Structure−Activity Analysis of the Effects of Lysophosphatidic Acid on Platelet Aggregation†

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ABSTRACT: Lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate or LPA) is a phospholipid mediator displaying numerous and widespread biological activities and thought to act via G-protein-coupled receptors. Here we have studied the effects on human platelets of a number of LPA analogues, including two enantiomers of both *N*-palmitoyl-(L)-serine-3-phosphate ((L) and (D)NAPS for *N*-acyl-phosphoserine) and 2-(*R*)-*N*-palmitoyl-norleucinol-1-phosphate ((*R*) and (*S*)PNPA), cyclic analogues of 1-acyl-*sn*-glycero-3-phosphate (cPA) and of 1-*O*-hexadecyl-*sn*-glycero-3-phosphate (cAGP), sphingosine-1-phosphate (SPP), as well as two palmitoyl derivatives of dioxazaphosphocanes bearing either a P−H or a P−OH bond (DOXP−H and DOXP−OH, respectively). Nine of these compounds induced platelet aggregation with the following order of potency: SPP < cAGP < DOXP−OH < (L)NAPS = (D)NAPS < (*R*)PNPA = (*S*)PNPA < LPA < AGP, EC_{50} varying between 9.8 nM and 8.3 μM. Two of these compounds (SPP and cAGP) appeared as weak agonists inducing platelet aggregation to only 33% and 41%, respectively, of the maximal response attained with LPA and other analogues. In cross-desensitization experiments, all of these compounds specifically inhibited LPA-induced aggregation, suggesting that they were all acting on the same receptor(s). In contrast, cPA and DOXP−H did not trigger platelet aggregation but instead specifically inhibited the effects of LPA in a concentration-dependent manner. The inhibitory action of cPA did not vary with the acyl chain length or the presence of a double bond and did not involve an increase in cAMP. These data thus confirm the lack of stereospecificity of platelet LPA receptor(s). In addition, since the order of potency of some analogues is different from that described in other cells, our results suggest that platelets contain (a) pharmacologically distinct receptor(s) whose molecular identity still remains to be established. Finally, this unique series of compounds might be used for further characterization of other endogenous or recombinant LPA receptors.

Lysophosphatidic acid (LPA)[1] is now recognized as an authentic phospholipid mediator produced mainly by platelets during blood coagulation (*1*−*4*) or accumulating in various biological fluids including serum, aged plasma, normal follicular fluid, pathological cerebrospinal fluid, and ascites induced by ovary tumors (*3*−*11*). It evokes a number of biological responses such as smooth muscle contraction, in vivo vasoactive effects, platelet aggregation, stimulation or inhibition of proliferation, promotion of tumor invasion, dedifferentiation of neural cells and reorganization of cytoskeleton, chemotaxis, activation of membrane ion channels, as well as closure of gap junctions (*12*−*19*). LPA was proposed to act via a specific membrane receptor, which was first identified by affinity cross-linking labeling (*20*) and was suggested to belong to the large family of G-protein-coupled receptors, on the basis of a number of studies dealing with cell signaling (*12*−*19*). Presently available data indicate that LPA receptors might be coupled to heterotrimeric proteins G_q, G_i, and G_{13} (*18*, *21*). This point of view was recently confirmed by the cloning of several cDNA coding for putative LPA receptors, which can be classified into two groups based on sequence similarities: the "edg family" (endothelium differentiation gene) comprises at least four members displaying some homology with cannabinoid and melanocortin receptors (*22*−*27*), whereas a cDNA cloned from *Xenopus laevis* oocytes suggests a closer similarity with

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[1] Abbreviations: LPA, lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate); AGP, alkyl glycero-phosphate (1-*O*-alkyl-*sn*-glycero-3-phosphate); cPA, cyclic PA (1-acyl-*sn*-glycero-2,3-cyclic phosphate); cAGP, cyclic alkyl glycero-phosphate (1-*O*-alkyl-*sn*-glycero-2,3-cyclic phosphate); NAPS, *N*-acyl phosphoserine (2-*N*-acyl-serine-3-phosphate); PNPA, palmitoyl norleucinol phosphate (2-*N*-palmitoyl-norleucinol-1-phosphate); SPP, sphingosine 1-phosphate; DOXP−H, dioxazaphosphocane bearing a P−H bond; DOXP−OH, dioxazaphosphocane bearing a P−OH bond; nano-ESI (nanoscale electrospray ionization); EC_{50}, efficient concentration giving half-maximal effect; IC_{50}, inhibitory concentration giving half-maximal effect.

