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Farnesyl Phosphates Are Endogenous Ligands of Lysophosphatidic Acid Receptors: Inhibition of LPA GPCR and Activation of PPAR

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Abbreviations used:

ACox acetylCoA oxidase

ACox-Rluc acyl-coenzyme A oxidase-luciferase

AGP alkyl-glycerophosphate

ATP definition

$[Ca^{2+}]_i$ intracellular Ca^{2+} concentration

FAP fatty alcohol phosphate

FDP farnesyl diphosphate

FMP farnesyl phosphate

FR farnesol

GPCR G protein-coupled receptor

LDL low-density lipoproteins

LPA lysophosphatidic acid

PPAR peroxisome proliferator-activated receptor

PPRE PPAR response element

Rosi rosiglitazone

SREBP sterol regulatory element binding protein

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Abstract

Oligoprenyl phosphates are key metabolic intermediates for the biosynthesis of steroids, the side chain of ubiqinones, and dolichols and the posttranslational isoprenylation of proteins. Farnesyl phosphates are isoprenoid phosphates that resemble polyunsaturated fatty alcohol phosphates, which we have recently shown to be the minimal pharmacophores of lysophosphatidic acid (LPA) receptors. Here we examine whether farnesyl phosphates can interact with the cell surface and nuclear receptors for LPA. Both farnesyl phosphate and farnesyl diphosphate potently and specifically antagonized LPA-elicited intracellular Ca^{2+}-mobilization mediated through the LPA_3 receptor, while causing only modest inhibition at the LPA_2 receptor and no measurable effect at the LPA_1 receptor. Farnesol also inhibited LPA_3 but was much less effective. The estimated dissociation constant of LPA_3 for farnesyl phosphate is 48 ± 12 nM and 155 ± 30 nM for farnesyl diphosphate. The transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) binds to and is activated by LPA and its analogs including fatty alcohol phosphates. We found that both farnesyl phosphate and diphosphate, but not farnesol, compete with the binding of the synthetic PPARγ agonist [^{3}H]rosiglitazone and activate the PPARγ-mediated gene transcription. Farnesyl monophosphate at 1 μM, but not diphosphate, activated PPARα and PPARβ/δ reporter gene expression. These results indicate new potential roles for the oligoprenyl phosphates as potential endogenous modulators of LPA targets and show that the polyisoprenoid chain is recognized by some LPA receptors.

Keywords

farnesyl phosphate; isoprenoid; LPA; lysophospholipid; GPCR; PPARγ

INTRODUCTION

The oligoprenyl farnesyl diphosphate (FDP) is a key intermediate in the biosynthesis of steroids, carotenoids, the side chain of ubiqinones, and polyisoprenoids, as well as the donor of the farnesyl group for isoprenylation of many proteins, including the βγ subunit of heterotrimeric G proteins and the small GTPases, Ras and Rho. FDP is synthesized from dimethylallyl diphosphate via two sequential condensation steps with isopentenyl diphosphate catalyzed by FDP synthase (for a review:[1]). In addition to de novo synthesis, FDP can be formed from farnesol (FR) via two consecutive phosphorylation reactions catalized by farnesol kinase and farnesyl phosphate (FMP) kinase [2,3], responsible for the endogenous production of both FMP and FDP.

Lysophosphatidic acid (1-radyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA) is a lipid mediator and intracellular messenger regulating fundamental cellular events including cell proliferation, survival, differentiation, the actin cytoskeleton, cell migration, and Ca^{2+} homeostasis [4-6]. LPA at the cell surface acts on distinct plasma membrane receptors that belong to the G protein-coupled receptor superfamily (GPCR). There are three well-characterized LPA GPCRs in the endothelial differentiation gene family designated as $LPA_{1/2/3}$ [7]. Recently, GPR23 and GPR92 have been identified in the purinergic receptor cluster as additional LPA GPCRs, whose function remains to be elucidated [8-10]. The nuclear transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) has been identified as an intracellular target of LPA [11], and activation of PPARγ by select forms of LPA, particularly alkyl-

glycerophosphate (AGP), which accumulates in oxidatively modified low-density lipoprotein (LDL), has been implicated in vascular smooth muscle dedifferentiation and neointima formation [12,13].

From a chemical point of view, oligoprenyl phosphates are similar to fatty alcohol phosphates (FAP). We have shown that FAPs satisfy the minimal structural requirements of the LPA pharmacophore at both LPA GPCRs and PPARγs [14,15]. With modifications to the length and saturation of the hydrocarbon chain, as well as to the bond of the phosphate group, we identified agonists and antagonists with subtype selectivity at the different the LPA GPCRs. Many of the FAP derivatives also activate PPARγ-regulated reporter genes in CV-1 cells [14]. Furthermore, dioctylglycerol pyrophosphate is an effective antagonist of LPA_3 and to a lesser degree of LPA_1 [16] and is more effective than dioctyl phosphatidic acid, indicating that LPA GPCRs not only tolerate but prefer the pyrophosphate moiety similar to that present in FDP.

In the present study, we examined the hypothesis that farnesyl phosphates as polyunsaturated, naturally occurring analogs that resemble FAP could activate or inhibit the LPA GPCRs and the nuclear LPA receptor PPARγ. Neither FR, FMP, nor FDP activated the plasma membrane LPA receptors, whereas they all showed potent competitive antagonistic effects against LPA at the LPA_3 receptor, a weak but significant inhibition at the LPA_2 receptor, and no effect at LPA_1. FR only weakly inhibited LPA_3 but had no effect on LPA_2. FR, FMP, and FDP did not interfere with the activation of the ATP receptor in the same cells. Both lipids competed with [^{3}H]rosiglitazone (Rosi) binding to purified recombinant PPARγ protein and also weakly activated this transcription factor as judged by the activation of the PPARγ-regulated acetylCoA oxidase (ACox) reporter gene. FMP was also effective, albeit weakly, in activating PPARα and PPARβ/δ reporter gene expression. These findings raise the possibility that isoprenyl phosphates, in addition to LPA analogs, might endogenously modulate the function of receptors that we currently associate only with LPA and also provide new insights concerning the types of hydrocarbons tolerated by LPA targets.

EXPERIMENTAL PROCEDURES

Materials

Oleoyl-LPA was purchased from Avanti Polar Lipids; Fura-2 AM was from Molecular Probes. All other reagents were purchased from Sigma-Aldrich Chemical Co. and Fisher Scientific and were used without further purification.

FMP and FDP (Fig. 1) were prepared by chemical phosphorylation of FR as described earlier [17]. Lipids were dissolved in methanol at a concentration of 10 mM, then an appropriate volume of these stock solutions was dried in glass vials and taken up in modified Krebs solution (10 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 6 mM glucose), also containing 1 mM BSA, to yield a final lipid concentration of 1 mM. Cells were treated with the appropriate dilution of this 1 mM lipid-BSA stock solution.

