THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 44, Issue of October 31, pp. 43214–43223, 2003

Printed in U.S.A.

Lipid Phosphate Phosphatases Regulate Lysophosphatidic Acid Production and Signaling in Platelets

STUDIES USING CHEMICAL INHIBITORS OF LIPID PHOSPHATE PHOSPHATASE ACTIVITY*

Received for publication, June 24, 2003, and in revised form, August 7, 2003
Published, JBC Papers in Press, August 8, 2003, DOI 10.1074/jbc.M306709200

Susan S. Smyth‡§, Vicki A. Sciorra¶, Yury J. Sigal‖, Zehra Pamuklar‡, Zuncai Wang‖, Yong Xu, Glenn D. Prestwich**, and Andrew J. Morris‖‡‡**

*From the ¶Howard Hughes Medical Institute, University of California, San Diego, La Jolla, California 92093-0668, the **Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah 84108, and the Departments of ‡Medicine, Division of Cardiology and ‖Cell and Developmental Biology, University of North Carolina, Chapel Hill, North Carolina 27599-7090*

Blood platelets play an essential role in ischemic heart disease and stroke contributing to acute thrombotic events by release of potent inflammatory agents within the vasculature. Lysophosphatidic acid (LPA) is a bioactive lipid mediator produced by platelets and found in the blood and atherosclerotic plaques. LPA receptors on platelets, leukocytes, endothelial cells, and smooth muscle cells regulate growth, differentiation, survival, motility, and contractile activity. Definition of the opposing pathways of synthesis and degradation that control extracellular LPA levels is critical to understanding how LPA bioactivity is regulated. We show that intact platelets and platelet membranes actively dephosphorylate LPA and identify the major enzyme responsible as lipid phosphate phosphatase 1 (LPP1). Localization of LPP1 to the platelet surface is increased by exposure to LPA. A novel receptor-inactive *sn*-3-substituted difluoromethylenephosphonate analog of phosphatidic acid that is a potent competitive inhibitor of LPP1 activity potentiates platelet aggregation and shape change responses to LPA and amplifies LPA production by agonist-stimulated platelets. Our results identify LPP1 as a pivotal regulator of LPA signaling in the cardiovascular system. These findings are consistent with genetic and cell biological evidence implicating LPPs as negative regulators of lysophospholipid signaling and suggest that the mechanisms involve both attenuation of lysophospholipid actions at cell surface receptors and opposition of lysophospholipid production.

Lysophosphatidic acid (1-acylglycerol-3-phosphate, LPA)[1] is the prototypic member of a class of receptor-active lipid phosphoric acid mediators that function as intercellular signaling molecules. LPA and the structurally related compound sphingosine 1-phosphate (S1P) are present in biological fluids at physiologically relevant concentrations and exert many of their effects on target cells by binding to specific heterotrimeric GTP-binding protein (G-protein)-coupled cell surface receptors. Responses to stimulation with LPA and S1P include growth, differentiation, chemotaxis, smooth muscle contraction, and changes in cell morphology and motility through alterations in organization of the actin cytoskeleton (1–3).

Blood plasma contains physiologically relevant levels of LPA (4). Because of the multiple actions of LPA on cells of the vasculature, a growing body of evidence suggests that LPA plays an important role in blood clotting, wound healing, and tissue regeneration (3). The mitogenic and chemotactic actions of LPA may also participate in the recruitment of monocytes and macrophages and aberrant stimulation of endothelial and vascular muscle cell growth that underlie intimal hyperplasia, formation of an atherosclerotic plaque, and progression of atherosclerosis (5). Elevated plasma levels of LPA have also been associated with ovarian and certain other gynecological cancers, where LPA may participate in autocrine or paracrine stimulation of tumor cell growth (6).

LPA is a major growth factor in serum where it is produced by pathways that are initiated by platelet activation. Platelets also appear to play a central role in the production of LPA in the blood (7, 8). One pathway for LPA synthesis involves the actions of a secretory phospholipase A_2 on phosphatidic acid (PA)-enriched membrane microparticles that are released from activated platelets (9). Another pathway involves formation of lysophosphatidylcholine by lecithin cholesterol acyl transferase or release of this lipid by platelets and other blood cells, which is then converted to LPA by the lysophosphatidylcholine-selective lysophospholipase D activity of autotaxin (10–12).

Platelets are highly responsive to LPA. Exposure of resting platelets to LPA elicits rapid shape change, actin reorganization, aggregation, and fibronectin matrix assembly (3, 5, 13). Enzymatic dephosphorylation terminates the signaling actions of LPA (14). The major activities responsible are lipid phosphate phosphatases (LPPs), which are integral membrane enzymes with specificity for LPA, S1P, and related lipid monophosphates (15–18). LPPs localize to the plasma membrane and to intracellular membrane compartments. When in the plasma membrane, the active sites of these enzymes are oriented toward the extracellular space (18–20). Genetic and cell biological evidence identifies roles for LPPs in both intracellular lipid metabolism and as negative regulators of lysophospholipid signaling, although the mechanisms involved remain to be firmly established (19, 21–25).

Here we show that a specific LPP isoform, LPP1, is strongly

* This work was supported in part by National Institutes of Health Grants GM 50388 and CA 12451 (to A. J. M.), HL070304 and DK064183 (to S. S. S.), and NS029632 (to G. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A Scholar of the American Society of Hematology.
‡‡ To whom correspondence should be addressed. Tel.: 919-843-5001; Fax: 919-966-1856; E-mail: ajmorris@med.unc.edu.

[1] The abbreviations used are: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; PA, phosphatidic acid; LPP, lipid phosphate phosphatase; TRAP, thrombin receptor-activating peptide; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; RT, room temperature.

expressed in platelets and that its localization and activity are regulated by platelet agonists. Newly synthesized chemical inhibitors identify LPP1 as a global regulator of LPA production and signaling in platelets. The dual function of LPP1 as a regulator of both LPA production and responsiveness may explain the complex and pleiotropic phenotypes associated with overexpression, misexpression, or down-regulation of this class of enzymes in many systems (19, 21, 25–28).

EXPERIMENTAL PROCEDURES

General Procedures and Reagents—Unless otherwise stated, reagents used in this study were from previously identified sources. SDS-PAGE and Western blotting were performed as described previously (18, 20, 29).

Expression and Purification of LPP Enzymes—LPP1, LPP2, LPP3, and His6-tagged LPP1 were expressed in insect cells using baculovirus vectors, and membranes were prepared from these cells and used as sources of recombinant LPP proteins for Western blots and activity assays as described previously (18, 20, 29).

Preparation of Substrates—Unlabeled PA and LPA with acyl chain configurations as specified were from Avanti Polar Lipids (Alabaster, AL). Radiolabeled lipid substrates were prepared as described previously (18). 1-Oleoyl[oleoyl-9,10-^{3}H(N)]lysophosphatidic acid was obtained from PerkinElmer Life Sciences, Boston, MA.

Lipid Phosphatase Assays—Dephosphorylation of radiolabeled or unlabeled lipid substrates presented in detergent micelles with Triton X-100 or complexed with fatty acid-free BSA as described previously (18, 20).

Synthetic LPA and PA Analogs—The synthesis of the compounds used in this study has been described in detail elsewhere (30–32).

Isolation of Human Platelets and Microvesicles—Blood was collected from healthy aspirin-free donors, and platelet-rich plasma was prepared by centrifugation. For most experiments, platelets were separated from citrated plasma by gel-filtration through Sepharose 2B in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 10 mM HEPES, 0.35% BSA, 0.1% glucose, pH 7.4). Aggregation of gel-filtered platelets ($1–3 \times 10^8$/ml) was performed as previously described (33). For isolation of microvesicles, platelets were resuspended in Tyrode's buffer containing 2 mM $CaCl_2$ and incubated with 19 μM A23187 and 100 nM phorbol 12-myristate 13-acetate at 37 °C for 30 min. Intact platelets were sedimented at $2000 \times g$ for 20 min at room temperature, and microvesicles were recovered by centrifugation of the supernatant at $100,000 \times g$ for 20 min.

Antibodies—A peptide corresponding to residues 254–267 (FFKERTSFKERKE) of the sequence of human LPP1 was synthesized and used as a conjugated antigen for production of an affinity-purified rabbit antibody using the procedure previously described for the LPP3 antibody (20).

Surface-labeling Platelets with Biotin—Washed platelets were suspended in Tyrode's buffer at 5×10^8/ml and then mixed with an equal volume of Sulfo-*N*-Hydroxysuccimido-biotin (1 mg/ml) in Tyrode's buffer. The mixture was incubated at room temperature with constant rocking for 30 min. Platelets were washed twice with Tyrode's buffer containing 1 mM glycine and then suspended in buffer A and 2% Triton X-100 and incubated at 4 °C for 1 h with constant rocking. The material was centrifuged at $25,000 \times g$ at 4 °C for 30 min. The supernatant was mixed with ~8 units of avidin-Sepharose and rocked at 4 °C for 1 h. The resin was sedimented by gentle centrifugation and washed six times by resuspension in ~100 volumes of ice-cold lysis buffer containing 2% Triton X-100. The avidin-Sepharose was resuspended in lysis buffer for determination of LPA phosphatase activity or boiled in SDS-PAGE sample buffer for analysis of proteins by Western blotting.