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the receptors of platelet-activating factor, another phospholipid mediator (*28*). This heterogeneity is reinforced by the fact that some of the edg family genes (edg-1 and edg-3) actually correspond to receptors for sphingosine 1-phosphate (SPP), a LPA analogue also considered as a novel lipid mediator acting mainly by an extracellular action (*24*−*26*, *29*).

Such a complexity of LPA and SPP receptors was actually suggested in previous studies dealing with the characterization of the biological effects of LPA and some of its analogues. For instance, the use of cPA led to predict the presence in *Xenopus laevis* of at least two receptors recognizing either LPA alone or LPA and cPA (*30*). Moreover, whereas SPP receptors do not appear generally to respond to LPA, at least three types of receptors were characterized, based on their ability to cross-react or not with sphingosylphosphorylcholine (*31*).

When focusing on platelets, only feline, human, and sheep cells (in the order of decreasing sensitivity) respond to LPA or analogues by undergoing significant aggregation (*7*, *32*−*36*). This typical hemostatic function requires activation of a number of signaling pathways, including phospholipases A_2 and C, protein kinase C, and protein tyrosine phosphorylation (*37*−*41*). Human platelet aggregation can be triggered not only by LPA itself but also by a number of analogues such as acetal phosphatidic acid (*42*, *43*), AGP and its 2-acetyl derivative (*34*), 2-*N*-acyl-aminoethanol-1-phosphate (*44*), and SPP (*45*). In addition, platelets have allowed to discover the first specific inhibitors of LPA, that is, NAPS and *N*-acyl-phospho-(L)-tyrosine, which was then extended to *Xenopus laevis* oocytes (*44*, *46*−*47*). These various studies have reported a number of observations which differ from conclusions obtained with other cells: (i) activation of platelets by AGP did not bring evidence for a stereospecific recognition of LPA by its receptor (*34*), in contrast to a recent study dealing with the effects of (D) and (L)NAPS on the human breast cancer cell line MDA MB231 (*48*); (ii) AGP, which was shown to act by a mechanism different from platelet-activating factor, revealed 30-fold more potent than LPA (*34*), while in contrast 4−5-fold higher concentrations of AGP, compared to LPA, were required to induce calcium mobilization in the epidermal cell line A431 (*49*); and (iii) cross-desensitization experiments suggested that SPP may act on the same platelet receptor as LPA, which is not supported by functional analysis of other SPP receptors present in other cells (*45*).

The present study deals with the structure−activity analysis of the effects of various LPA analogues on platelet aggregation. Besides fully confirming that platelet LPA receptor is not stereospecific, our data bring further arguments to the view that platelets possess (a) pharmacologically distinct receptor(s). Finally, they allow us to describe a novel type of LPA specific inhibitor.

MATERIALS AND METHODS

Materials

Palmitoyl LPA was purchased from Alexis Corporation. Sphingosine-1-phosphate (SPP), human α-thrombin, phospholipase D from *Streptomyces chromofuscus*, (L) and (D)-norleucine, myristic, palmitic, stearic and oleic anhydrides, *O*-phospho (L) and (D)-serine, L-α-glycerophosphate di-(monocyclohexylammonium) salt, and human fibrinogen (fraction 1) were obtained from Sigma.

Methods

General. The concentration of phospholipids in ethanolic solutions was determined on the basis of their phosphorus content (*50*). Thin-layer chromatography was performed on Merck silica gel K60 plates. Mass spectra were recorded on a NERMAG R10-10 spectrometer. NMR measurements ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a BRUCKER 250 MHz spectrometer. The chemical shifts are expressed in parts per million using TMS as the internal standard (for ^1H and ^{13}C data) and 85% H_3PO_4 as the external standard (for ^{31}P data).