Measurement of intracellular Ca^{2+} concentration

RH7777 cells stably transfected with either LPA_1, LPA_2, or LPA_3 and PC3 prostate cancer cells endogenously expressing all three EDG-family LPA GPCRs were plated on poly-D-lysine-coated black-wall clear-bottom 96-well plates (Becton Dickinson) at a density of 5×10^4 cells/well and cultured overnight in DMEM containing 10% FBS, penicillin/streptomycin (Sigma), and G418 (Gibco). The culture medium was then replaced with a modified Krebs solution (10 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 6 mM glucose), and the cells were serum starved 6-8 h. Subsequently,

the cells were loaded with 2 μM Fura-2 AM for 40 min in modified Krebs medium containing 2% pluronic acid. The dye loading medium was removed and replaced with 100 μl of modified Krebs medium per well prior to analysis using a FlexStation II robotic plate fluorimeter (Molecular Devices, [14,18]). Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by measuring the ratio of emitted light intensity at 520 nm excited alternately at 340 or 380 nm. Each well was monitored for 80 s. The test compound (50 μl) was added automatically after 15 s of baseline measurement. Ca^{2+} transients were quantified with the SoftMax Pro software calculating the difference between maximum and baseline ratio values for each well [14,18].

Competition displacement assay of [^{3}H]Rosi from purified recombinant PPARγ

Recombinant His_6-PPARγ fusion protein or His_6 containing the thrombin recognition site in an empty vector control was expressed in BL-21 (DE3) cells and processed as previously described [12]. Transformed BL-21 cells were induced by 0.3 mM IPTG (Fischer Scientific) for 12 h at 25°C and collected by centrifugation. Recombinant LBD-PPARγ was extracted with lysis buffer (50 mM HEPES, pH 6.8, 200 mM NaCl, 5 mM DTT, 1 mM PMSF, 0.5% Triton X-100, and 10% glycerol) using centrifugation at 12,000×g for 20 min. The lysate supernatant (1 ml) was incubated with 50 μl of TALON metal affinity resin (BD Bioscience) at 4°C for 1 h in lysis buffer. The resin was washed three times with wash buffer (50 mM HEPES, pH 6.8, 200 mM NaCl, 5 mM DTT, 10% glycerol, and 5 mM imidazole) and eluted with 150 mM imidazole in the wash buffer. The LBD-PPARγ protein was quantified using the Bradford protein assay and Coomassie blue staining (Pierce). For binding assay, 1 μg His_6-LBD-PPARγ protein was incubated at 18°C for 1 h in 200 μl of 50 mM HEPES, pH 6.8, buffer containing 100 mM NaCl, 5 mM EDTA, and 5 mM DTT in the presence of 5 nM [^{3}H]Rosi with FR, FMP, or FDP. The radioligand-LBD fusion protein complex was washed five times with wash buffer (50 mM HEPES, pH 6.8, 100 mM NaCl, 5 mM EDTA, 5 mM DTT), and the bound radioligand was quantified by scintillation counting.

PPARα/β/γ activation reporter gene assays

Determining PPARγ activation in CV-1 or B103 cells (the latter lacks endogenous LPA GPCRs and PPARs, [12]) transfected with PPARγ and an acyl-coenzyme A oxidase-luciferase (PPRE-ACox-Rluc) reporter gene construct was performed as previously reported [12,13]. Briefly, CV-1 cells were plated on 96-well plates (1×10^4 cells per well) in DMEM supplemented with 10% FBS. The next day, cells were transiently transfected with 125 ng of pGL3-PPRE-ACox-Rluc, 62.5 ng of pcDNA3.1-PPARγ, and 12.5 ng of pSV-β-galactosidase (Promega) by using LipofectAMINE 2000 (Invitrogen). One day after transfection, cells were treated with OptiMEM (Invitrogen) containing 10 μM or 1μM DMSO or test compound dissolved in DMSO for 20 h. Luciferase and β-galactosidase activities were measured with the Steady-Glo® Luciferase Assay System (Promega) and the Galacto-Light Plus™ System (Applied Biosystems), respectively. Samples were run in quadruplicate, and the data are representative of at least two independent transfections. For assaying the regulation of the PPARγ target gene CD36, CV-1 cells were transfected with a CD36 promoter-luciferase reporter that contained its PPRE (CD36 -273) or a mutant in which the PPRE site was deleted (CD36 -261). All cells were cotransfected with SV40-β-galactosidase for normalization of expression and also with PPARγ to augment its low endogenous expression in CV1 cells. The cells were treated for 20 h with 10 μM Rosi, FR, FMP, or FDP before the ratio of luciferase to β-galactosidase was determined and normalized to vehicle-treated cells.

PPARγ activation was also performed using B103 cells lacking endogenous PPARγ expression and transfected with the same constructs as previously reported [12]. For PPARα activation, 62.5 ng of pG4M-PPARα-ligand binding domain and 12.5 ng of pSV-β-galactosidase or 125 ng of 17m5×-βGLOB-Luc were used. Similarly, for PPARβ, we used 62.5 ng of pG4M-

PPARβ-LBD plasmid DNA. Twenty-four hours after transfection, cells were treated with Opti-MEM (Invitrogen) containing 1μM or 10μM test compound dissolved in DMSO and cultured for an additional 20 h. Luciferase and β-galactosidase activities were measured with Steady-Glo Luciferase Assay System (Promega) and the Galacto-Light Plus system (Applied Biosystems, Foster City, CA), respectively. Samples were run in quintuples, and the mean ± standard deviation was calculated. Data are representative of at least two independent transfections.

Statistical analysis

Data are expressed as the average ± standard deviation. Significant difference between two experimental groups was determined by the Student's *t*-test at a P value of 0.05. EC_{50} and IC_{50} values were calculated by fitting a sigmoid function to data points by using the nonlinear curve-fitting feature of KaleidaGraph (Synergy Software, Essex Junction, VT).

RESULTS

FMP and FDP inhibit LPA-elicited intracellular Ca^{2+}-responses elicited by distinct LPA GPCRs

To test the ability of FMP and FDP to interact with LPA GPCRs, we used rat hepatoma cells (RH7777) stably expressing the LPA_1, LPA_2, and LPA_3 receptors [11,12]. RH7777 cells represent an ideal transfection host for LPA GPCRs since they do not respond to high concentrations of LPA Ca^{2+} transients. In the transfectants, LPA at nanomolar concentrations elicit intracellular Ca^{2+}-transients by activating these GPCRs. When applied alone up to 30 μM, the highest concentration tested, neither FR, FMP, nor FDP evoked any Ca^{2+} response in wild type or LPA GPCR transfected cell line (data not shown). This finding suggests that FR, FMP, and FDP do not activate LPA GPCRs or any other endogenous receptor coupled to Ca^{2+} responses expressed in the RH7777 cell line.

To investigate further the potential antagonistic effect of FR, FMP, and FDP, these lipids were applied together with oleoyl-LPA. As shown in Fig. 2A, 30 μM FMP or FDP almost completely abolished $[Ca^{2+}]_i$ transients elicited by 1 μM LPA in the LPA_3-expressing cells when applied at a concentration of 30 μM. In contrast, this high ligand concentration had no significant effect on the LPA response of the LPA_1 expressing cells. The inhibitory effect of farnesyl phosphates did not affect the $[Ca^{2+}]_i$ transients evoked by ATP acting on endogenous purinergic receptors expressed in RH cells (Fig. 2), indicating that FMP or FDP in concentrations as high as 30 μM does not interfere with the activation of ATP receptors. The EC_{50} values of the LPA-elicited $[Ca^{2+}]_i$ transients are 125 nM and 85 nM for heterologously expressed LPA_1 and LPA_3, respectively. Thus, 1 μM LPA evoked a submaximal response in these experiments. In the case of the LPA_2-expressing cells, the EC_{50} value of the LPA response was 13 nM; hence, we applied 30-50 nM LPA to evoke a submaximal response when evaluating the inhibitory effects of farnesyl phosphates. As shown in Fig. 2B, both FMP and FDP applied at 10 μM partially inhibited the $[Ca^{2+}]_i$ transients elicited by 30 nM LPA. This is a unique observation, since the FAPs showed no inhibitory effect at LPA_2 [11]. To further characterize the effect of the polyisoprenoid chain at LPA GPCRs, we also tested FR against near EC_{50} concentrations of LPA for the three receptors. Fig. 2C shows that FR failed to affect the LPA_1 and LPA_2 responses but slightly inhibited the LPA_3 response at high micromolar concentrations; FR had no effect on the ATP response (data not shown).

