDP phosphatase that is functionally distinct from even the closely related lipid phosphatases, SPP1, LPP1, LPP2, and LPP3. In future studies, as more becomes known about PDP1's full function, other possible substrates in addition to PSDP may be identified.

Isoprenoids play diverse roles as molecular signals in the regulation of cell function in health and disease, including, but not limited to isoprenylation, formation of ubiquinone and dolichols, and modification of tRNA (23-26). Structure-activity relationships have determined that the diphosphate moiety is important for PSDP's bioactivity as an inhibitor of neutrophil activation and signaling (13,15-17). Consistent with a role in cellular activation, levels of PSDP rapidly and transiently decrease and PSMP levels increase within seconds after cells encounter agonist, concurrent with activation and deactivation of functional responses (15,16). Upon PMN activation by leukotriene B_4, PSDP directly interacts with phosphoinositol 3-kinase to potently inhibit its activity and further superoxide anion generation (17). These kinetics suggest a PSDP phosphatase as a critical point of regulation for the rapid activation of cells by soluble stimuli. Here, cell activation with PMA led to concentration-dependent increases in both PSDP phosphatase activity and phosphorylation of a 32 kDa protein with properties on western analysis consistent with PDP1. In addition, the expression of PDP1 siRNA in HEK293 cells led to concomitant decreases in PDP1 RNA, protein, and phosphorylation as well as decreased PSDP phosphatase activity, indicating that PDP1 plays a pivotal role in PMA initiated PSDP remodeling. Of interest, unbiased approaches to exploring cell signaling networks has also uncovered stimulus triggered phosphorylation of PDP1 after exposure to epidermal growth factor (27). Together with prior reports (9,13,15-17,28), these findings support roles in addition to cholesterol biosynthesis for PSDP in signal transduction and point to PDP1 as a pivotal checkpoint for these PIPP remodeling events in cell activation.

PSDP structural mimetics have been prepared and screened for bioactivity by inhibition of *in vitro* cell activation (13,17). These bioactive mimetics also potently block acute inflammation associated with peritonitis and acute lung injury (13,17). The PIPP analogs most active *in vivo* are a (Z)-PSDP isomer and a PSDP amino bis-phosphonate analog. Characterization of PDP1 as a PSDP phosphatase enabled determination of the capacity of these PIPP mimetics to resist phosphatase-based inactivation and these PSDP mimetics proved to be neither PDP1 substrates nor inhibitors. Together, these results suggest that these bioactive mimetics can resist metabolic inactivation by PDP1 *in vivo*, providing a rationale for their superior counter-regulatory properties.

In summary, rhPDP1 displayed catalytic properties of an LPT with a distinct substrate preference for PSDP, and cell activation was associated with PDP1 dependent PIPP remodeling

of PSDP to PSMP. These results are the first evidence that PDP1 is critical to agonist-initiated PIPP remodeling in cells and provides a potential new counter-regulatory signaling checkpoint in cell activation.

Acknowledgments

We thank Dr. Andrew Morris (University of Kentucky) for PDP1 antibody and helpful discussion, Dr. Charles N. Serhan (Brigham and Women's Hospital, Harvard Medical School) for critical review of the manuscript and William Cullen for assistance in manuscript preparation.

The work was supported in part by NIH grants HL68669, 5T32HL007633-22, and P50-DE016191.