Sucrose Density Gradient Fractionation—Outdated platelets were washed and disrupted by nitrogen cavitation as previously described (33). A stepwise sucrose gradient (from 0.8 to 2.0 M sucrose in increments of 0.2 M) was used to subfractionate the platelet lysate. The platelet lysate was layered on top of the gradient and centrifuged at $100,000 \times g$ and 4 °C for 60 min in a SW-41 rotor (Beckman Instruments; Palo Alto, CA). A volume containing 30 μg of the indicated fractions was separated on a 10% SDS-PAGE. The relative concentrations of the well-characterized platelet membrane proteins GPIIIa, α-granule P-selectin, and LPP1 were determined by immunoblotting and scanning densitometry.

Enzymatic Deglycosylation—Proteins were subjected to enzymatic deglycosylation using previously described procedures or modifications of procedures provided by the manufacturer (20). The enzymes used were obtained from Calbiochem, San Diego, CA.

Fluorescence Microscopy—Fibrinogen (100 μg/ml) was immobilized on Lab-Tek chamber slides (Nunc, Inc., Rochester, NY) overnight at 4 °C, and nonspecific sites were blocked with BSA. Gel-filtered platelets (25,000–50,000/ml in Tyrode's buffer containing 1 mM $MgCl_2$) were incubated with the fibrinogen-coated slides in the absence or presence of 10 μM ADP or 1 μM LPA for 30 min at room temperature (~22 °C). The wells were washed by inversion, and the adherent platelets were stained for 30 min at RT with either monoclonal antibody 10E5 against $\alpha_{IIb}\beta_3$ or monoclonal antibody 6F1 against $\alpha_2\beta_1$, or control antibody (34). Platelets were fixed with 4% paraformaldehyde in PBS for 20 min at RT and permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. After washing, the fixed, permeabilized platelets were incubated in the dark with TRITC-phalloidin (0.025 μg/ml in PBS, Sigma Chemical Co.) antibodies specific for LPP1 or LPP3 and the appropriate fluorescein isothiocyanate-labeled secondary antibody. Slides were mounted with Prolong AntiFade (Molecular Probes) and examined with a Nikon Eclipse 800 microscope. Digital images were captured and scored by a blinded reviewer for the number of adherent platelets/high power field and the percentage of spread platelets/high power field. Spread platelets were identified by TRITC-phalloidin staining of actin fibers.

Rho Activation Assays—Platelets were incubated in 100-mm diameter dishes that had been coated with 100 μg/ml fibrinogen as described above. Rho activation was measured using a rhotekin binding assay (Upstate Biotechnology Inc.) following the manufacturer's instructions. Total and active Rho were detected by SDS-PAGE and Western blotting of total lysate and rhotekin-bound proteins, respectively, and quantitated by scanning densitometry.

Measurement of LPA Production by Platelets—LPA production by washed platelets was determined using minor modifications of a recently published procedure (10). In brief, gel-filtered platelets were washed and then resuspended in phosphate-free Tyrode's buffer containing 2 mCi of $[^{32}P]PO_4^{2-}$ and 1 μM prostaglandin E1 at 2×10^9 platelets/ml. The platelet suspension was incubated for 3 h at 37 °C in a shaking water bath. Platelets were sedimented by gentle centrifugation, the labeling medium was removed, and the platelets were washed twice in Tyrode's buffer containing 1 μM prostaglandin E1 before resuspension in this buffer to a final density of 2×10^8 platelets/ml. 0.5-ml incubations were initiated by combining 0.25 ml of platelet suspension with 0.25 ml of Tyrode's buffer supplemented with 2 mM $CaCl_2$ and containing combinations of 10 μM TRAP (thrombin receptor-activating peptide SFLLRN) with 10 μM XY-14. 37 °C incubations in a gently shaking water bath were terminated by microcentrifugation. Lipids were extracted from the supernatant and separated by thin layer chromatography as described (10). $[^{32}P]$LPA was identified by co-migration with a radiolabeled standard run in parallel and quantitated using a STORM PhosphorImager system.

Statistical Analysis—Results are expressed as the mean ± S.D. unless otherwise indicated and, where appropriate, analyzed by Student *t* test.

RESULTS

Characterization of LPP Activities in Intact Platelets and Platelet-derived Fractions—When gel-filtered human platelets were incubated with 10 μM $[^{32}P]$oleyl-LPA in a final concentration of 0.1% fatty acid-free BSA, we observed rapid time-dependent release of ammonium molybdate-extractable $[^{32}P]PO_4^{2-}$, which occurred without significant uptake of the substrate by the platelets. When this experiment was repeated using a mixture of $[^{32}P]$oleyl-LPA and oleyl-LPA that was labeled with ^{3}H in the acyl chain, we found that greater than 95% LPA was converted to a stoichiometric mixture of monoacylglycerol and PO_4^{2-} and that the kinetics of accumulation of these products were identical. Taken together, these results show that intact platelets contain a surface phosphatase that actively dephosphorylates LPA to generate the receptor-inactive molecule monoacylglycerol. To probe the substrate specificity of this phosphatase activity, we examined dephosphorylation of LPA, PA, and S1P by intact platelets, platelets that had been disrupted by brief probe sonication, and platelet-derived membrane microvesicles. Intact platelets dephosphorylated all three substrates, and the rate of dephosphorylation of LPA was approximately four times greater than that ob-

TABLE I

Properties of LPP activities in intact platelets and platelet fractions

Freshly isolated gel-filtered platelets prepared in the presence of PGI2 were incubated at 37 °C in Tyrode's solution containing 1 μM [^{32}P]oleyl-LPA. Dephosphorylation was quantitated by measuring formation of [^{32}P] PO_4^{2-}. In one series of assays, the platelets were disrupted by brief probe sonication before addition to the assays. In another series, platelets were stimulated with phorbol ester and Ca^{2+} ionophore and sedimented by centrifugation. LPA phosphatase activity was determined in membrane microvesicles released by the activated platelets, which were recovered from the supernatant. These are representative data from one series of experiments using platelets from the same donor. We did not observe significant differences in lipid phosphatase activities in platelet samples isolated from different donors.

	Phosphatase activity (mean ± S.D., n = 3)		
	LPA	PA	S1P
	pmol/min/10^9 platelets		
Intact platelets	4.62 ± 0.16	1.20 ± 0.03	0.76 ± 0.13
Sonicated platelets	8.66 ± 0.17	1.21 ± 0.05	3.78 ± 0.14
Microvesicles	1.71 ± 0.13	0.15 ± 0.03	0.10 ± 0.01

TABLE II

Biochemical characterization of LPP activities in platelet fractions

Washed platelets were disrupted by sonication and separated into cytosolic and membrane fractions by differential centrifugation. Phosphatase activities were determined using radiolabeled substrates presented in mixed micelles with Triton X-100 as described in the text. The final Triton X-100 concentration was 3.2 mM, and the final substrate concentration was 100 μM. Assays contained 5 mM EDTA or 5 mM $MgCl_2$. In some cases samples were preincubated with 5 mM NEM before addition to the assays. The data shown were obtained using fractions isolated from platelets obtained from the blood bank and are representative of three separate experiments.

		Phosphatase activity (mean ± S.D., n = 3)		
	Substrate	$-Mg^{2+}$	$+Mg^{2+}$	+NEM
		pmol/mln/10^6 platelets		
Cytosol	PA	0.02 ± 0.01	0.14 ± 0.02	0.02 ± 0.01
	LPA	0.21 ± 0.2	1.80 ± 0.3	0.19 ± 0.03
	S1P	ND	ND	ND
Membranes	PA	8.9 ± 1.2	9.0 ± 1.3	7.6 ± 2.1
	LPA	14.1 ± 0.36	13.1 ± 2.0	12.9 ± 1.5
	S1P	2.8 ± 0.2	2.7 ± 0.3	2.3 ± 0.3

served with PA and six times greater than observed with S1P. When platelets were disrupted by sonication, the rate of dephosphorylation of LPA was approximately doubled suggesting that most of the activity responsible was at the platelet surface. The microvesicle preparations contained ~20% of the LPA phosphatase activity of the platelets from which they were derived (Table I). Using mixed substrate and Triton X-100 micelles, we found that LPA, PA, and S1P phosphatase activities were predominantly membrane-associated in platelets, were stimulated by Triton X-100, were independent of Mg^{2+}, and were insensitive to inhibition by *N*-ethylmaleimide (NEM), all of which are characteristic properties of the LPP enzymes (14) (Table II).