To quantify their inhibitory potency at LPA_2, we measured the effect of FMP and FDP in the 0.3-30 μM concentration range (Fig. 3A) against 50 nM LPA in LPA_2-expressing cells. Although both lipids inhibited the LPA response. however, even at 30 μM, the highest concentration applied, they failed to achieve complete inhibition. Assuming that both lipids

would fully inhibit the response at higher concentrations, we estimated their apparent IC_{50} values at 21 ± 3 μM and 46 ± 6 μM for FMP and FDP, respectively, by fitting a declining sigmoid function to the data.

In the inhibition experiments, we found that FR and farnesyl phosphates inhibited the LPA-elicited $[Ca^{2+}]_i$ transients in LPA_3-expressing cells (Fig. 2). To determine the IC_{50} values of FMP and FDP at LPA_3, we applied these ligands in the 0.3-30 μM concentration range against 300 nM oleoyl-LPA. As shown in Fig. 3B, both FMP and FDP decreased dose-dependently the LPA response and achieved a complete inhibition at 10 μM concentration. In contrast, FR did not inhibit LPA_3 completely, even at the highest 30 μM concentration tested (Fig. 2C). The IC_{50} values were first calculated by fitting a sigmoid function to the data. Because the Hill coefficient did not differ significantly from unity, we fixed its value as 1 and repeated the fit with a declining hyperbole, which yielded apparent IC_{50} values of 161 ± 31 nM and 517 ± 60 nM for FMP and FDP, respectively.

To study the mechanism of inhibition, we also determined the dose-response curves for oleoyl-LPA in LPA_3-expressing cells in the presence of farnesyl phosphates. As shown in Fig. 4A, the dose-response curve of LPA shifted to the right in the presence of FMP at both 300 nM or 3 μM concentrations, increasing the EC_{50} from 85 ± 8 nM to 378 ± 28 nM and 1430 ± 200 nM, respectively. At 10 μM, the highest concentration tested, LPA could overcome the inhibition of FMP at both concentrations. Fig. 4B shows that FDP also caused a right-shift in the dose-response curve of LPA with EC_{50} values of 170 ± 9 nM, 480 ± 46 nM, and 1330 ± 140 nM for LPA alone or in the presence of 300 nM or 3 μM FDP, respectively. LPA at a concentration of 10 μM also could overcome the inhibition at both concentrations of FDP.

These data together are consistent with a competitive mechanism of inhibition for both farnesyl phosphates. Based on the dose-response and inhibition curves of LPA in the absence and presence of the inhibitors, we estimated the equilibrium dissociation constants for FMP and FDP at the LPA_3 receptor by using the Cheng-Prusoff relationship [19]: $K_B = IC_{50}/(1+(A/EC_{50})$, where K_B denotes the dissociation constant of the inhibitor and A is the concentration of the agonist used in establishing the inhibition curve. Taking the average of the two EC_{50} values of LPA from Figs. 4A and 4B and the IC_{50} values from Fig. 3, we estimated the dissociation constant K_B of FMP and FDP at LPA_3 to be 48 ± 12 nM and 155 ± 30 nM, respectively.

To evaluate whether FMP and FDP can inhibit LPA GPCRs endogenously expressed in PC3 prostate cancer cells, we applied LPA-elicited Ca^{2+}-mobilization assays as previously described [11,12]. Indeed, both FMP and FDP showed dose-dependent partial inhibition of the LPA response (Fig. 5). These results extend our observations obtained in RH7777 cells heterologously expressing a combination of these LPA GPCRs.

Farnesyl phosphates activate PPARγ

LPA, its alkyl ether analog AGP, and FAP (Fig. 1) have recently been shown to be agonists of PPARγ [12]. We evaluated whether FR and farnesyl phosphates also can interact with PPARγ. First, we determined the ability of FR, FMP, and FDP to compete with binding of $[^3H]$Rosi, a well-characterized selective agonist of PPARγ. Fig. 6A shows that both farnesyl phosphates dose-dependently displaced Rosi from the purified ligand binding domain of PPARγ, but the displacement was not complete even at 50 μM, the highest concentration tested. In contrast, FR was much less potent, displacing only 25% of $[^3H]$Rosi at 10 μM, the highest concentration tested. Fitting a sigmoid function to the displacement curves yielded apparent IC_{50} values of 19 ± 7 μM and 19 ± 2 μM for FMP and FDP, respectively. To study the functional consequences of farnesyl phosphate binding to PPARγ, the activity of this transcription factor was monitored in CV-1 cells transfected with a PPRE-ACox-luc reporter construct. As shown

in Fig. 6B, at a concentration of 10 μM, FR, FMP, and FDP weakly but significantly activated PPARγ; nonetheless, Rosi was more efficacious. Interestingly, FR was more efficacious than FMP and FDP, yet it bound less weakly to the isolated PPARγ ligand binding protein. This apparent contradiction between binding and activation could indicate that the extracellularly applied FR once internalized becomes phosphorylated and converted to a an intermediate with a higher potency and/or efficacy.

The scavenger receptor CD36 is a PPRE-regulated target gene of PPARγ with a PPRE between -273 and -261. The CD36 PPRE-Luc and its truncation mutant CD36-261-Luc constructs were transfected into CV-1 cells. The cells were exposed to 10 μM Rosi, FMP, or FDP, and the induction of the reporter gene was determined (Fig. 6C). Neither compound activated the truncated CD36 construct. However, all three activated the transcription of the construct with the intact PPRE at a comparably low level. These data support the hypothesis that extracellularly applied farnesyl phosphates can regulate PPARγ-mediated gene transcription as shown for the examples of acetyl-CoA oxidase and CD36 scavenger receptor genes.

PPARγ shares considerable homology with PPARα and PPARβ/δ, thereby raising the possibility that farnesyl phosphates can activate other members of the PPAR family. This hypothesis was tested using B103 cells transiently transfected with either PPARα, PPARβ/δ, or PPARγ reporter genes. We chose the B103 cell line for these studies due to the lack of expression of the different LPA GPCRs and PPARs [12]. The cells expressing the individual PPAR constructs were exposed to selective agonists of the respective nuclear hormone receptor (positive control) and either FMP, FDP, or AGP, and transcriptional activity was determined using normalized luciferase expression. As shown in Fig. 7, FMP at 1 μM weakly activated all three PPARs. In contrast, FDP at 1 μM activated only PPARγ and at 10 μM also activated PPARα. Both farnesyl phosphates activated PPARγ at both concentrations to an extent that was comparable to that elicited by AGP. These results provide further support to the hypothesis that farnesyl phosphates are capable of interacting with members of the PPAR family in B103 cells that lack LPA GPCRs of the EDG family. Nonetheless, the direct interaction between the farnesyl phosphates and the ligand binding domain of PPARα and PPARβ/δ will have to be addressed in pending studies so that a meaningful concentration of the farnesyl phosphates in the same cellular compartment can be established.