References

1. Serhan CN. Lipoxins and aspirin-triggered 15-epi-lipoxin biosynthesis: an update and role in anti-inflammation and pro-resolution. Prostaglandins & Other Lipid Mediators 2002;68-69:433–455. [PubMed: 12432935]
2. Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. Nat Rev Drug Discov 2004;3:401–416. [PubMed: 15136788]
3. Nathan C. Points of control in inflammation. Nature 2002;420:846–852. [PubMed: 12490957]
4. Brindley DN, Waggoner DW. Mammalian lipid phosphate phosphohydrolases. Journal of Biological Chemistry 1998;273:24281–24284. [PubMed: 9733709]
5. Cowart LA, Obeid LM. Yeast sphingolipids: Recent developments in understanding biosynthesis, regulation, and function. Biochim Biophys Acta 2007;1771:421–431. [PubMed: 16997623]Epub 2006 Aug 2010
6. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nature Reviews Immunology 2005;5:560–570.
7. Saba JD, Hla T. Point-counterpoint of sphingosine 1-phosphate metabolism. Circ Res 2004;94:724–734. [PubMed: 15059942]
8. Sigal YJ, McDermott MI, Morris AJ. Integral membrane lipid phosphatases/phosphotransferases: common structure and diverse functions. Biochemical Journal 2005;387:281–293. [PubMed: 15801912]
9. Fukunaga K, Arita M, Takahashi M, Morris AJ, Pfeffer M, Levy BD. Identification and functional characterization of a presqualene diphosphate phosphatase. Journal of Biological Chemistry 2006;281:9490–9497. [PubMed: 16464866]
10. Stukey J, Carman GM. Identification of a novel phosphatase sequence motif. Protein Science 1997;6:469–472. [PubMed: 9041652]
11. Faulkner A, Chen X, Rush J, Horazdovsky B, Waechter CJ, Carman GM, Sternweis PC. The LPP1 and DPP1 gene products account for most of the isoprenoid phosphate phosphatase activities in Saccharomyces cerevisiae. J Biol Chem 1999;274:14831–14837. [PubMed: 10329682]
12. Carlo T, Levy BD. Chemical mediators and the resolution of airway inflammation. Allergol Int 2008;57:299–305. [PubMed: 18946231]Epub 2008 Nov 2001
13. Levy BD, Hickey L, Morris AJ, Larvie M, Keledjian R, Petasis NA, Bannenberg G, Serhan CN. Novel polyisoprenyl phosphates block phospholipase D and human neutrophil activation in vitro and murine peritoneal inflammation in vivo. British Journal of Pharmacology 2005;146:344–351. [PubMed: 16041402]
14. Samuelsson, B. Les Prix Nobel: Nobel Prizes, Presentations, Biographies and Lectures. Almqvist & Wiksell; Stockholm: 1982. From studies of biochemical mechanisms to novel biological mediators: prostaglandin endoperoxides, thromboxanes and leukotrienes; p. 153-174.
15. Levy BD, Petasis NA, Serhan CN. Polyisoprenyl phosphates in intracellular signalling. Nature 1997;389:985–990. [PubMed: 9353124]
16. Levy BD, Fokin VV, Clark JM, Wakelam MJ, Petasis NA, Serhan CN. Polyisoprenyl phosphate (PIPP) signaling regulates phospholipase D activity: a ‘stop’ signaling switch for aspirin-triggered lipoxin A4. Faseb J 1999;13:903–911. [PubMed: 10224233]

17. Bonnans C, Fukunaga K, Keledjian R, Petasis NA, Levy BD. Regulation of phosphatidylinositol 3-kinase by polyisoprenyl phosphates in neutrophil-mediated tissue injury. Journal of Experimental Medicine 2006;203:857–863. [PubMed: 16567384]

18. Roberts R, Sciorra VA, Morris AJ. Human type 2 phosphatidic acid phosphohydrolases. Substrate specificity of the type 2a, 2b, and 2c enzymes and cell surface activity of the 2a isoform. Journal of Biological Chemistry 1998;273:22059–22067. [PubMed: 9705349]

19. Waggoner DW, Gomez-Munoz A, Dewald J, Brindley DN. Phosphatidate phosphohydrolase catalyzes the hydrolysis of ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate. Journal of Biological Chemistry 1996;271:16506–16509. [PubMed: 8663293]

20. Le Stunff H, Peterson C, Thornton R, Milstien S, Mandala SM, Spiegel S. Characterization of murine sphingosine-1-phosphate phosphohydrolase. Journal of Biological Chemistry 2002;277:8920–8927. [PubMed: 11756451]

21. Huitema K, van den Dikkenberg J, Brouwers JF, Holthuis JC. Identification of a family of animal sphingomyelin synthases. EMBO Journal 2004;23:33–44. [PubMed: 14685263]

22. Theofilopoulos S, Lykidis A, Leondaritis G, Mangoura D. Novel function of the human presqualene diphosphate phosphatase as a type II phosphatidate phosphatase in phosphatidylcholine and triacylglyceride biosynthesis pathways. Biochim Biophys Acta 2008;1781:731–742. [PubMed: 18930839]

23. Sacchettini JC, Poulter CD. Creating isoprenoid diversity. Science 1997;277:1788–1789. [PubMed: 9324768]

24. Holstein SA, Hohl RJ. Isoprenoids: remarkable diversity of form and function. Lipids 2004;39:293–309. [PubMed: 15357017]