Immunological Analysis of LPPs in Platelets—We previously reported a selective antibody for LPP3 (20). We raised an antibody against LPP1 by immunization of rabbits with a peptide corresponding to a unique internal sequence (Fig. 1*A*). This antibody selectively recognizes LPP1 expressed in sf9 cells using a baculovirus vector in Western blots (Fig. 1*B*) and efficiently immunoprecipitates LPP1 but not LPP2 or LPP3 activity from detergent extracts of membranes from insect cells infected with these vectors (Fig. 1*C*). Detergent extracts from platelet membranes were analyzed by immunoprecipitation using the LPP1 and LPP3 antibodies. Although immunoprecipitation using an irrelevant control antibody resulted in no loss of LPP activity from the extracts, immunoprecipitation with the LPP1 antibody resulted in depletion of 87 ± 0 3.1% of

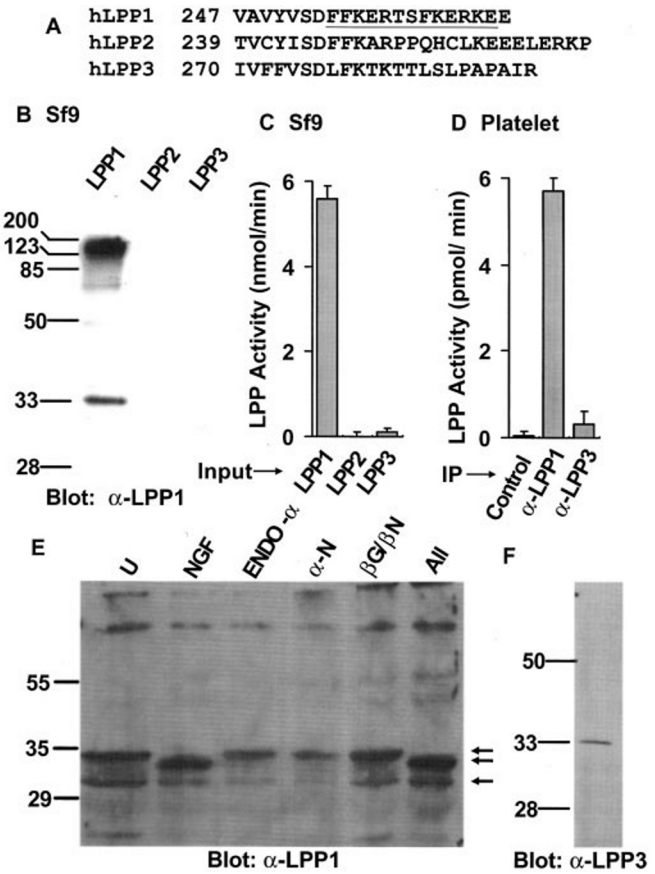

FIG. 1. **Immunological analysis of LPP expression in platelets.** *A*, the sequences of human LPP1, LPP2, and LPP3 are shown aligned. The sequence used for the synthetic peptide used to generate the LPP1-selective antibody is *underlined*. *B*, detergent extracts of membranes from sf9 cells expressing LPP1, LPP2, and LPP3 were separated by SDS-PAGE on a 12.5% gel. Immunoreactive proteins were visualized by Western blotting using the LPP1-selective antibody as described in the text. *C*, LPP1, LPP2, and LPP3 were expressed in insect cells using baculovirus vectors, and membrane proteins were extracted with detergent and subjected to immunoprecipitation using the LPP1-selective antibody. LPP activity was determined in the immune complexes as described in the text. The data shown are means ± S.D. of triplicate assays from a single experiment. *D*, platelet membrane proteins were extracted with detergent and subjected to immunoprecipitation using control, LPP1-, and LPP3-selective antibodies. LPP activity was determined in the immune complexes as described in the text. The data shown are means ± S.D. of triplicate assays from a single experiment. *E*, platelet membrane proteins were extracted with detergent and separated by SDS-PAGE on a 12.5% gel before Western blotting using an LPP1-selective antibody as described in the text. Samples were untreated (*U*) or treated with protein deglycosylation enzymes as indicated: *NGF*, endo-α-*N*-acetylgalactosaminidase; *ENDO*-α, α2–3,6,8,9-neuraminidase; α-*N*, β*G*/β*N*, β1,4-galactosidase, or β-*N*-acetylglucosaminidase, or all these enzymes in combination. *F*, platelet membrane proteins were extracted, separated by SDS-PAGE, and analyzed by Western blotting with an LPP3-selective antibody.

LPP activity. By contrast, immunoprecipitation with the LPP3 antibody depleted only 4.5 ± 0.7% of the activity (means ± S.D., n = 3). In agreement with these findings, LPA phosphatase activity in the immune complexes from the LPP1 immunoprecipitation was ~16-fold higher than that recovered after immunoprecipitation with the LPP3 antibody (Fig. 1*D*). Western blot analysis of detergent extracts of platelet membranes using the LPP1 antibody described above detected a strongly reacting protein species with a molecular mass of ~34 kDa and a less abundant species with a molecular mass of ~31 kDa. LPPs are extremely hydrophobic and it is likely that the minor immunoreactive species with higher molecular weight represent aggregated forms of the proteins. Human LPP1 has a

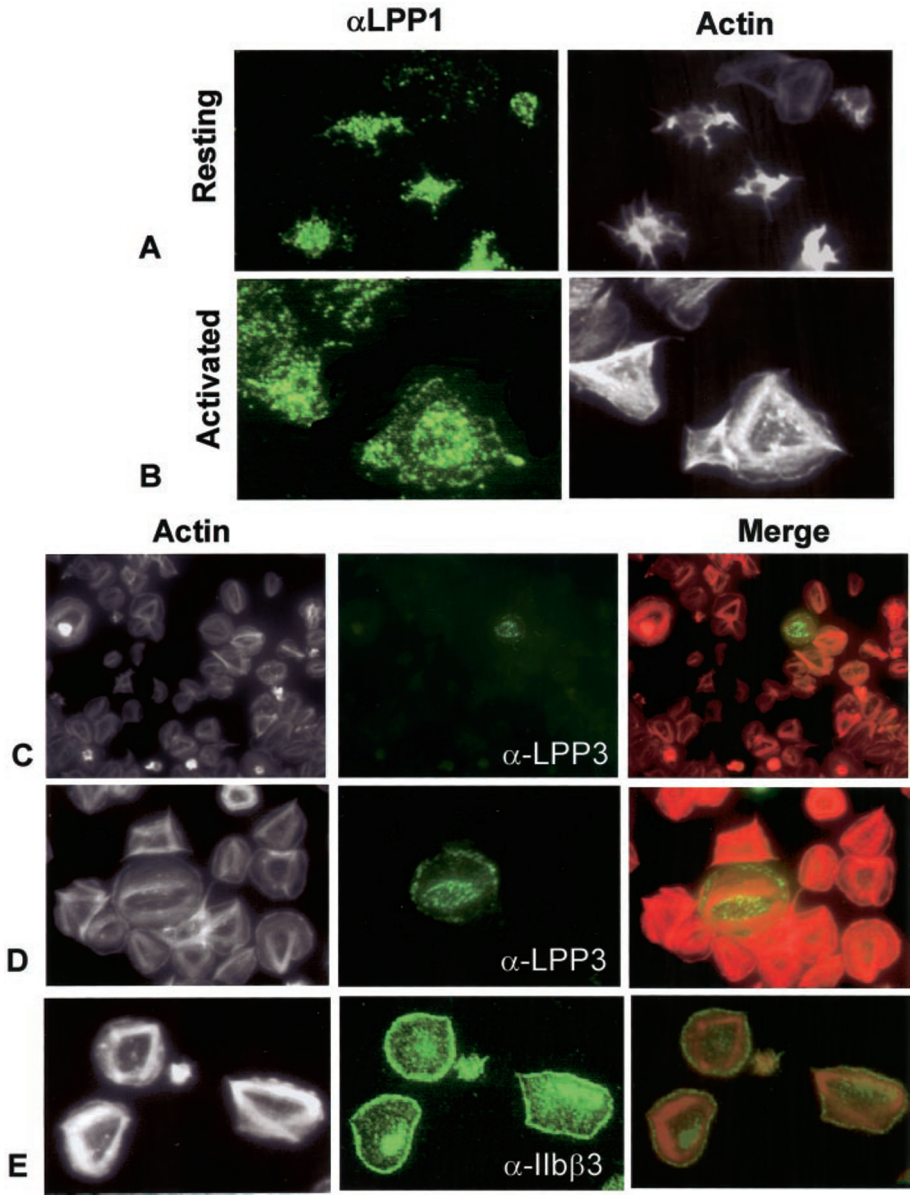

FIG. 2. **Localization of LPP1 and LPP3 in resting and LPA-stimulated platelets.** Platelets were incubated with fibrinogen-coated slides under resting conditions (*A*) or with stimulation by 1 μM LPA (*B*). The platelets were fixed and stained with antibodies against LPP1. Immunoreactive proteins were visualized using a fluorescein isothiocyanate-conjugated secondary antibody (*right panel*), and actin organization was simultaneously analyzed by staining with Rhodamine-conjugated phalloidin (*Left panel*). *C–E*, platelets were incubated with fibrinogen-coated slides in the presence of 1 μM LPA. Platelets were fixed and stained with antibodies against LPP3 or $\alpha_{IIb}\beta_3$ as indicated. Actin organization was simultaneously visualized by staining with rhodamine-conjugated phalloidin.