We also tested FMP and FDP for the inhibition of lysophospholipase D (autotaxin), which has been shown to be inhibited by LPA, S1P [21], FAP [14], and cyclic phosphatidates [22]. We followed procedures described by Baker et al. [22] but could not detect any inhibition to the isoprenyl phosphates applied up to 10 μM (data not shown).

DISCUSSION

We have shown that the endogenous lipids FR, FMP, and FDP can modulate LPA signaling, because both are potent antagonists of oleoyl-LPA at the LPA_3 receptor but cause only modest inhibition of LPA_2 and no detectable effect on LPA_1. The steady-state human plasma concentration of FDP is ~7 ng/ml (~15 nM), which is sufficiently high to modulate LPA_3 [20]. This discovery originated from our previous work that identified FAP as the minimum pharmacophore of LPA GPCRs with activity at the nuclear hormone receptor PPARγ. Thus, LPA GPCRs might be controlled not only by naturally occurring agonists but also by antagonists such as FMP and FDP. We propose that polyisoprenoid backbone-containing ligands offer a new hydrocarbon chain variant for the synthesis of LPA GPCR ligands. The two farnesyl phosphates exert a competitive mechanism of inhibition. With their strong preference for LPA_3 combined with their nanomolar IC_{50}, they might be useful tools in testing physiological and pathological responses mediated by this receptor subtype. Interestingly, the branched polyunsaturated farnesyl phosphates also inhibited LPA_2, an inhibition we have not

observed for the FAP-containing linear aliphatic chains with the single exception of a phosphonate analog with 14 carbons [14,15]. Thus, the farnesol backbone indeed provides a novel hydrocarbon sidechain in synthetic consideration of LPA_2-selective compounds. Our earlier findings underlined the importance of the bond linking the phosphate group to the hydrocarbon chain in determining the ligand properties for the LPA_2 receptor. Combined with our present finding, we now know that the phosphonate group is not the only unique motif of the pharmacophore that can target this receptor subtype.

We also included lysophospholipase D/autotaxin an important enzyme that is the source as well as the target of LPA [21][22]. However, none of the farnesyl phosphates inhibited the enzyme, indicating that this LPA target recognizes LPA, FMP, and FDP distinctly.

We have also showed here that FR, FMP, and FDP are able to activate the nuclear hormone receptor PPARγ. FMP, in particular, also activated PPARα- and PPARβ/δ-dependent transcription, which are important transcription factors regulating lipid metabolism. Though both farnesyl phosphates can bind to PPARγ with high affinity in vitro and can activate it, activation requires micromolar local concentrations. FR was much less potent than FMP or FDP in the ligand binding assays (Fig.6A) but more potent in the reporter gene assay (Fig.6B). Thus, if FR was to be the active form, it should be much less active than its phosphorylated analogs in the reporter gene assay. This is clearly not the case. Thus, it seems that FR once its taken up into the cell, it may become phosphorylated, which in turn could explain its higher potency in the reporter gene assay and add support to the bona fide activation of PPARγ by FMP and FDP.

The steady-state cellular concentration of FDP is estimated to be low, but the inhibition of the FDP metabolizing enzyme squalene synthase, which controls the main metabolic flux of FDP to sterol synthesis, can lead to substantial elevation in FDP concentration [23]. Besides *de novo* synthesis by FDP synthase, farnesyl phosphates can also be generated by the action of kinases on farnesol. Rat liver microsomal and peroxisomal fractions are able to phosphorylate free farnesol to its diphosphate ester in a CTP-dependent manner [3]. FMP is synthesized in the presence of ATP, while the phosphorylation of FMP to FDP depends on CTP [2]. Consequently, rat liver microsomes contain two enzymes for the consecutive phosphorylation of farnesol to FDP, thereby generating both FMP and FDP intracellulalry.

The peroxisome is the major site of the synthesis of FDP from mevalonate, since all of the cholestrogenic enzymes involved in this conversion are localized in the peroxisome [24]. Our data indicate that FMP, the substrate of FDP synthase, can regulate the expression of all three PPARs. Furthermore, activation of PPARα by fibrates induced FDP synthase gene expression in both hepatocytes and in mouse liver. This effect appears to be dependent on the cellular sterol level, possibly through sterol regulatory element binding protein (SREBP)-mediated transcriptional activation[25]. FDP synthase carries a 20-amino acid region that is required for the peroxisomal localization of the enzyme [26], which sets up a potential feed-forward loop between FMP/FDP and the regulation of peroxisomes. These reports in the literature combined with our present findings concerning the potential regulatory role of farnesyl phosphates of the PPARs raise the possibility that these molecules may serve as endogenous modulators of peroxisomal cholesterol synthesis. However, the experimental scrutiny of such a hypothesis is beyond the scope of the present study. Again, we are unaware of reports in the literature that can either support or rule out intracellular concentrations of FR, FMP, and FDP necessary to exert a major influence on PPAR signaling; hence, the in vivo concentrations of these farnesyl phosphates will have to be addressed in future studies. Nonetheless, we underline the importance of our competition binding results that showed a strong competition between Rosi and farnesyl phosphates in the low nanomolar range (Fig. 6A).

FDP and geranylgeranyl diphosphate (geranyl diphosphate in plants) are considered branching point intermediates of the isoprenoid pathway. FDP synthase generates FDP from dimethylallyl diphosphate via two sequential condensations with isopentenyl diphosphate. This well-characterized enzyme [1] generates “branch-point products” that include sterols, carotenoids, ubiquinone side chains, long-chain polyprenols and dolichols, and farnesylated proteins and farnesylated heme [27]. FDP can be reversibly converted to farnesol, which stimulates the differentiation of epidermal keratinocytes via PPARα [28] and has been shown to activate the farnesoid X receptor, another member of the nuclear hormone receptor superfamily [29,30] that regulates bile acid metabolism [31]. We found that FMP and FDP weakly activate PPARα transcription, but we do not know at the present time whether this is a direct effect or via farnesol produced by the dephosphorylation of FMP and FDP. Since FR was less effective in binding and activation than its phosphorylated analogs, we cannot discard the idea that FMP and FDP can act as bona fide ligands. The level of FDP is regulated by different interconnected mechanisms, with the presumed purpose of maintaining the necessary level of this important lipid that fulfills a central location in the isoprenoid pathway. For example, mevalonate depletion, a consequence of the inhibition of HMG-CoA reductase, decreases the level of FDP but also can result in the upregulation of FDP formation. Also, an interplay is possible between farnesol and FDP on fatty acid metabolism as PPARα activators induce hepatic FDP synthase gene expression in rodents [32]. Another consequence of mevalonate depletion is the upregulation of Ras protein and some related small GTPases [33-35], which are also mediated by the FDP/farnesol system. In CaCo-2 colon epithelial cells, depletion of mevalonate upregulated fatty acid synthesis, which could be prevented selectively by FDP [36].