25. Schmidt RA, Schneider CJ, Glomset JA. Evidence for post-translational incorporation of a product of mevalonic acid into Swiss 3T3 cell proteins. J Biol Chem 1984;259:10175–10180. [PubMed: 6565705]

26. Takada K, Aksentijevich I, Mahadevan V, Dean JA, Kelley RI, Kastner DL. Favorable preliminary experience with etanercept in two patients with the hyperimmunoglobulinemia D and periodic fever syndrome. Arthritis Rheum 2003;48:2645–2651. [PubMed: 13130485]

27. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 2006;127:635–648. [PubMed: 17081983]

28. Levy BD, Serhan CN. Polyisoprenyl phosphates: natural antiinflammatory lipid signals. Cellular and Molecular Life Sciences 2003;59:1–13.

Abbreviations

CSS2
: Candidate sphingomyelin synthase type 2

FDP
: farnesyl diphosphate

G6P
: glucose 6-phosphate

LPP
: lipid phosphate phosphatase

LPT
: lipid phosphate phosphatase/phosphotransferase

LPR/PRGs
: lipid phosphatase-related proteins/plasticity-related genes

LPA
: lysophosphatidic acid

PA
phosphatidic acid

PAP2A
phosphatidic acid phosphatase 2A

PPAPDC2
phosphatidic acid phosphatase type 2 domain containing 2

PDP1
polyisoprenyl diphosphate phosphatase 1

PSDP
presqualene diphosphate

PSMP
presqualene monophosphate

PKC
protein kinase C

S1P
sphingosine-1-phosphate

SPP
sphingosine-1-phosphate phosphatase

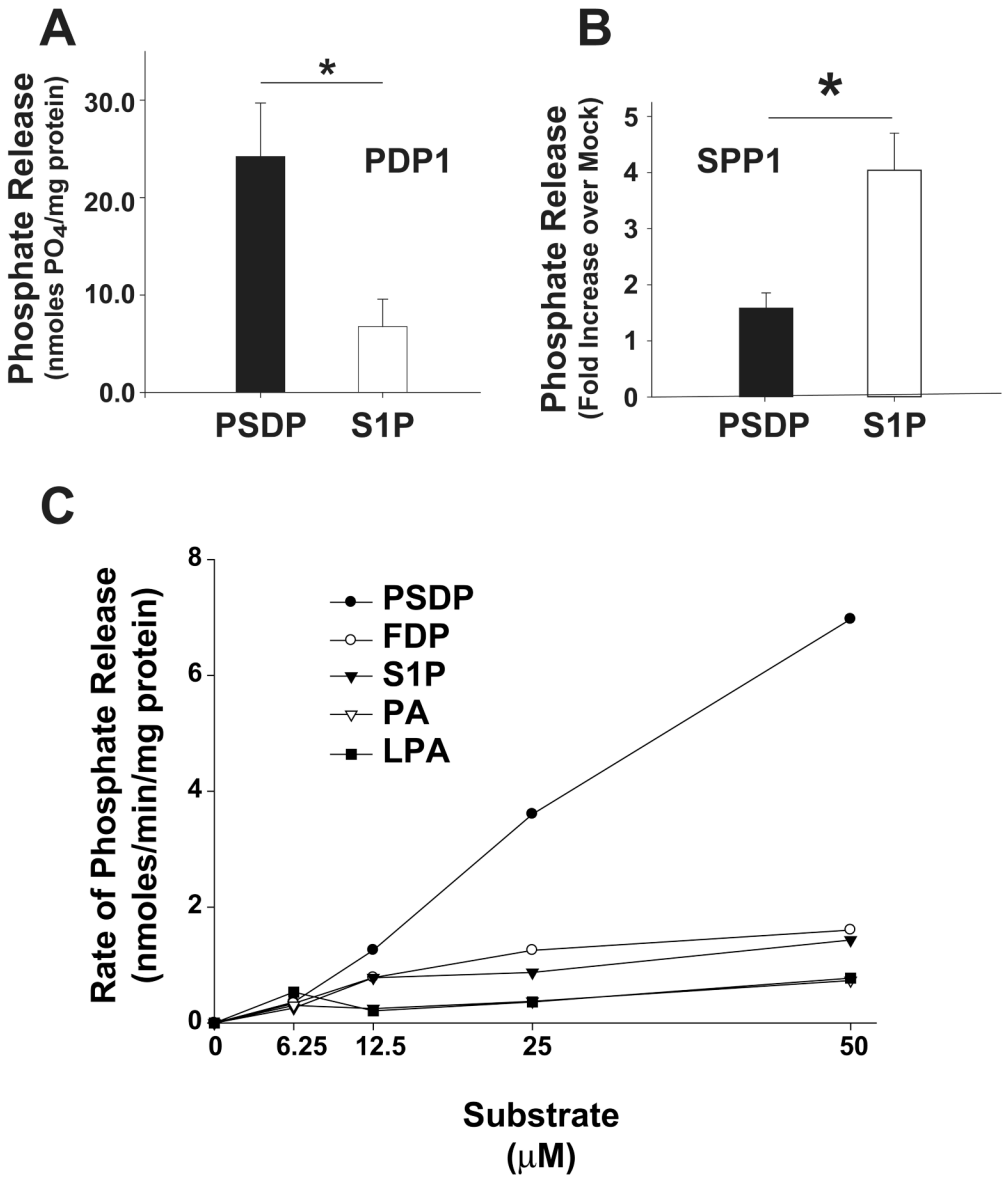

Figure 1.

PDP1 and SPP1 Substrate Preferences. *A*. rhPDP1 (2μg) was exposed to PSDP (20μM) or S1P (60μM) for 30min (37°C) and phosphate release determined (See Methods). *B*. Cell extracts from HEK293 cells transfected with hSPP1 or a mock control, were exposed to (30min, 37° C) either PSDP (20μM) or S1P (60μM) and phosphate release was determined. *C*. Concentration dependent phosphate release was determined for rhPDP1 and control Sf21 extracts (1μg) exposed to PSDP, FDP, S1P, PA or LPA for 30min (37°C) at a constant detergent to lipid molar ratio (12.5:1). The net rate of phosphate release was determined for rhPDP1 Sf21 extracts (See Methods). Values represent the mean ± SE. ($n \geq 4$, *$P < 0.05$)