predicted molecular mass of 31.9 kDa. All LPP isoforms contain a single functional consensus glycosylation sequence in the loop between transmembrane regions three and four. Glycosylated LPPs have been shown to be sensitive to treatment with *N*-glycanase and sialidase (14, 20). We found that treatment of samples with *N*-gycosidase F increased electrophoretic mobility of the ~34-kDa species, but no increase in mobility was observed when samples were treated with endo-α-*N*-acetylgalactosaminidase, α2–3,6,8,9-neuraminidase, β1,4-galactosidase, or β-*N*-acetylglucosaminidase. These results suggest that platelet LPP1 is modified by *N*-linked glycosylation. Mobility of the less abundant immunoreactive species was unaltered by these treatments, so this may represent a nonglycosylated form of the protein (Fig. 1*E*). We detected a less abundant ~35-kDa immunoreactive protein when platelet membrane extracts were probed using an LPP3-specific antibody. Taken together, these results suggest that LPP1 accounts for the major LPP activity in human platelets and platelets also express LPP3 at a much lower level (Fig. 1*F*).

Distinct Localization Patterns of LPP1 and LPP3 in Resting and LPA-stimulated Adherent Platelets—We used indirect immunofluorescence to examine the distribution of LPP1 in fixed platelets adherent to fibrinogen. These experiments revealed that in unstimulated platelets LPP1 localized to the platelet surface and possibly also intracellular membrane structures. Stimulation of platelets with LPA resulted in dramatic platelet spreading, which was accompanied by concomitant expansion of LPP1 distribution across the platelet surface (Fig. 2, *A* and *B*). LPP1 was not distributed evenly in resting or stimulated platelets but displayed a punctate localization pattern. LPP1 localization was distinct from that of two other platelet surface proteins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ (not shown, but see Fig. 2, *C–E*). Similar changes in LPP distribution were observed when platelets were stimulated with the thrombin receptor-activating peptide TRAP or with ADP (not shown). Unexpectedly, staining platelets with the LPP3 antibody revealed that the apparently low levels of expression of this LPP isoform observed by Western blotting arise not from uniformly low expression in the entire platelet population but because LPP3 is strongly expressed in ~5% of the platelets (Fig. 2, *C* and *D*). In LPA-activated platelets, LPP3 localized both to the center of the platelet and to the platelet periphery. Interestingly, the localization pattern of LPP3 was very similar to that of $\alpha_{IIb}\beta_3$ (Fig. 2*E*). LPP3 has recently been noted to contain an RGD sequence that interacts with β_3 integrins and was therefore postulated to function in cell-cell interactions (26). Our results are consistent

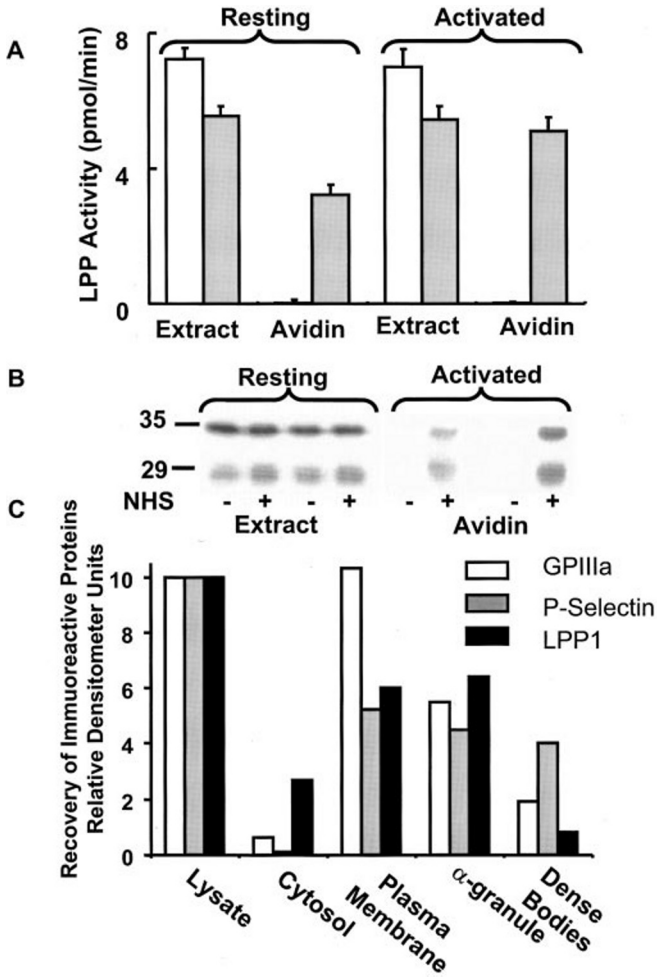

FIG. 3. **Capture of LPP activity and LPP1 protein after surface biotinylation of resting and LPA-stimulated platelets.** *A*, LPA phosphatase activities were determined in extracts and avidin-captured material from resting and LPA-activated platelets that had been incubated in the presence (*gray bars*) or absence (*white bars*) of sulfo-NHS-biotin. The data shown are means ± S.D. of triplicate determinations from a representative experiment. *B*, detergent-extracted proteins or avidin-captured proteins were obtained from resting or LPA-activated platelets that had been incubated in the presence or absence of sulfo-NHS-biotin as indicated. Proteins were separated by SDS-PAGE on a 12.5% gel and analyzed by Western blotting using an LPP1-selective antibody. *C*, platelet lysates were fractionated by sucrose density gradient centrifugation. Material from the indicated subfractions was separated by SDS-PAGE and immunoblotted for the of the well characterized platelet membrane proteins GPIIIa, the platelet α-granule membrane protein P-selectin, and LPP1. Results of scanning densitometry are expressed relative to the band density for each protein in total platelet lysate.

with an interaction between LPP3 and $\alpha_{IIb}\beta_3$ that may determine the lateral organization of LPP3 in the platelet membrane.

Recovery of LPP1 Protein and Activity after Surface Biotinylation of Resting and Stimulated Platelets—Because of their small size, application of high resolution imaging strategies to resolve internal and surface proteins in platelets is technically challenging. To directly evaluate localization of LPP1 to the platelet surface, we examined susceptibility of the protein to labeling with a cell impermeant biotin derivative. Although biotinylation resulted in a modest (~25%) decrease in LPP activity measured in detergent extracts from either resting or LPA-activated platelets, exposure to this reagent did not measurably effect the ability of our LPP1 antibody to detect the protein in Western blots (Fig. 3, *A* and *B*). 58% of the extracted LPP activity could be captured by avidin-Sepharose after surface biotinylation of resting platelets, and this value was increased to 93% in platelets that were stimulated with LPA. In agreement with these activity measurements, in comparison to resting platelets we saw a proportionate increase in the amount of immunoreactive LPP1 that was captured from LPA-stimulated platelets (Fig. 3, *A* and *B*). Taken together with our observations that intact platelets dephosphorylate LPA, these results indicate that LPP1 is present at the surface of resting platelets and suggest that the redistribution of LPP1 across the surface of activated platelets observed in the immunofluorescence studies is accompanied by an increase in surface localization of the protein. Fractionation of platelet membranes by sucrose density gradient centrifugation revealed that the majority of LPP1 was in plasma membrane and α-granule fractions (Fig. 3*C*). The presence of LPP1 in α-granules could explain the re-distribution of LPP1 after agonist stimulation of platelets, because these granules fuse with the plasma membrane following platelet activation.