LPA is the structurally simplest phospholipid with growth factor-like properties [4-6]. LPA targets include a set of plasma membrane receptors of the endothelial differentiation gene GPCR family [7], designated as $LPA_{1/2/3}$, GPR23 receptor [10], and GPR92 [8,9]. In addition, the transcription factor PPARγ was the first reported intracellular receptor for LPA [11]. PPARγ is an essential transcription factor in adipogenesis [37], and its activation by LPA has been implicated in neointima formation [13]. In our computationally guided rational drug discovery effort, we have identified the FAP scaffold as the minimal pharmacophore of the LPA receptors [14,15]. Now we expand this scaffold, with the inclusion of oligoprenyl phosphates, which are endogenous compounds with high similarity to polyunsaturated FAP. This inclusion raises the possibility that FMP and FDP also modulate biological targets that, up to the present time, were solely linked to LPA. Although modest, the activity of farnesyl phosphates on PPARγ might also provide a new potential physiological link between lysophospholipid and isoprenoid signaling. The recent finding that statins upregulate CD36 expression, which is a PPARγ-regulated gene [38], and our finding that it is upregulated by FMP and FDP in a PPARγ-dependent PPRE-mediated manner tend to support a potential cross-talk, since LPA up-regulates CD36 [13]. The equally exciting possibility that LPA analogs can activate farnesoid receptors remains to be elucidated in future experiments.

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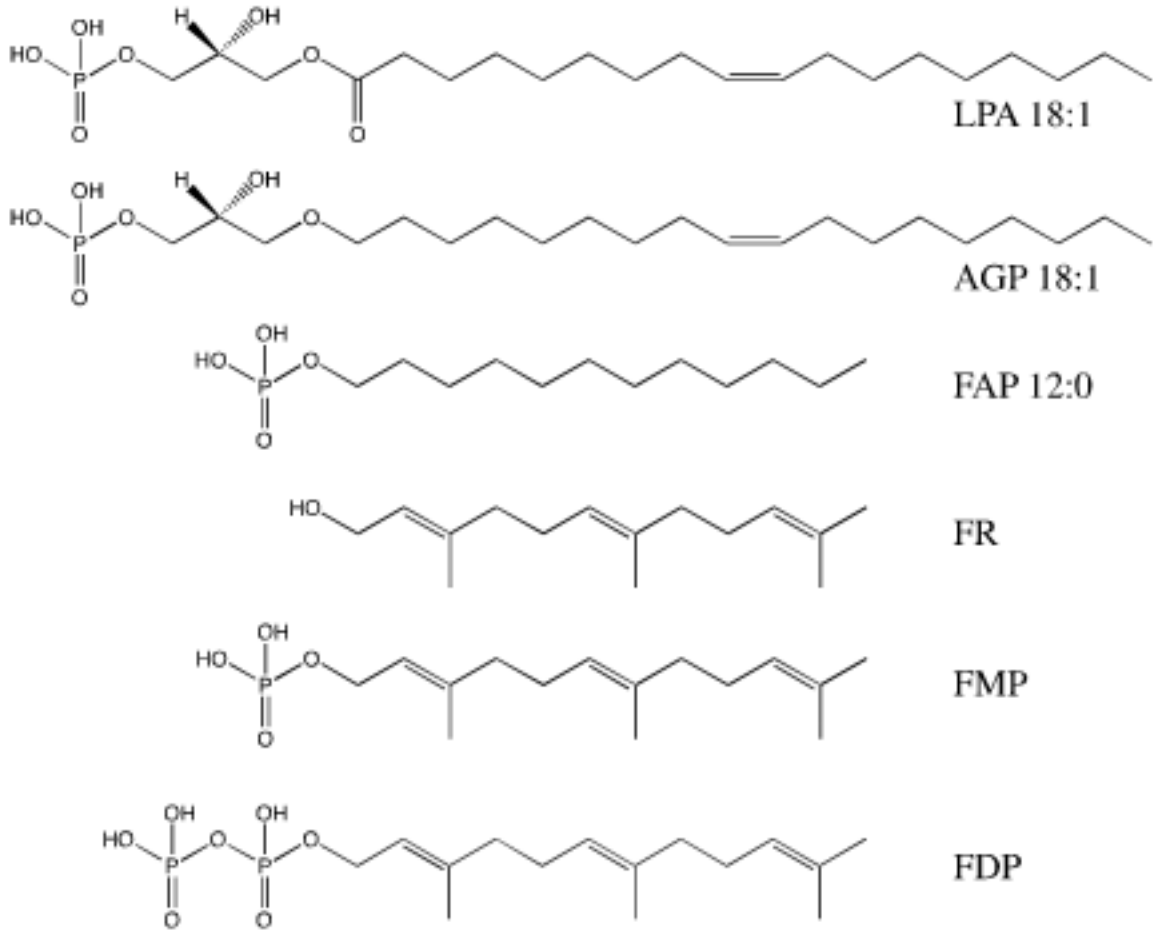

Fig. 1.
The structures of LPA, FAP, FR, FMP, and FDP.

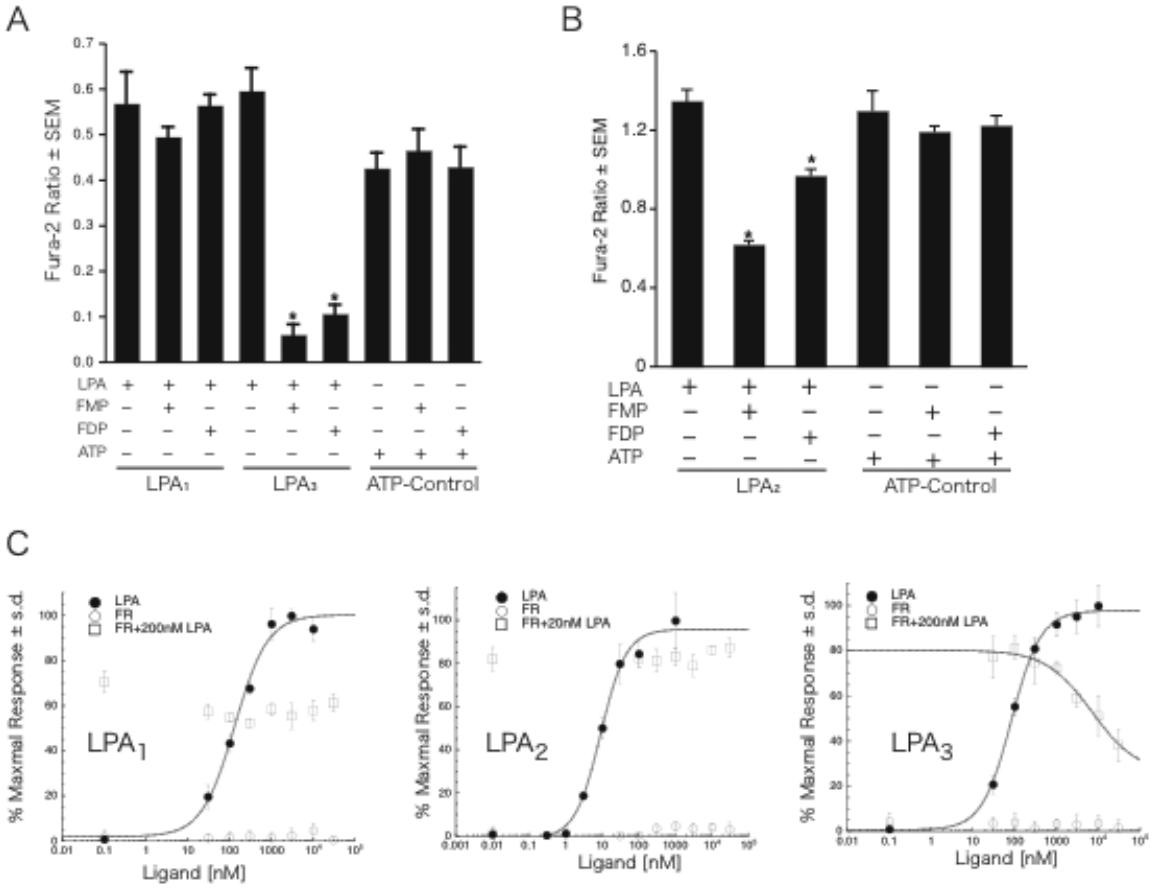

Fig. 2.