Mass Spectrometry Analysis of SPP. SPP (1 mM) was dissolved in ethanol without desalting or adding salt. Mass spectrometry was performed with an LCQ ion-trap spectrometer (Finningan MAT, San Jose, CA). A commercial nanospray ESI source (The Protein Analysis Co, Odense, Denmark) was used using glass capillaries (The Protein Analysis), which were positioned directly at a distance of 1 mm from the entrance hole of the heated transfer capillary with the help of a microscope. The capillaries were gold-coated for electrical contact. The glass capillaries were filled by gel loader tips with 5 μL of analyte solution. Nebulizer gas was not necessary in this spray mode. For the nanospray needle, voltage was set at 700 V. The heated transfer capillary was kept at a temperature of 180 °C. Mass spectra were averaged over 10 scans, and each scan (mass range 150−2000) was built-up from three microscans. In the positive ion polarity mode, SPP yielded an intense molecular ion (*m*/*z* 380.1) with excellent S/N ratio with nano-ESI. Due to the high concentration of the analyte, dimer, trimer, and tetramer ions were present in the spectrum. In the negative mode, the signal intensity of the major peak was 20-fold lower than in the positive mode. Two major peaks with a good S/N ratio, corresponding to the pseudo molecular ion ($[M - H]^-$; *m*/*z* 378.5) and a dimer could be detected. In both positive and negative mode, no additional molecule arose from the background, indicating that the sample was free of contaminants.

Synthesis of 2-N-Palmitoyl-norleucinol-1-phosphate (PNPA). (*R*) and (*S*)PNPA were obtained using the reaction of phospholipase D from *Streptomyces chromofuscus* with (*R*) and (*S*)PNPG (2-*N*-palmitoylnorleucinol-1-phosphoglycol), synthesized as described previously (*51*). In a typical reaction, 10 mg of PNPG was dispersed in 1 mL of Tris buffer (0.2 M Tris-HCl, pH 7.4, 1 mM $CaCl_2$) and 20 units of enzyme were added. After 30 min at 37 °C, the aqueous suspension was subjected to a Bligh and Dyer extraction with HCl as described (*1*, *52*). PNPA was purified by thin-layer chromatography in the solvent system chloroform/methanol/concentrated ammonia (65:25:5, v/v). PNPA exhibiting an R_f value of 0.15 was recovered by scraping off the plate and eluted using chloroform/methanol/water (1:2:0.8, v/v), yield = 45%.

Synthesis of 2-N-Palmitoyl-serine-3-phosphate (NAPS). *O*-Phospho-(L)-serine (2-amino-3-hydroxypropanoic acid 3-phosphate, 500 mg, 2.7 mmol) and palmitoyl chloride (657 mg, 2.4 mmol) were refluxed in tetrahydrofuran/water (1/1,

v/v, 6 mL) in the presence of triethylamine (835 mg, 6 mmol) during 18 h. Tetrahydrofuran was removed by evaporation, and the aqueous layer was washed with chloroform/methanol (2/1, v/v) with 1 M HCl. The organic phase was washed according to Folch et al. (*53*) in the presence of 1 M NaOH. NAPS was obtained by evaporation of the organic phase. Yield: 241 mg (23%). R_f = 0.25 in chloroform/methanol/water (65:35:8, v/v). ^{1}H NMR ($CDCl_3/CD_3OD$, 1/1): 0.62 (t, 3H, CH_3); 1 (s, 24H, CH_2); 1.37 (td, 2H, CH_2 β); 2.02 (t, 2H, CH_2 α); 4 (m, 2H, C*H*$_2$-OP); 4.3 (m, 1H, CH). The synthesis of the D-stereoisomer was carried out in the same way, using *O*-phospho-(D)-serine as a starting compound (yield: 35%), and its NMR spectra were identical to those of the L analogue.