Identification of Competitive Inhibitors of LPP Activity—We recently observed that a phosphothionate analog of LPA that has, by virtue of its resistance to LPP activity, been employed as a metabolically stable LPA receptor agonist and was an effective LPP inhibitor (6). This finding prompted us to search for compounds that inhibited LPA dephosphorylation without directly stimulating LPA signaling because of their obvious potential as tools to probe LPP function. We therefore explored activities of a series of recently synthesized fluorinated analogs of LPA and PA on LPP activity (30–32). Structures of four of these analogs termed XY-3, -4, -13, and -14 are shown in Fig. 4*A*. XY-3 and -4 are analogs of 2-oleoyl- and 2-palmitoyl-LPA with a difluoromethyl group at the *sn*-1 position but an unmodified phosphomonoester group. Compounds XY-13 and -14 are a pair of enantiomeric analogs of dioleoyl-PA with a phosphatase-resistant difluoromethylenephosphonate group replacing the *sn*-3 phosphate group. Detergent extracts of membranes from insect cells infected with baculovirus vectors for expression of human LPP1, LPP2, and LPP3 can be used for kinetic analysis of these enzymes using mixed micelles of detergent and phospholipid. In this assay system LPP activity is dependent on both the bulk and surface concentrations of substrates (18). At a detergent concentration of 3.4 mM, apparent K_m values for hydrolysis of LPA by these enzymes are in the range of 1.1–1.4 mol%. We therefore initially examined effects of a fixed concentration of 10 μM of each of these PA analogs on LPP activity measured at an initial concentration of 0.1, 1.0, and 10 mol% LPA (corresponding to final concentrations of 3.4, 34, and 340 μM LPA). XY-3 and XY-4 had no significant effect on LPP1 activity at high mol fractions of substrate and produced a very modest inhibition at low mol fractions of substrate. By contrast, compounds XY-13 and XY-14 produced a significant inhibition of LPP1 activity at all concentrations tested. Inhibition was more pronounced at lower LPA concentrations suggesting a competitive mode of inhibition (Fig. 4*B*). In this assay, XY-13 and -14 inhibited LPP2 and LPP3 activity in an identical manner to that observed for LPP1 (not shown). To examine the mechanism of this inhibition directly we determined LPP1 activity at increasing LPA concentrations in assays containing a fixed concentration of 3.4 mM detergent and increasing concentrations of XY-14. In this assay system, LPP1 activity increased with substrate concentration and the apparent K_m obtained from fitting the data to the Michaelis-Menten equation was 0.2 μM. Increasing concentrations of XY-14 produced a marked inhibition of LPP1 activity (Fig. 4*C*). These data suggest that XY-14 is acting as a pure competitive inhibitor of LPP1 activity, because inhibition was accompanied by an increase in apparent K_m without changing the apparent V_{max} of

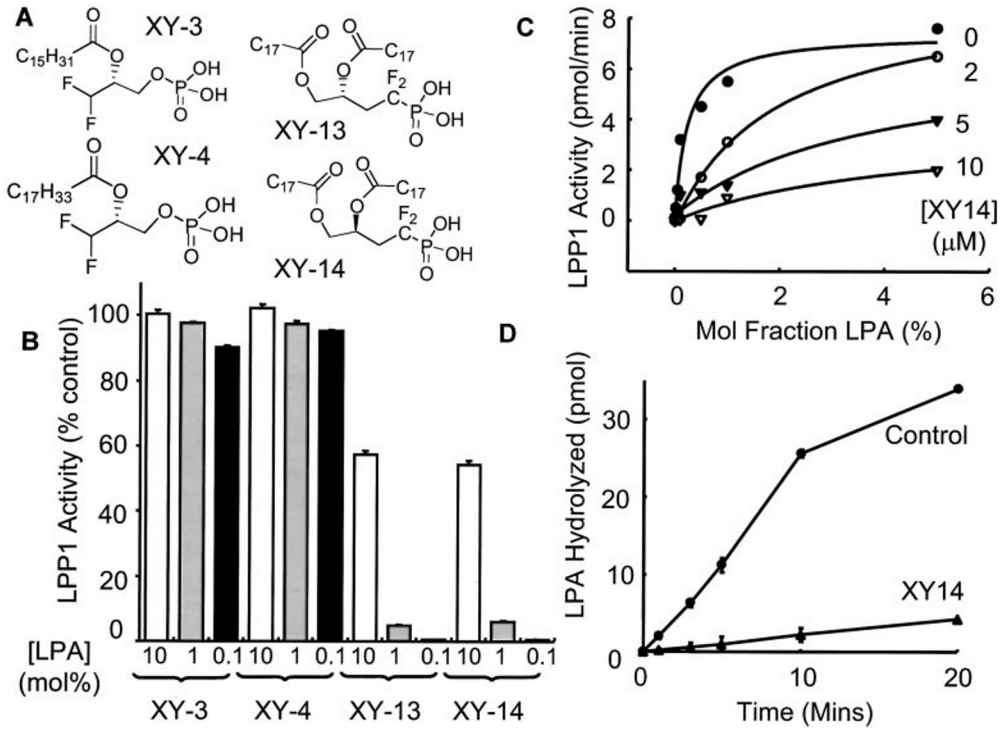

FIG. 4. **Inhibition of LPP1 activity by PA and LPA analogs.** *A*, structures of PA and LPA analogs used in this study. *B*, phosphatase activity of LPP1 in detergent extracts of membranes from baculovirus-infected sf9 cells was determined using LPA substrate presented as a component of mixed micelles of Triton X-100 at mol% values of 10, 1, and 0.1. The concentration of XY-3, -4, -13, and -14 was 10 μM. The activities shown are initial rates of substrate dephosphorylation expressed as a percentage of the rate of dephosphorylation observed in the absence of any inhibitor. The data shown are means ± S.D. of triplicate determinations. *C*, using detergent extracts from baculovirus-infected insect cell membranes as a source of enzyme, LPP1 activity was determined as the mol fraction of substrate in the mixed substrate, and Triton X-100 micelles were varied in the presence of increasing concentrations of XY-14. The activities are initial rates of dephosphorylation (less than 20% substrate hydrolysis) and are means of duplicate determinations. *D*, intact platelets were preincubated in Tyrode's solution containing 0.1 mg/ml BSA with (▲) or without (●), 10 μM XY-14 for 5 min before addition of 1 μM [^{32}P]LPA. Dephosphorylation was determined as described in the text. The data are means of duplicate determinations.

the enzyme. The K_i value for inhibition of LPP1 by XY-14 in this assay system obtained from the equation $K_m(\text{app}) = K_m/K_i[\text{I}] + K_m$ was 0.4 μM suggesting that XY-14 binds to the enzyme with a similar affinity to that of LPA itself. This finding is not surprising, because the previously measured K_m values for hydrolysis of PA and LPA by LPP1 are very similar (18). Similar results were obtained with XY-13 (not shown). Consistent with the results obtained using recombinant LPP1, XY-14 was an effective inhibitor of LPP activity measured in detergent extracts of platelet membranes and in LPP1 immunoprecipitates isolated from these extracts (not shown).

Although the detergent micelle assay system allowed us to determine the mechanism by which XY-14 inhibited LPP activity, use of the compound with intact platelets would necessarily require detergent-free conditions. Preincubation of washed platelets with 10 μM XY-14 in 0.1% BSA produced a decrease of ~10-fold in the rate of dephosphorylation of 1 μM LPA (Fig. 4*D*). However, consistent with the results obtained using recombinant LPP1 compounds, XY-3 and XY-4 had no significant effect on the rate of dephosphorylation of LPA by intact platelets (not shown).

None of the defined LPA receptors are responsive to PA, and all three LPA receptors show stereoselective recognition of LPA analogs at the *sn*-2 position of the glycerol backbone (35). It is therefore not surprising that at concentrations up to 10 μM XY-14 is not an agonist for LPA1, LPA2, or LPA3.[2] As described below, at concentrations up to 10 μM XY-14 does not activate platelet shape change or aggregation responses and can therefore be used to investigate the consequences of LPP1 inhibition on platelet function.

Inhibition of LPP1 Activity Amplifies Platelet Responses to LPA—Within seconds to minutes of exposure to exogenous LPA, platelets exhibit Rho-dependent actin reorganization and shape change and aggregation (36). We sought to determine whether inhibition of platelet LPP activity would alter these responses to LPA. To measure effects on actin reorganization, platelets were incubated with immobilized fibrinogen under conditions were unstimulated platelets adhere but the majority do not fully spread (which is defined here as being characterized by actin reorganization and the assumption of a polygonal shape). Platelet spreading is promoted by agonists. Maximally effective concentrations of ADP (10 μM) or LPA (1 μM) increased the number of spread platelets by 3.7-fold and 1.8-fold (p = 0.001 and p = 0.02 *versus* untreated platelets, respectively) (Fig. 5, *A–C*). At concentrations up to 10 μM, XY-14 alone had no effect on platelet spreading and did not significantly alter ADP-induced platelet spreading. However, in the presence of XY-14 LPA-stimulated platelet spreading was significantly (p = 0.02) enhanced (to a 2.5-fold increase over resting platelets), and we observed that under these conditions ~13% of the spread platelets were "large spread" platelets, defined as platelets with an area at least twice that of the mean of the area occupied by ADP-spread platelets (Fig. 5*D*). These "large spread" platelets were not observed under any of the other conditions tested (see Table III for summarized results from replicate experiments). To probe the mechanism responsible for this augmentation of platelet shape change responses to LPA by XY-14, we measured Rho activation in fibrinogen-adherent platelets under resting conditions or following exposure to ADP

[2] R. Janoz, G. D. Prestwich, A. J. Morris, and K. R. Lynch, unpublished observations.