Effects of FMP and FDP on the $[Ca^{2+}]_i$ response mediated through LPA GPCRs expressed in RH7777 cells. (Panel A). RH7777 cells stably expressing either LPA_1 or LPA_3 receptors were exposed to 1 μM oleoyl-LPA in the absence or presence of 30 μM FMP or FDP. Data shown are the averages of six determinations ± standard errors. Under these conditions, the LPA_1 response was not significantly affected, whereas LPA_3 was strongly inhibited by both lipids. The control shows the $[Ca^{2+}]_i$ responses evoked by 300 μM ATP acting through the endogenous purinergic receptors. At a concentration of 30 μM, neither FMP nor FDP caused any change in the ATP-evoked response. Asterisks denote significant differences evaluated by Student's *t*-test at a P value of 0.05. (Panel B). Effect of FMP and FDP on LPA-elicited intracellular $[Ca^{2+}]_i$ response in RH7777 cells expressing LPA_2. Fura-2-loaded RH7777 cells stably expressing LPA_2 receptor were exposed to oleoyl-LPA at a concentration of 30 nM in the absence or presence of 10 μM FMP or FDP. Data shown are the averages of six determinations ± standard errors. Under these conditions, the response was significantly inhibited but not abolished by both lipids. The 10 μM ATP-elicited $[Ca^{2+}]_i$ response again was unaffected by either lipid. Asterisks denote significant differences evaluated by Student's *t*-test at a P value of 0.05. (Panel C). Effects of FR on RH7777 cells expressing the three LPA GPCRs. Data points are the mean of three individual determinations ± SD. Note that only LPA_3 was affected by concentrations > 1μM of FR and the inhibition was not complete even at 10 μM.

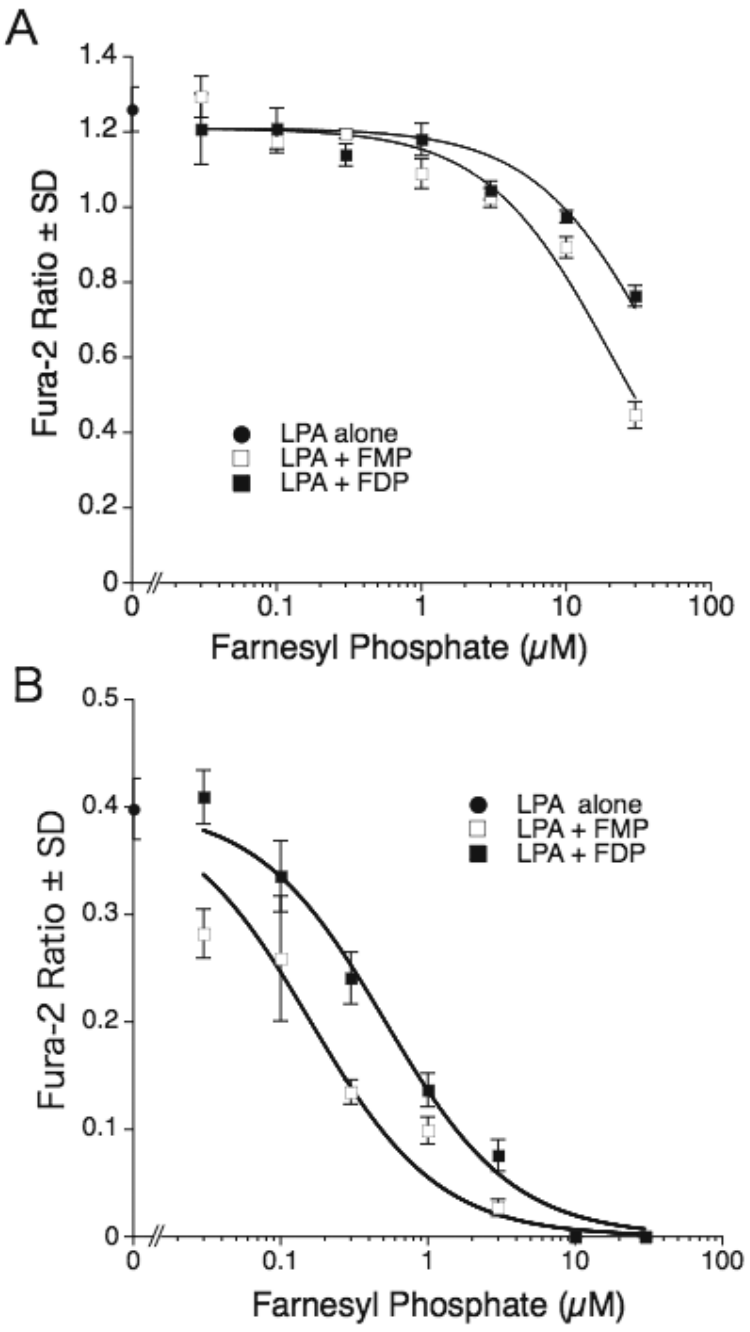

Fig. 3.

Dose-dependent inhibition of the LPA_2 and LPA_3 responses by FMP or FDP in RH7777 cells. (Panel A). RH7777 cells stably expressing LPA_2 were exposed to 50 nM oleoyl-LPA alone (filled circle) or 50 nM oleoyl-LPA mixed with increasing concentrations of either FMP (empty squares) or FDP (filled squares). Data points represent the averages of six determinations ± standard errors and were fitted to a displacement curve. The estimated apparent IC_{50} values were 21 ± 3 μM and 46 ± 6 μM for FMP and FDP, respectively. (Panel B). RH7777 cells stably expressing LPA_3 were exposed to 300 nM oleoyl-LPA alone (filled circle) or 300 nM oleoyl-LPA mixed with increasing concentrations of either FMP (empty squares) or FDP (filled squares). Data points representing the averages of six determinations

± standard errors were fitted to a displacement curve. The estimated IC_{50} values were 161 ± 31 nM and 517 ± 60 nM for FMP and FDP, respectively.