*Synthesis of Palmitoyl-*L*-α-glycero-1,2-cyclic Phosphate (cPA).* The synthesis of cPA was achieved as described by Lapidot et al. (*54*) except the purification step. To a solution of L-α-glycerophosphate di(monocyclohexylammonium) salt (174 mg, 0.47 mmol) in water (1.5 mL) were added 2 M NH_4OH (1.5 mL) and dimethylformamide (2.5 mL). Then a solution of dicyclohexylcarbodiimide (495 mg, 2.4 mmol) in *tert*-butyl alcohol (0.7 mL) was added, and the reaction was refluxed for 2 h at 135 °C. Solvents were evaporated, and water was added. After extraction with ether, the aqueous layer was filtered and evaporated. The residue (yield 91%) was dissolved in dry pyridine (30 mL) with palmitic anhydride (643.2 mg, 1.3 mmol) and was stirred for 36 h at room temperature. Solvent was removed and the residue was isolated on silica gel plates (chloroform/methanol/water, 65:25:4, v/v). cPA exhibiting an R_f value of 0.56 was recovered by scraping the silica gel and extracted by chloroform/methanol/water (1:2:0.8, v/v). Yield: 40%.^{1}H NMR ($CDCl_3$): 0.85 (t, 3H, CH_3); 1.24 (s, 26H, CH_2); 1.86 (m, 11H, CH_2 cyclohexylNH_3); 2.3 (t, 2H, C*H*$_2$CO); 3.1 (m, 3H, NH_3); 3.97 (q, 1H, C*H*$_2$−OCO); 4.22 (m, C*H*$_2$−OP (2H) and C*H*$_2$−OCO (1H)); 4.6 (m, 1H, C*H*-OP). ^{31}P NMR ($CDCl_3$): 17.57. The same procedure was applied for the synthesis of cPA bearing a myristoyl, stearoyl, or oleoyl chain, using the corresponding fatty acid anhydride in the acylation reaction. The purified compounds displayed very similar ^{1}H and ^{31}P NMR spectra (not shown).

*Synthesis of 1-O-Hexadecyl-*L*-α-glycero-1,2-cyclic Phosphate (cAGP).* To a solution of hexadecanol (1.28 g, 5.32 mmol) in pyridine was added methane sulfonyl chloride (950 mg, 8 mmol), and the mixture was stirred 5 h at room temperature. The reaction was quenched by adding 15 mL of ether and 12 mL of water. After the ether phase was washed, the solvent was removed and hexadecyl methane sulfonate (mp 45 °C) was obtained in an 86% yield. To a solution of (*R*)1,2-*O*-isopropylideneglycerol (0.36 mg, 2.7 mmol) in xylene with KOH was added hexadecyl methane sulfonate (1.8 mmol), and the reaction was refluxed for 5 h at 140 °C. Xylene was distilled, water and ether were added, and the organic layer was dried over anhydrous potassium carbonate. The solvent was removed, and the residue was hydrolyzed, refluxing 3 h at 100 °C in methanol with 1 M HCl. After extraction with water/ether (1:1, v/v), the organic phase was washed and the solvent was evaporated (yield: 97%). Then, to a solution of 1-*O*-hexadecyl-*sn*-glycerol (411.4 mg, 1.3 mmol) in pyridine, was added phenyl dichlorophosphate (548.6 mg, 2.6 mmol) in dichloromethane, and the reaction mixture was stirred 1 day at room temperature. Water was added, and the reaction mixture was submitted to a Folch partition (*53*). The residue was hydrogenolyzed with platinium oxide (PtO_2) in ethanol/dichloromethane (1:1, v/v). PtO_2 was filtered off, and the crude product was isolated on silica gel plates (chloroform/methanol/water, 65:25:4, v/v). cAGP exhibiting an R_f value of 0.37 was recovered by scraping the silica gel and extracted with chloroform/methanol/water (65:25:4, v/v). Yield = 40%. ^{1}H NMR ($CDCl_3/CD_3OD$, 1:1): 0.71 (t, 3H, CH_3); 1.09 (s, 24H, CH_2); 1.37 (dd, 2H, C*H*$_2$CH$_2$−O); 3.3 (m, 5H, C*H*$_2$O−C*H*$_2$α and $CH_2\gamma$); 3.8 (m, 1H, C*H*$_2\gamma$); 4.3 (m, 1H, C*Hβ*). ^{31}P NMR ($CDCl_3$): 15.52. Mass spectrometry (DCI/NH_3): *m*/*z* =396 (38%) MNH_4^+.