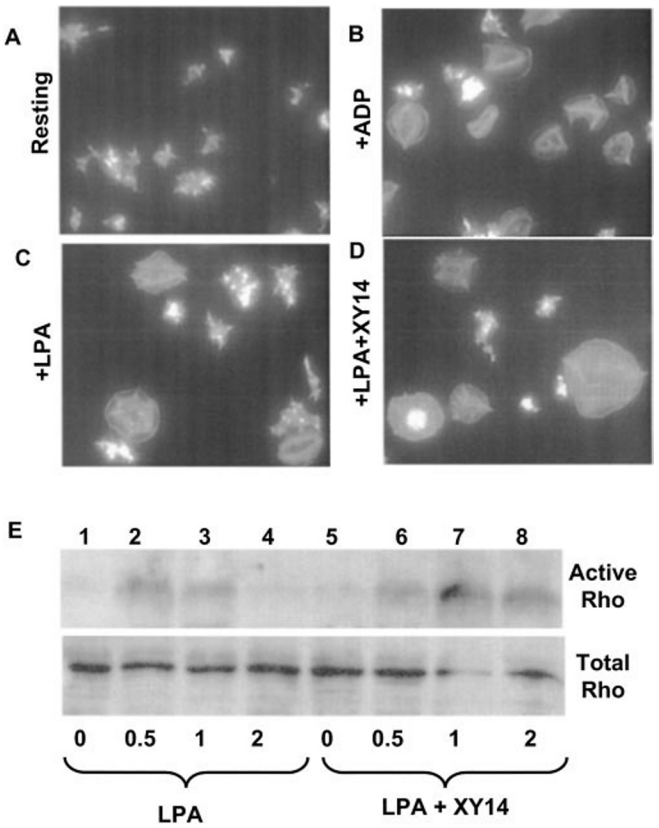

FIG. 5. **Effect of XY-14 on platelet shape change and Rho activation responses to agonists.** Platelets were incubated with fibrinogen-coated slides under resting conditions (*A*) or with stimulation by 10 μM ADP (*B*) or 1 μM LPA (*C*) alone or in combination with 10 μM XY-14 (*D*). Spread platelets, which assume a polygon shape with a number of filopodia-like projections, were identified by TRITC-phalloidin staining of actin fibers. A "large spread" platelet is marked by an *arrowhead* in *panel D*. *E*, Rho activation was determined in resting adherent platelets or adherent platelets treated with ADP LPA or LPA plus XY14 using a rhotekin binding assay as described in the text. Total and activated Rho proteins were separated by SDS-PAGE and detected by Western blotting. Total and activated Rho were quantitated by scanning densitometry. Increases in active Rho (expressed as a fraction of total Rho) were normalized to the fraction of Rho in the active state in resting platelets.

TABLE III
Effect of XY-14 on platelet shape change responses to agonists

Platelets were incubated with fibrinogen-coated slides under resting conditions or with stimulation by 1 μM ADP or 1 μM LPA alone or in combination with 10 μM XY-14. Spread platelets, which assume a polygon shape with a number of filopodia-like projections, were identified by TRITC-phalloidin staining of actin fibers. A "large spread" platelet is marked by an *arrowhead* in *panel D* of Fig. 5. The table summarizes data obtained from a series of experiments obtained using platelets from six separate donors.

Additions: LPA, ADP (1 μM); XY-14 (10 μM)	Spread platelets (mean ± S.D.)	Large spread platelets (mean ± S.D.)
	%	
Resting	10.8 ± 3.2	0
ADP	39.6 ± 3.9	0
ADP plus XY-14	37.1 ± 3.3	0
LPA	18.4 ± 2.8	0
XY-14	9.0 ± 3.5	0
LPA plus XY-14	25.3 ± 7.1	3.2 ± 0.4

or LPA in the presence or absence of XY-14 using a rhotekin binding assay. Exposure of platelets to ADP or LPA increases the fraction of platelet Rho in the activated state. LPA-induced Rho activation was significantly greater ($p = 0.05$) in the presence of XY-14 (Fig. 5*E*). Potentiation of LPA-induced Rho activation by XY-14 may therefore underlie the enhanced platelet shape change responses observed in the presence of the LPP inhibitor.

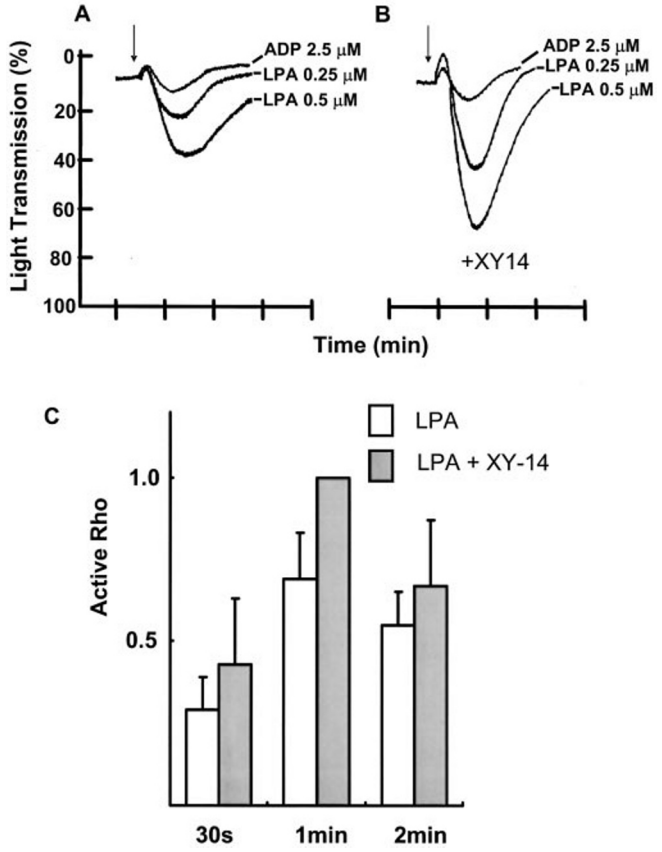

FIG. 6. **Effect of XY-14 on agonist-stimulated platelet aggregation and Rho activation.** Platelet aggregation responses were measured using the procedures described in the text. Platelets were stimulated with 2.5 μM ADP and the indicated concentrations of LPA in the absence (*A*) or presence (*B*) of 2.5 μM XY-14. The time scale markers represent minute intervals. The data shown are representative of sets of experiments performed using platelets obtained from six separate donors. *C*, time courses of Rho activation were measured in platelets in suspension that were stimulated with LPA in the presence or absence of XY-14 as indicated. Total and activated Rho proteins were resolved by SDS-PAGE and visualized by Western blotting and quantitated by scanning densitometry. Increases in active Rho (expressed as a fraction of total Rho) were normalized to the fraction of Rho in the active state in platelets stimulated for 1 min with LPA in the presence of XY-14. The data shown are means ± S.D. of triplicate determinations.

Next, we tested the effects of XY-14 pretreatment on LPA- or ADP-induced platelet aggregation. Platelets isolated from multiple donors were stimulated with LPA. As has been observed by others, stimulation of aggregation by LPA was concentration-dependent (Fig. 6). In all but three platelet preparations, we observed that preincubation with XY-14 resulted in an 2- to 3-fold potentiation of the aggregation response to LPA (Fig. 6, *A* and *B*). It is presently unclear why platelets from three donors were much less responsive to LPA. In all cases, XY-14 alone had no effect on platelet aggregation or on aggregation induced by ADP (Fig. 6, *A* and *B*). We also measured LPA-induced Rho activation in platelets that displayed a potentiation of LPA-induced aggregation by XY-14. The results obtained show that LPA-induced Rho activation is both greater and more persistent in the presence of XY-14 (Fig. 6*C*). These results show that XY-14 selectively potentiates platelet shape change and aggregation responses to LPA and suggest that the mechanism may involve enhanced activation of Rho.