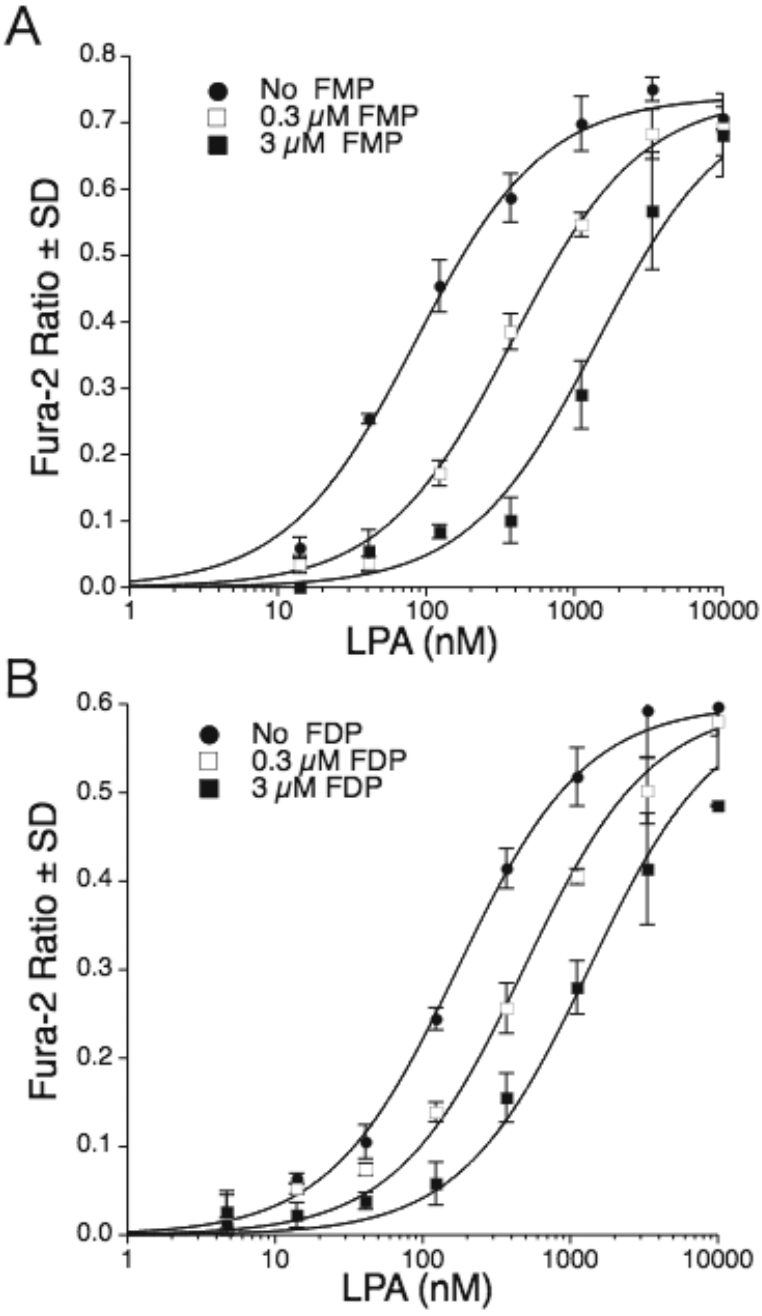

Fig. 4.

Characterization of the mechanism of inhibition of FMP and FDP on LPA_3. Oleoyl-LPA was exposed to RH7777 cells stably expressing LPA_3 in the absence or presence of farnesyl phosphates. Data points represent the averages of four determinations ± standard errors and were fitted to a hyperbole by nonlinear curve fitting. (Panel A). Filled circles, LPA alone; empty squares, LPA plus 0.3 μM FMP; and filled squares, LPA plus 3 μM FMP. The estimated EC_{50} values were 85 ± 8 nM, 378 ± 28 nM, and 1430 ± 200 nM for LPA alone, LPA plus 0.3 μM FMP, and LPA plus 3 μM FMP, respectively. (Panel B). Filled circles, LPA alone; empty squares, LPA plus 0.3 μM FDP; and filled squares, LPA plus 3 μM FDP. The estimated

EC_{50} values were 170 ± 9 nM, 480 ± 46 nM, and 1330 ± 140 nM for LPA alone, LPA plus 0.3 μM FDP, and LPA plus 3 μM FDP, respectively.

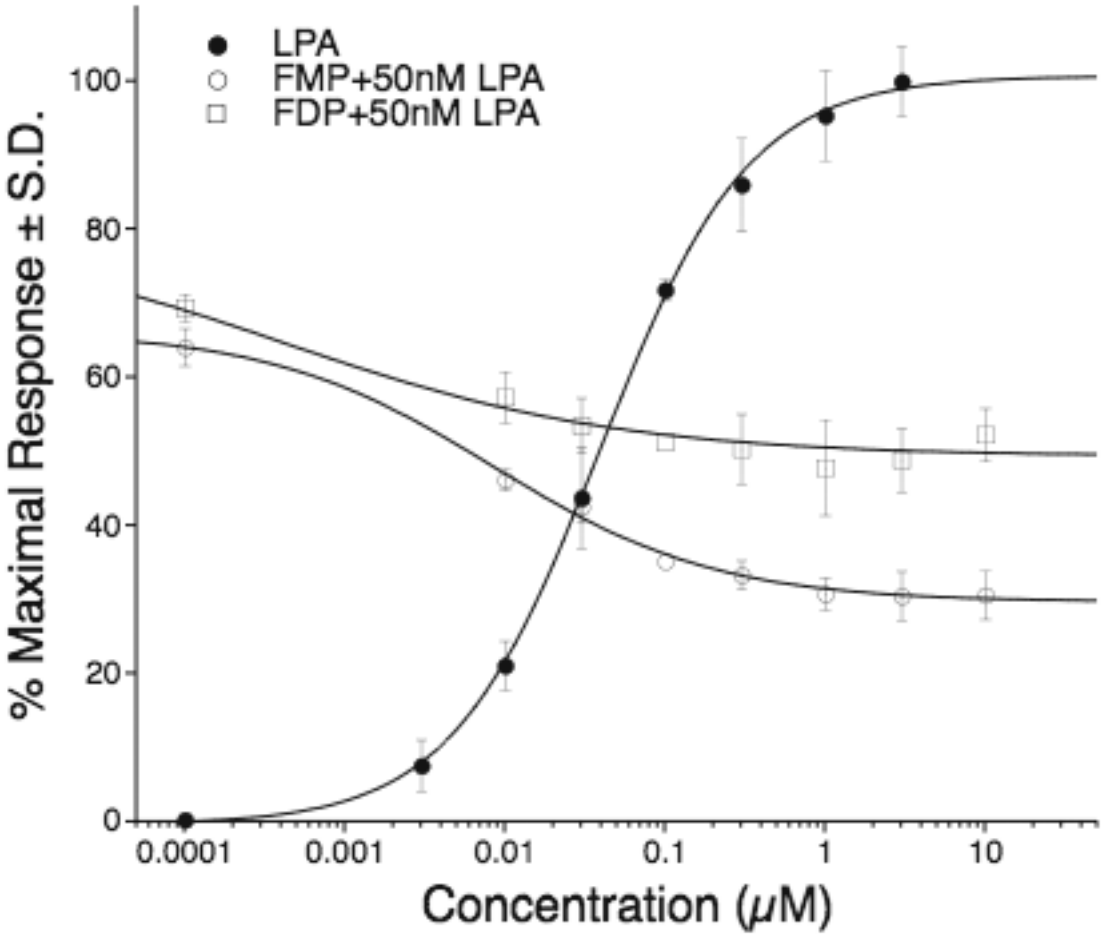

Fig. 5.