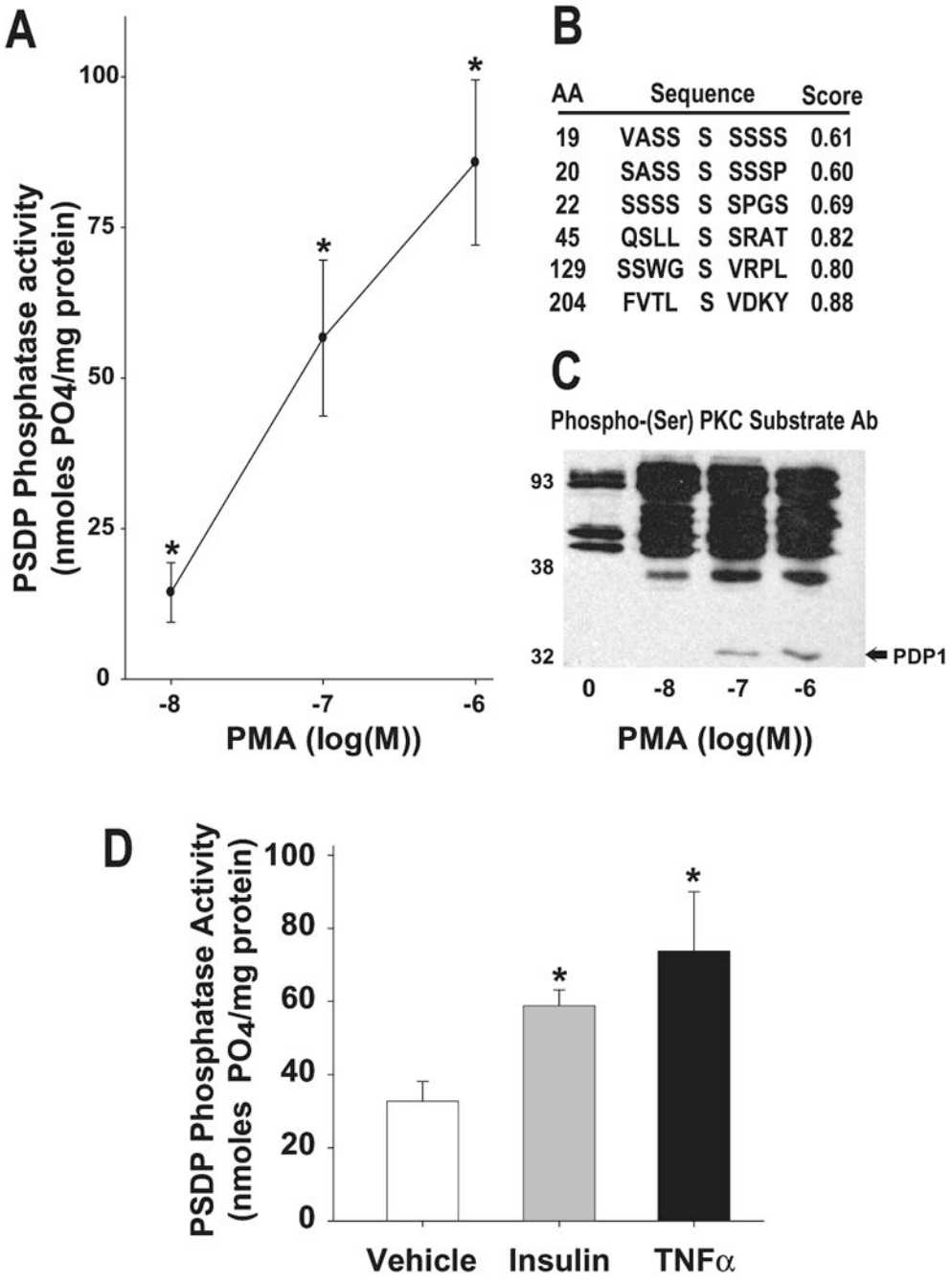

Figure 2.

Agonist Induced Increase in Cellular PSDP Phosphatase Activity. HEK293 cells were exposed (10min, 37°C) to (A) PMA (10^{-8}-10^{-6} M) or vehicle (0.1% EtOH) or (D) TNFα (10ng/ml), insulin (25μM), or vehicle (PBS) and cell extracts were assayed for PSDP (20 μM) phosphatase activity in 0.1% Triton-X 100. Values represent the mean ± SEM for n=3. *P<0.05 relative to vehicle. *B*. Potential PKC phosphorylation sites in hPDP1 amino acid sequence motifs are indicated with a score generated by the NetPhos K 1.0 computer algorithm (http://www.cbs.dtu.dk/services/NetPhosK/). *C*. Western analysis of cell extracts from HEK293 cells exposed (10min, 37°C) to PMA (10^{-8}-10^{-6}M) or vehicle (0.1% EtOH) probed

with a Phospho-(Ser) PKC substrate antibody (1:1000 dilution, Cell Signaling Technology). Molecular weight markers (left) and PDP1 (right) are indicated.