Inhibition of LPP1 Activity Amplifies Agonist-promoted LPA Production by Platelets—Thrombin-stimulated platelets gener-

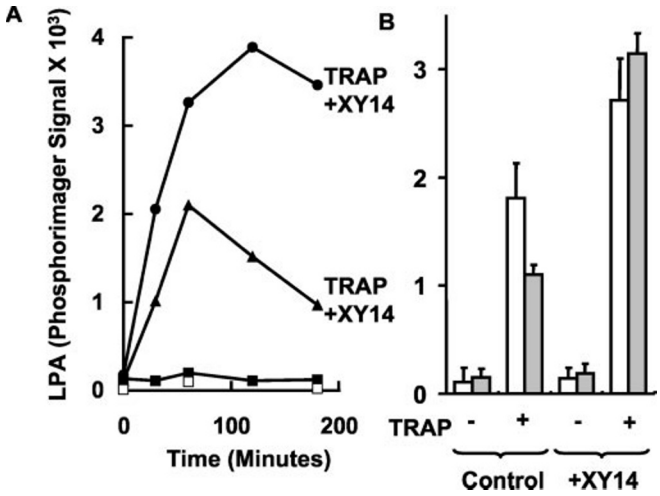

FIG. 7. **Effect of XY-14 on LPA production by agonist stimulated platelets.** *A*, platelets were labeled with [^{32}P]PO_4^{2-}, washed, and then incubated with vehicle (□), 10 μM XY-14 (■), or 10 μM TRAP alone (▲) or in combination with 10 μM XY-14 (●). Radioactivity associated with LPA released into the incubation medium was quantitated by PhosphorImager scanning after separation by thin layer chromatography. The data are single LPA determinations and representative of three separate experiments. *B*, platelets were incubated for 60 min (*white bars*) or 120 min (*gray bars*) in the presence or absence of 10 μM TRAP with and without 10 μM XY-14 as indicated, and LPA production was determined. The data shown are means ± S.D. of triplicate determinations from a single experiment.

ate LPA, which accumulates in the incubation medium (10). LPA production by thrombin-stimulated platelets is, in comparison to other indices of platelet activation, relatively slow being maximal after 1–2 h. [^{32}P]PO_4^{2-}-labeled platelets were treated with vehicle, 10 μM XY-14 alone, and thrombin receptor agonist peptide (TRAP) in the presence and absence of 10 μM XY-14. LPA production in the incubation medium was determined by lipid extraction and thin layer chromatography with quantitation made using a PhosphorImager. XY-14 had no effect on LPA production in these assays, but, in agreement with reports from several other groups, TRAP stimulated an accumulation of LPA in the incubation medium that was maximal after 1 h and then declined over the second hour of incubation (10). In the presence of XY-14, TRAP-stimulated LPA production was both more rapid and persistent. In the presence of XY-14, maximal LPA accumulation was increased ~1.8-fold (Fig. 7*A*). In a second experiment we measured LPA production 60 and 120 min after stimulation with vehicle or TRAP in the presence or absence of XY-14. In this experiment at 60 min TRAP-stimulated LPA production was increased by 1.5-fold in the presence of XY-14, and the XY-14-dependent increase in LPA production was more pronounced (3-fold) at 120 min (Fig. 7*B*). These results suggest that LPP activity opposes pathways responsible for production of LPA by agonist-activated platelets.

DISCUSSION

Although the LPP enzymes are structurally simple proteins that catalyze well defined enzymatic reactions, ongoing studies continue to associate them with a surprisingly complex range of biological functions. At the cellular level, overexpression studies suggest that LPPs 1–3 act as both negative regulators of LPA and S1P signaling and have roles in metabolism of intracellular lipid messengers (23, 37). At the organismal level, inactivation or misexpression of two *Drosophila* LPP enzymes alters gem cell migration in early development, which has been suggested to involve localized changes in LPP-catalyzed degradation of an as yet unidentified lipid signaling molecule (21). Although genetic inactivation of LPP2 in mice produces no obvious phenotype, inactivation of the LPP3 gene results in early embryonic lethality resulting from defects in vascularization and patterning (27, 38). Furthermore, there are hints that LPPs may have actions that are independent of their catalytic activities. Effects of LPP3 inactivation in mice are similar to those seen when wnt signaling is up-regulated and LPP3 inhibits wnt signaling in cultured cells (27). LPP3 has also been shown to serve as an integrin ligand that regulates cell-cell interactions through a unique RGD sequence in the second extracellular loop (26). Clearly, more subtle approaches are required to separate activity-dependent and independent functions of these proteins. We report the identification of chemical inhibitors of LPP activity that can be used to probe the role hydrolysis of phospholipid substrates by endogenously expressed LPPs. Results using one such compound to investigate effects of inhibition of LPP activity in platelets identify roles for LPPs in control of both platelet responses to LPA and of LPA production by activated platelets. These findings are significant, because they suggest a physiological role for LPPs in controlling platelet and by implication possibly also vascular function. Our results also provide new insight into the physiological function of LPPs that may explain some of their complex biological activities.

Platelets are highly responsive to LPA and play both direct and indirect roles in the mechanisms responsible for LPA synthesis in the blood. We found that platelets actively dephosphorylate LPA. Immunological analysis reveals that LPP1 is strongly expressed by platelets and that expression of LPP3 is restricted to a subpopulation of ~5% of platelets. Heterogeneous has been observed for other platelet surface proteins and may relate to differences in platelet age and agonist responsiveness (39). Indirect immunofluorescence studies reveal that the localization of LPP1 and LPP3 is different in LPA-activated platelets, and comparisons with resting platelets suggest that LPP1 redistributes across the platelet surface when agonist-activated platelets spread on a fibrinogen surface. LPA increases the amount of LPP1 activity and protein accessible to labeling by a cell-impermeant biotin derivative suggesting that platelet activation is accompanied by translocation of LPP1 to the platelet surface which may have a role in adaptive responses to activation of platelets by lysophospholipid agonists.

Cell surface localization has been reported for both overexpressed and endogenous LPPs 1–3 in several mammalian cell types (18–20, 23, 24). Plasma membrane localization LPP1 has been associated with increased cell surface LPP activity and specific decreases in rapid signaling responses to activation of LPA and S1P receptors (19, 22). In cases where overexpression of LPP1 resulted in attenuation of rapid LPA signaling responses (*i.e.* responses that are manifest over seconds to minutes), notably phospholipase C activation and consequent Ca^{2+} mobilization, the effects were observed under conditions where the fraction of extracellularly added LPA degraded was much too small to account for the decreased response. These findings led to the proposition that LPPs restrict access of a localized pool of LPA to LPA receptors possibly by controlling LPA levels associated with the outer leaflet of the plasma membrane (22, 37). Here we found that preincubation of platelets with a fluorophosphonate analog of PA under conditions that substantially inhibit LPP activity resulted in a potentiation of two platelet functional responses to LPA, aggregation and shape change. In both cases these effects were observed when platelets were stimulated with maximally effective concentrations of LPA and, based on the duration of these assays and on the small numbers of platelets used, even in the absence of the inhibitor, hydrolysis of LPA in the incubation media would be

less than 0.1% of the total present at the beginning of the experiment. Because we used concentrations of LPA that are maximally effective in the absence of the inhibitor, this result also makes it unlikely that the potentiation observed is due to a nonspecific increase in bioavailability of LPA. Our findings are, therefore, consistent with this "restricted access" model and extend the overexpression studies that data in support of this model to show that LPP inhibition potentiates rapid LPA signaling responses to LPA in a manner that cannot be accounted for by preservation of LPA in the medium. Testing this model will be challenging; levels of cell surface-associated LPA are small and, once LPA partitions into the plasma membrane, dephosphorylation of LPA is rapid (29).

Other work has shown that overexpression of LPPs results in attenuation of cell growth and survival responses to LPA, which occur with much longer time courses than the rapid responses discussed above (6, 24). In comparison to LPA, LPP-resistant LPA analogs have been found to exhibit increased potency in cell proliferation assays that likely results from their resistance to metabolic degradation (24). Transient or stable overexpression of LPP3 in cultured ovarian cancer cells results in decreased growth in a colony forming assay and an increase in the rate of apoptosis. Most strikingly, co-culture of LPP3-expressing cells with their parental counterparts decreased growth of the parental cells. This action of LPP3 can most simply be explained by a decrease in levels of an extracellular mediator, most likely LPA, because the decrease in growth could be rescued by addition of a hydrolysis-resistant LPA analog (6). These results indicate that LPPs can also modulate LPA signaling "in *trans*"; *i.e.* LPP activity in one cell can alter LPA signaling in another. Our observation that inhibition of LPP1 activity potentiates LPA production by platelets suggests that this "*trans*" action of LPPs involves the degradation of extracellular LPA and possibly also the opposition of pathways responsible for LPA production. Precisely how LPP inhibition potentiates LPA production by activated platelets is not clear. The simplest explanation would be that the role of LPP in the process is indirect: in the presence of an LPP inhibitor LPA produced in response to agonists is simply protected from degradation. Alternatively, one of the mechanisms proposed to account for LPA production by isolated platelets is the hydrolysis of phosphatidic acid by phospholipase A_2. Evidence that the source of this PA is micoparticles released by activated platelets has been presented, and our finding that LPP activity is enriched in these microparticles suggests the possibility that inhibition of LPP activity could produce elevated LPA production through increases in PA levels.