Synthesis of DOXP−H. Methyl aminodiol (5.54 g, 33.86 mmol) in toluene (80 mL) was refluxed with 5.3 g (32.5 mmol) of tris(dimethylamino)phosphine. Solvent was removed, and bicyclophosphane was distilled under reduced pressure (46−48 °C under 10^{-2} Torr, yield 65%). To 1 g of bicyclophosphane (5.29 mmol) in 20 mL of acetonitrile was added 1.42 g (5.29 mmol) of palmitic acid, and the precipitated crystalline product was filtered off and recrystallized in ethyl acetate (mp 65 °C, yield 65%). ^{1}H NMR ($CDCl_3$): 0.80 (t, 3H, $^3J_{HH}$ = 7, CH_3 palm.); 1.1 (s, 24H, 12 CH_2 palm.); 1.48 (2H, $CH_2\beta$); 2.29 (m, 2H, CH_2CO); 1 (s, 3H); 1.2 (s, 3H); 1.3 (s, 3H); 1.4 (s, 3H); (4 CH_3); 3.05(d, 1H, $^2J_{HH}$ = − 15.8, NC*H*H); 3.2 (d, 1H, $^2J_{HH}$ = − 15.8, NCH*H*); 3.62 (d, 1H, $^2J_{HH}$ = − 17.8, NC*H*H); 4 (d, 1H, $^2J_{HH}$ = − 17.8, NCH*H*); 6.66 (d, 1H, $^1J_{HP}$ = 705.8, *H*P). ^{13}C NMR ($CDCl_3$): 14.1 (s, CH_3 palm.); 25.3 (d, $^3J_{CP}$= 7.0); 27.4 (d, $^3J_{CP}$= 7.2); 27.9 (d, $^3J_{CP}$= 7.0); 28.1 (d, $^3J_{CP}$= 8.0); 58.14 (s); 58.85 (s); 82.4 (d, $^2J_{CP}$= 9.1); 84.7 (d, $^2J_{CP}$= 9.1); 174 (s). ^{31}P NMR ($CDCl_3$): −5.6. $^1J_{PH}$ = 720 Hz. $C_{24}H_{48}NO_4P$ calcd (%): C, 64.68; H, 10.85; N, 3.14. (445.628) exp (%): C, 64.74; H, 10.81; N, 3.17.

Synthesis of DOXP−OH. To a solution of DOXP−H (1 g, 2.24 mmol) in pyridine was added 570 mg (2.24 mmol) of I_2 in pyridine/H_2O (98/2, v/v), and the reaction mixture was stirred 1 h at room temperature. Then, 20 mL of $CHCl_3$ was added and the solution was washed with 10% H_2SO_4, carbonate water, and water and dried with Na_2SO_4. DOXP−OH was obtained by precipitation in pentane (yield 25%). ^{1}H NMR ($CDCl_3$): 0.82 (t, 3H, CH_3 palm.); 1.19 (s, 24H, 12 CH_2 palm.); 1.35 (s, 6H, CH_3 cycl.); 1.42 (s, 6H, CH_3 cycl.); 1.57 (m, 2H, $CH_2\beta$CO); 2.27 (t, 2H, CH_2CO); 1 (s, 3H); 3.40−4.20 (m, 4H, NC*H*$_2$); 8.8 (m, 1H, O*H*). ^{13}C NMR ($CDCl_3$): 14.40 (s, CH_3 palm.); 23.13−33.96 (s, CH_2 palm.); 26.44 and 27.48 (CH_3 cycl.); 51.86 and 56.22 (2s, NCH_2); 81 (d, $^2J_{CP}$ = 7.66, COP); 82.89 (d, $^2J_{CP}$ = 7.8, COP); 173.74 (CO). ^{31}P NMR ($CDCl_3$): −11.2. Mass spectrometry (DCI/NH3): *m*/*z* = 462 (21%) MH^+.

Determination of Platelet Aggregation. Fresh blood from drug-free volunteers was drawn onto 0.129 M sodium citrate (1 vol for 9 vol of blood), and platelets were isolated at room temperature as described previously (*34*, *55*). Briefly, anticoagulated blood was centrifuged at 120*g* for 10 min. Platelet-rich plasma was acidified at pH 6.4 with acid-citrate-dextrose (ACD, 85 mM trisodium citrate, 111 mM glucose, 65 mM citric acid) and centrifuged at 1000*g* for 15 min, and sedimented platelets were suspended in Hepes buffer (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose, 10 mM Hepes pH 7.35) at 3×10^8 cells/mL. All of the experiments were performed in a platelet aggregometer