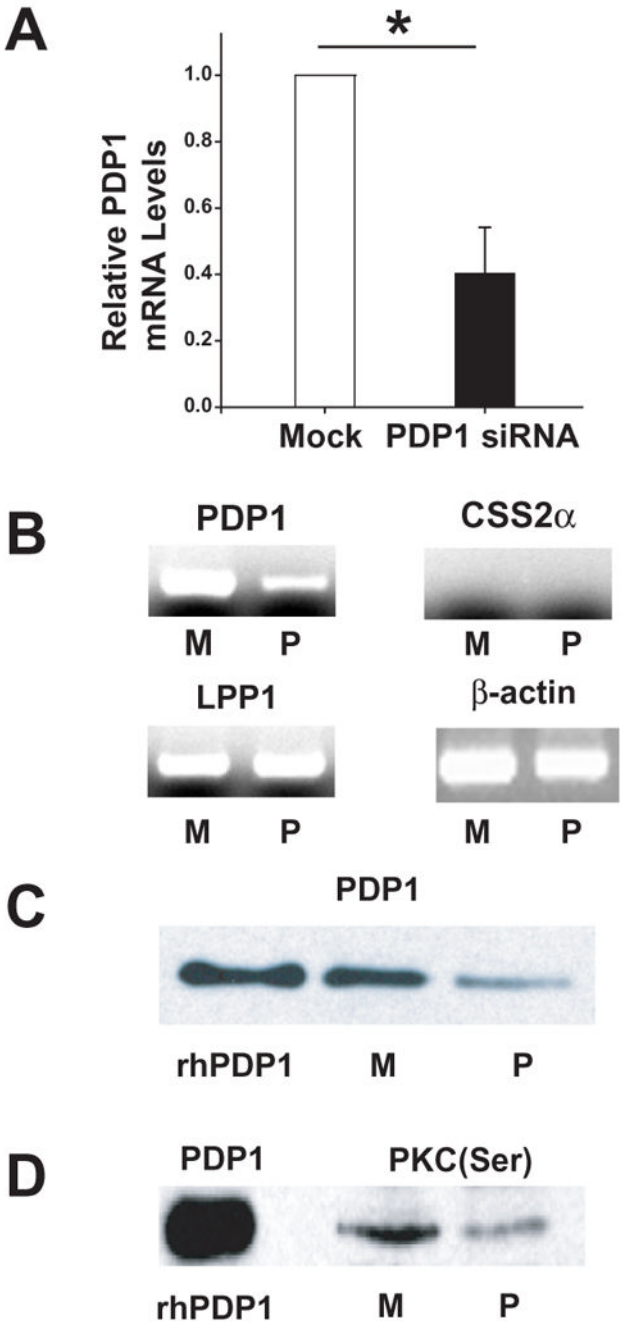

Figure 3.

Decreased Cellular PDP1 expression by siRNA. HEK293 cells were stably transfected with either a control vector (Mock or M) or a PDP1 siRNA construct (P) (See Methods). *A*. PDP1 RNA levels, relative to β-actin, were measured by semi-quantitative RT-PCR and densitometry. Values for Mock were arbitrarily set at 1.0. Values for PDP1 siRNA cells represent the mean ± SEM for n=3. *P<0.05. *B*. Expression of the closely related LPT genes, CSS2α and LPP1 as well as β-actin was determined by RT-PCR. *C*. Protein levels were determined by Western analysis of rhPDP1 (1μg) or protein extracts from Mock (M) or PDP1 siRNA (P) transfected cells (60μg) using PDP1 specific antisera (See Methods). Representative of n=4.