FMP and FDP inhibit the activation of endogenous LPA responses in PC3 cells. PC3 cells were loaded with Fura-2AM and exposed to increasing concentrations of oleoyl-LPA alone or FMP or FDP added to 50 nM LPA. Note that both farnesyl phosphates caused a dose-dependent but partial inhibition of the LPA-induced Ca^{2+} mobilization, which is consistent with the expression of multiple LPA GPCRs in these cells. Data points represent the mean ± SD of quadruplicates.

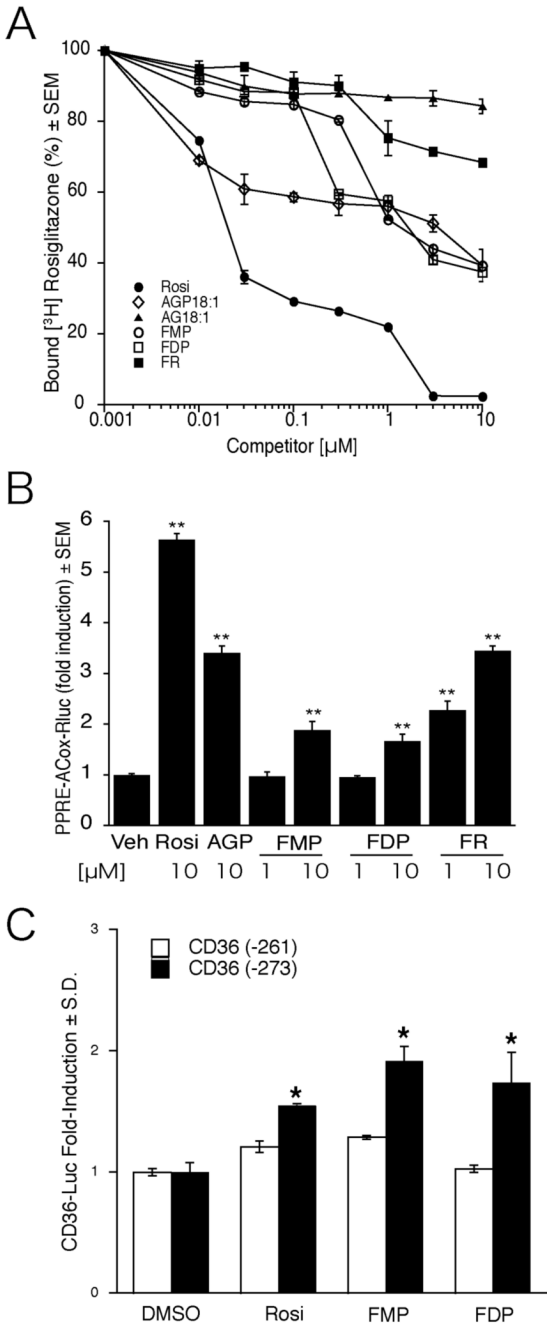

Fig. 6.

Effect of FR and farnesyl phosphates on PPARγ. (Panel A). Displacement of 5 nM [^{3}H] Rosi from the PPARγ ligand binding domain by FMP or FDP. Data shown are the averages of four determinations ± standard error. Displacement curves were fitted to the data points by using nonlinear curve fitting and yielded apparent IC_{50} values of 19 ± 7 μM and 19 ± 2 μM for FMP and FDP, respectively. FR showed only a 25% displacement at the highest concentration tested. (Panel B). The activation of PPARγ was monitored in CV-1 cells cotransfected with the PPARγ plus PPRE-ACox-luc reporter gene. Data shown are the averages of four determinations ± standard error. FMP and FDP at a concentration of 10 μM elicited a significant increase in the reporter gene transcription. However, Rosi but not FR, FMP, or FDP

significantly activated the reporter gene at a concentration of 1 μM. Asterisks denote significant differences over vehicle evaluated by Student's *t*-test at a P value of 0.01. (Panel C). FMP and FDP activate CD36 in a PPRE-dependent manner. CV-1 cells were transfected with a CD36 promoter-luciferase reporter that contained its PPRE (CD36 -273) or one that did not (CD36 -261). All cells were cotransfected with SV40-β-galactosidase and PPARγ. The cells were treated for 20 h with 10 μM Rosi, FMP, and FDP before the ratio of luciferase to β-galactosidase activity was determined and normalized to vehicle-treated cells. Asterisks denote significant activation at $P < 0.05$.

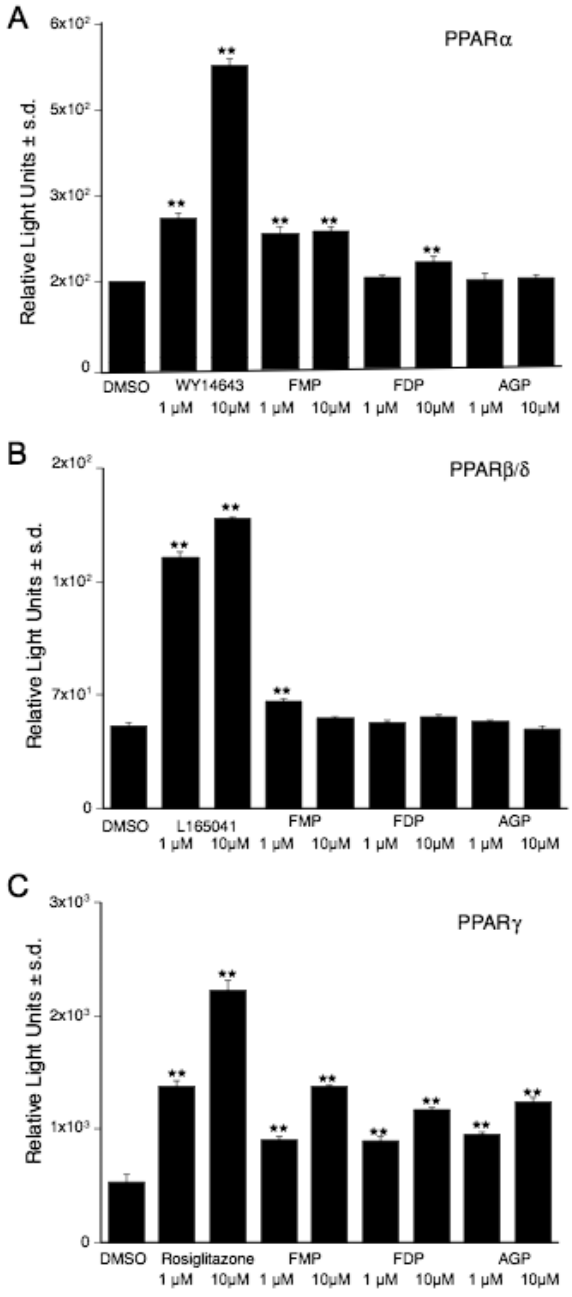

Fig. 7.

FMP and FDP activate PPARs expressed in B103 cells, which lack LPA GPCRs. LPA GPCRs lacking B103 cells were transfected with either PPARα, PPARβ/δ, or PPARγ and the appropriate PPRE-luciferase reporter gene. The cells were incubated with vehicle (DMSO), WY14643, L165041, Rosiglitazone (positive controls for PPARα, β/δ, and γ, respectively), or FMP, FDP, and AGP18:1 at 1 μM or 10 μM (n=5). All values are expressed as mean ± SEM. *, P<0.05 and **, p<0.01, significant differences over vehicle control using Student's *t*-test.