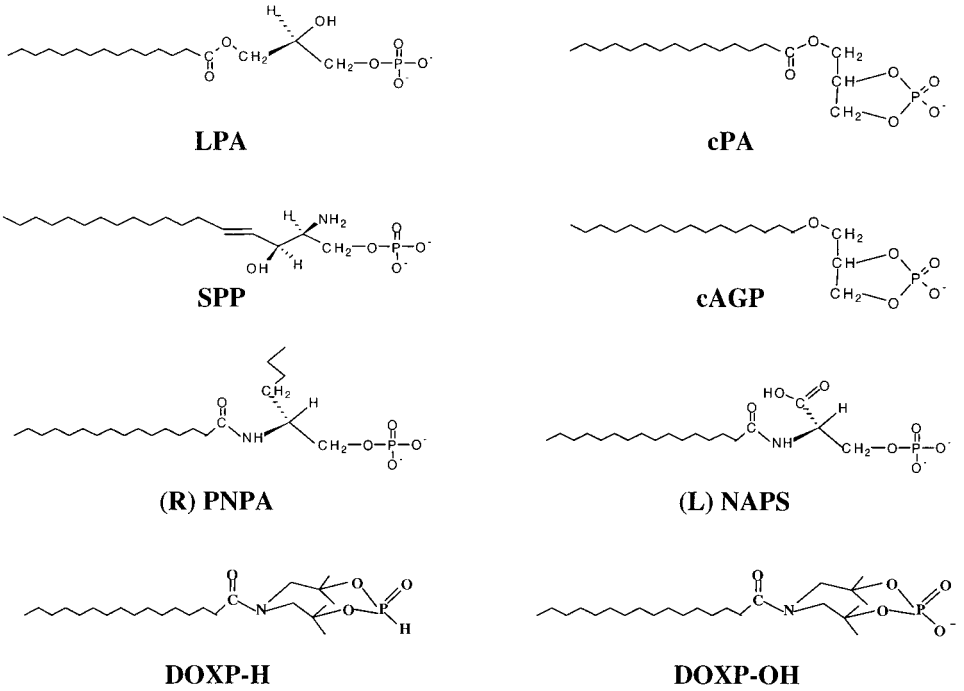

FIGURE 1: Chemical structure of LPA and its analogues.

(Payton Ionotrance) at 37 °C under stirring (900 rpm) by the turbidimetric method of Born (*56*). The various phospholipids were added directly to the platelet suspensions as ethanolic solutions under a minimal volume (4 μL for 400 μL of platelet suspension). When this method was used, platelets were fully responsive to LPA (*34*, *55*) and at variance with Benton et al. (*57*), addition of millimolar calcium concentrations was rather inhibitory. Under these conditions, aggregation depended on cyclooxygenase products and on released ADP. Indeed, indomethacin (40 μM) converted stable aggregation into reversible aggregation, in agreement with previous data (*38*), whereas a mixture of creatine phosphate (5 mM) and creatine phosphokinase (40 units/mL) abolished platelet response to LPA. In contrast, creatine phosphate or creatine phosphokinase alone was without effect.

To study the inhibitory effect of some of the phospholipids, we added these at various concentrations to platelet suspensions 1 min before LPA (1 μM), SPP (5 μM), cAGP (5 μM), or thrombin (0.2 IU/mL) under continuous stirring.

For cross-desensitization experiments, two different procedures were applied. In the first one, used in experiments depicted in Figures 2 and 3, a given phospholipid displaying agonistic activity was added to an unstirred platelet suspension at 37 °C, that is, under conditions where aggregation did not occur. After 1 min, stirring was turned on, followed immediately by the addition of a second phospholipid. One minute later, thrombin (0.2 IU/mL) was also added to verify that platelets remained fully responsive to a second unrelated agonistic compound. In the second procedure (see Figure 6), platelets were incubated for 5 min at 37 °C in the presence of a given phospholipid (5 μM). Then 0.2 vol of ACD was added, followed by centrifugation at 1000*g* for 15 min. Platelets were suspended in Hepes buffer, and aliquots were incubated at 37 °C under stirring with subsequent addition of fibrinogen (0.5 mg/mL, final concentration), of a second phospholipid (5 μM) and of thrombin (0.2 IU/mL) as above. Indeed, preliminary experiments indicated that cell washing required the addition of fibrinogen for platelets to keep their responsiveness to LPA.

RESULTS

Induction of Platelet Aggregation by Various LPA Analogues. Figure 1 gives the structures of the various compounds used in the present investigation. Almost all of them roughly meet the criteria defined in previous studies for LPA receptor agonists, that is, a long aliphatic chain and a free phosphate group, but this might not be always sufficient to reach biological activity (*44*, *49*).