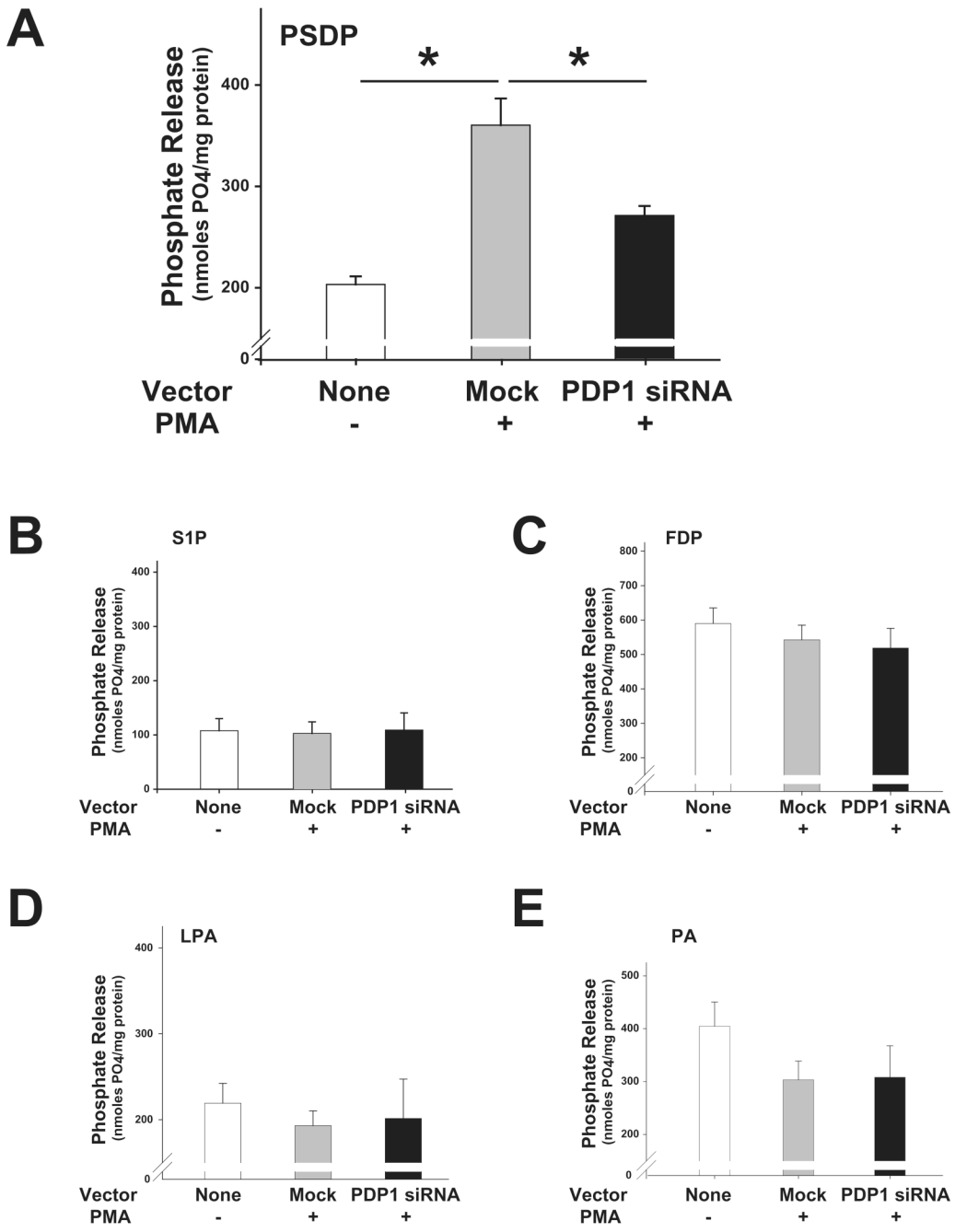

Figure 4.

Decreased Cellular Expression of PDP1 Blocks Agonist Induced PSDP Phosphatase Activity. Phosphate release from (A) PSDP (20μM), (B) S1P (60μM), (C) FDP (20μM), (D) LPA (20μM), or (E) PA (20μM) was determined in cell extracts from untransfected HEK293 cells not exposed to PMA (None) or PMA activated (10^{-7}M, 10min, 37°C) cells that were stably transfected with either a mock vector (Mock) or hPDP1 siRNA. Values represent mean ± SEM. (n≥3, *P<0.01)