Our finding that LPPs can oppose LPA production may explain some intriguing information about LPP function from developmental studies in *Drosophila*. In the developing fly embryo the germ cells are specified on the surface, and these cells move into the gut upon gastrulation. The germ cells migrate actively through the gut and then through the mesoderm to pair with their somatic counterparts to form the gonad. Two *Drosophila* LPP homologs, *Wunen-1* and *Wunen-2*, are normally expressed in tissues that repel germ cells. Germ cell migration and survival are disrupted in a *Drosophila* mutant, wunen, in which these genes are inactivated. Ectopic expression of either Wunen gene in the mesoderm repels migrating germ cells (21, 25). Wunen-2 is a functional LPP that hydrolyzes LPA, and the integrity of its catalytic domain is required for these effects (Starz-Gaiano, *et al.* (21)).[3] The simplest explanation of these data is that catalytic activity of the wunen genes destroys or opposes the synthesis of an as yet undefined factor that is required for germ cell survival and migration. XY-14 inhibits hydrolysis of LPA by Wunen-2,[4] and it will be interesting to see if exposure of *Drosophila* embryos to this compound recapitulates the wunen germ cell migration phenotype.

The identification of LPP1 as a pivotal regulator of LPA production and signaling in platelets suggest that this enzyme may function to control circulating LPA levels and possibly also regulate localized changes in LPA levels resulting from platelet activation. Because of the well defined actions of LPA on platelets, various inflammatory cell types, vascular smooth muscle cells, and endothelial cells, there is considerable interest in the possibility that LPA functions in vascular responses to injury and may also be a relevant thrombo- and atherogenic molecule in cardiovascular disease (3, 5). The lack of LPA receptor-selective agonists and antagonists and the apparent functional redundancy among the known LPA receptors inferred from gene inactivation studies in mice is hampering efforts to test these ideas explicitly (40). Assignment of specific responses to particular LPA receptor subtypes continues to be a challenge, and although platelets are clearly highly responsive to LPA the identity of the receptor involved is unclear (24). Similarly, although acute vascular responses have been observed in response to injection of LPA (41), the physiological sites of LPA synthesis and action in the cardiovascular system are not known (3). The LPP inhibitor reported here may prove to be a valuable tool to potentiate LPA signaling in response to endogenously formed LPA and thereby address this critical and longstanding issue.

REFERENCES

1. Hla, T., Lee, M. J., Ancellin, N., Paik, J. H., and Kluk, M. J. (2001) *Science* **294,** 1875–1878
2. Fukushima, N., Ishii, I., Contos, J. J., Weiner, J. A., and Chun, J. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 507–534
3. Tigyi, G. (2001) *Prostaglandins* **64,** 47–62
4. Baker, D. L., Desiderio, D. M., Miller, D. D., Tolley, B., and Tigyi, G. J. (2001) *Anal. Biochem.* **292,** 287–295
5. Siess, W. (2002) *Biochim. Biophys. Acta* **1582,** 204–215
6. Tanyi, J. L., Morris, A. J., Wolf, J. K., Fang, X., Hasegawa, Y., Lapushin, R., Auersperg, N., Sigal, Y. J., Newman, R. A., Felix, E. A., Atkinson, E. N., and Mills, G. B. (2003) *Cancer Res.* **63,** 1073–1082
7. Gerrard, J. M., and Robinson, P. (1989) *Biochim. Biophys. Acta* **1001,** 282–285
8. Eichholtz, T., Jalink, K., Fahrenfort, I., and Moolenaar, W. H. (1993) *Biochem. J.* **291,** 677–680
9. Fourcade, O., Simon, M. F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L., and Chap, H. (1995) *Cell* **80,** 919–927
10. Sano, T., Baker, D., Virag, T., Wada, A., Yatomi, Y., Kobayashi, T., Igarashi, Y., and Tigyi, G. (2002) *J. Biol. Chem.* **277,** 21197–21206
11. Umezu-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G. B., Inoue, K., Aoki, J., and Arai, H. (2002) *J. Cell Biol.* **158,** 227–233
12. Aoki, J., Taira, A., Takanezawa, Y., Kishi, Y., Hama, K., Kishimoto, T., Mizuno, K., Saku, K., Taguchi, R., and Arai, H. (2002) *J. Biol. Chem.* **277,** 48737–48744
13. Olorundare, O. E., Peyruchaud, O., Albrecht, R. M., and Mosher, D. F. (2001) *Blood* **98,** 117–124
14. Brindley, D. N., and Waggoner, D. W. (1998) *J. Biol. Chem.* **273,** 24281–24284
15. Kai, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1996) *J. Biol. Chem.* **271,** 18931–18938
16. Kai, M., Wada, I., Imai, S., Sakane, F., and Kanoh, H. (1997) *J. Biol. Chem.* **272,** 24572–24578
17. Hooks, S. B., Ragan, S. P., and Lynch, K. R. (1998) *FEBS Lett.* **427,** 188–192
18. Roberts, R., Sciorra, V. A., and Morris, A. J. (1998) *J. Biol. Chem.* **273,** 22059–22067
19. Jasinska, R., Zhang, Q. X., Pilquil, C., Singh, I., Xu, J., Dewald, J., Dillon, D. A., Berthiaume, L. G., Carman, G. M., Waggoner, D. W., and Brindley, D. N. (1999) *Biochem. J.* **340,** 677–686
20. Sciorra, V. A., and Morris, A. J. (1999) *Mol. Biol. Cell* **10,** 3863–3876
21. Zhang, N., Zhang, J., Purcell, K. J., Cheng, Y., and Howard, K. (1997) *Nature* **385,** 64–67
22. Xu, J., Love, L. M., Singh, I., Zhang, Q. X., Dewald, J., Wang, D. A., Fischer, D. J., Tigyi, G., Berthiaume, L. G., Waggoner, D. W., and Brindley, D. N. (2000) *J. Biol. Chem.* **275,** 27520–27530
23. Alderton, F., Darroch, P., Sambi, B., McKie, A., Ahmed, I. S., Pyne, N., and Pyne, S. (2001) *J. Biol. Chem.* **276,** 13452–13460
24. Hooks, S. B., Santos, W. L., Im, D. S., Heise, C. E., Macdonald, T. L., and

[3] Y. J. Sigal, A. J. Morris, and R. Lehmann, unpublished results.

[4] Y. J. Sigal, A. J. Morris, G. D. Prestwich, and R. Lehmann, unpublished observations.

Lynch, K. R. (2001) *J. Biol. Chem.* **276,** 4611–4621
25. Starz-Gaiano, M., Cho, N. K., Forbes, A., and Lehmann, R. (2001) *Development* **128,** 983–991
26. Humtsoe, J. O., Feng, S., Thakker, G. D., Yang, J., Hong, J., and Wary, K. K. (2003) *EMBO J.* **22,** 1539–1554
27. Escalante-Alcade, D., Hernandez, L., Le Stunff, H., Maeda, R., Cheng, J.-R., Sciorra, V. A., Daar, I., Spiegel, S., Morris, A. J., and Stewart, C. L. (2003) *Development* **130,** 4623–4637
28. Luquain, C., Sciorra, V. A., and Morris, A. J. (2003) *Trends Biochem. Sci.* **28,** 377–383
29. Roberts, R. Z., and Morris, A. J. (2000) *Biochim. Biophys. Acta* **1487,** 33–49
30. Xu, Y., and Prestwich, G. D. (2002) *Org. Lett.* **4,** 4021–4024
31. Xu, Y., and Prestwich, G. D. (2002) *J. Org. Chem.* **67,** 7158–7161
32. McIntyre, T. M., Pontsler, A. V., Silva, A. R., St Hilaire, A., Xu, Y., Hinshaw, J. C., Zimmerman, G. A., Hama, K., Aoki, J., Arai, H., and Prestwich, G. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 131–136
33. Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) *J. Clin. Invest.* **72,** 325–338
34. Broekman, M. J. (1992) *Methods Enzymol.* **215,** 21–32
35. Lynch, K. R., and Macdonald, T. L. (2002) *Biochim. Biophys. Acta* **1582,** 289–294
36. Schwartz, M. A., and Shattil, S. J. (2000) *Trends Biochem. Sci.* **25,** 388–391
37. Pilquil, C., Singh, I., Zhang, Q., Ling, Z., Buri, K., Stromberg, L. M., Dewald, J., and Brindley, D. N. (2001) *Prostaglandins* **64,** 83–92
38. Zhang, N., Sundberg, J. P., and Gridley, T. (2000) *Genesis* **27,** 137–140
39. Dale, G. L., Friese, P., Batar, P., Hamilton, S. F., Reed, G. L., Jackson, K. W., Clemetson, K. J., and Alberio, L. (2002) *Nature* **415,** 175–179
40. Yang, A. H., Ishii, I., and Chun, J. (2002) *Biochim. Biophys. Acta* **1582,** 197–203
41. Tokumura, A., Kume, T., Fukuzawa, K., and Tsukatani, H. (1981) *J. Pharmacol. Exp. Ther.* **219,** 219–224