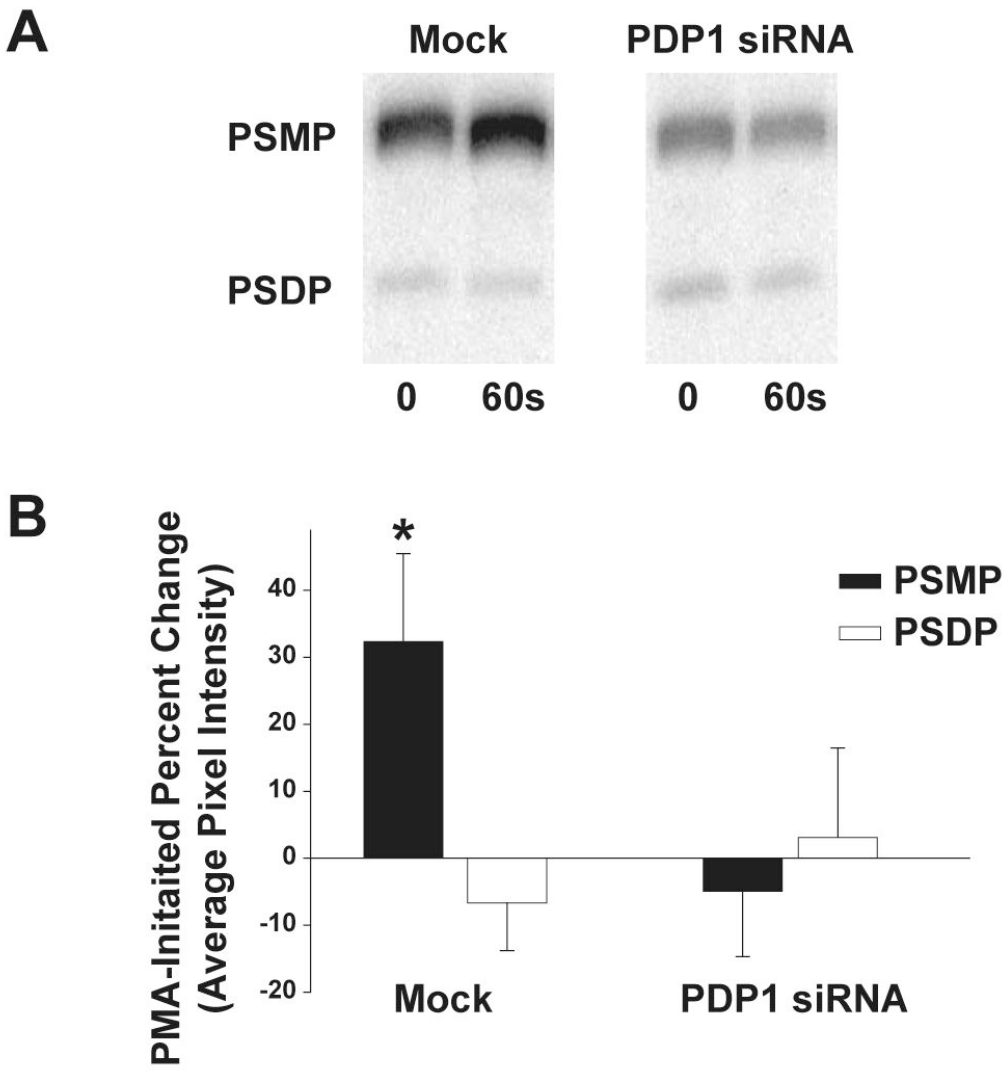

Figure 5.

Decreased Cellular Expression of PDP1 Blocks Agonist Induced PSDP dephosphorylation to PSMP. Mock and PDP1siRNA cells were exposed (0-60s, 37°C) to PMA (2μM). Lipids were extracted and analyzed by TLC (See Methods). Materials with the same Rf values as PSDP and PSMP were quantitated by phosphoimaging. (A) Image of [^{32}P]-labelled PSDP and PSMP. Representative of n=3. *B.* PMA-initiated change of [^{32}P]-labelled PSDP and PSMP amounts as measured by phosphoimaging. Values represent the mean ± SEM (n=3, *P<0.05).

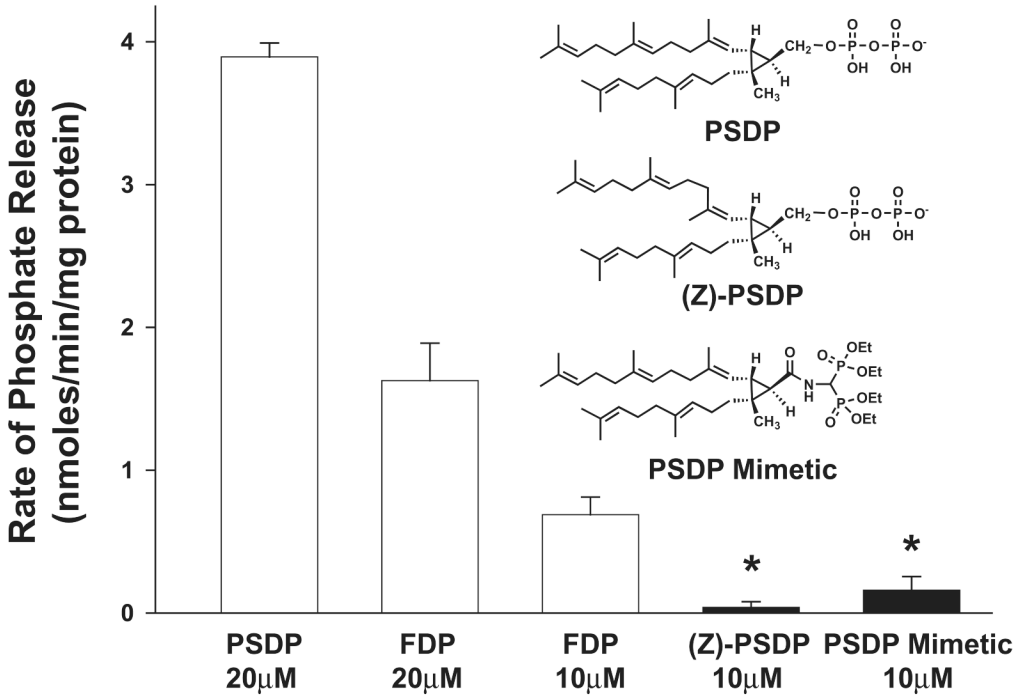

Figure 6.

PSDP Structural Mimetics Resist PDP1 Phosphatase Activity. rhPDP1 (2μg) was exposed to PSDP (20μM), FDP (10-20μM), or either the (Z)-PSDP or PSDP amino bis-phosphonate structural mimetic (Inset) (10μM) in 0.1% Triton-X 100 for 30min (37°C) and phosphate release determined (See Methods). Values represent the mean ± SE. (n=3, *P<0.04 vs. PSDP and both 10 and 20 μM FDP).