(*R*) and (*S*)PNPA induced a stable platelet aggregation, which was very similar to that induced by LPA (Figure 2A). Although the compound bearing the (*R*) configuration of natural phospholipids appeared somewhat more active at low concentration, the dose−response curve presented in Figure 2C did not reveal any gross difference between the two enantiomers. Moreover, cross-desentitization could be demonstrated between LPA, (*R*)PNPA, and (*S*)PNPA. Indeed, as illustrated in Figure 2B, platelets treated with LPA in the absence of stirring did not aggregate, owing to the lack of cell to cell contact. When stirring was then applied after 1 min to the same platelet suspension, aggregation did not occur even upon addition of (*R*)PNPA, (*S*)PNPA (Figure 2B), or LPA (not shown), whereas thrombin remained able to induce a full platelet aggregation. Finally, previous addition of (*R*)PNPA desensitized platelets to a second addition of (*S*)PNPA and *vice versa* (Figure 2B).

Very similar data were observed for (D) and (L)NAPS, which revealed an equal potency at inducing platelet aggregation (Figure 3A), whereas both compounds displayed cross-desentization with LPA (Figure 3B). This observation was rather puzzling, since NAPS was previously described as a specific inhibitor of platelet aggregation induced by AGP (*44*). Sugiura et al. (*44*) used platelet suspensions containing

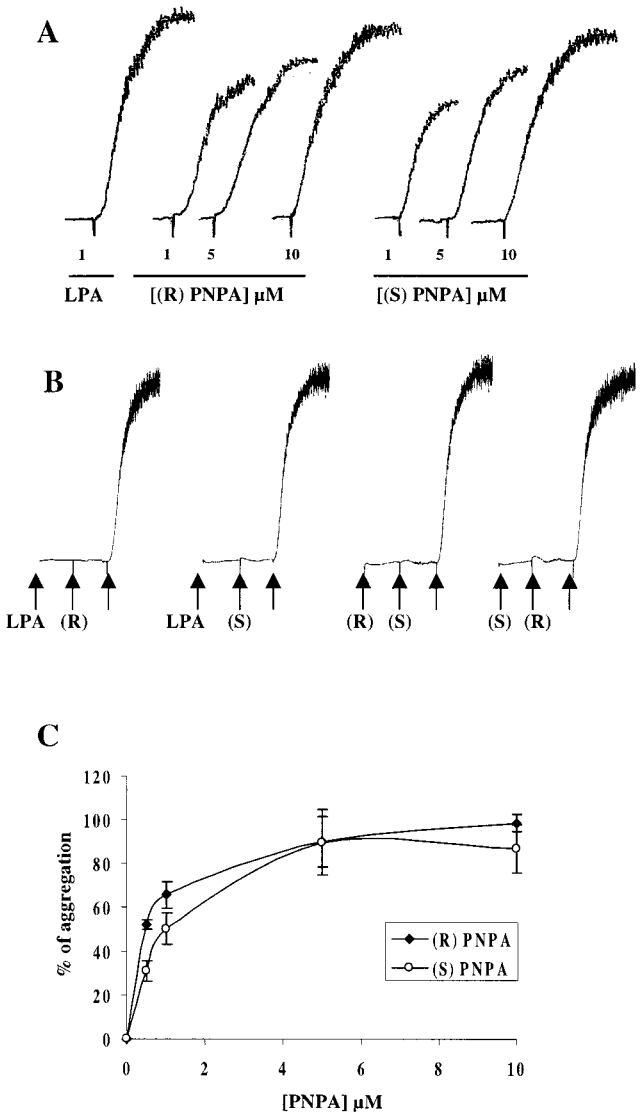

FIGURE 2: Effects of (*R*) and (*S*)PNPA on human platelets. (A) Typical aggregation traces in response to addition of LPA (1 μM) and (*R*) or (*S*)PNPA (1−10 μM). (B) Cross-desensitization between LPA and (*R*) and (*S*)PNPA. The first agonist (1 μM) was added to unstirred platelet suspension, briefly mixed, and incubated for 1 min. Stirring was then turned on, followed by the addition of the second analogue (1 μM) and, after 1 min, of thrombin (0.2 IU/mL, nonlabeled arrows). **(**C**)** Dose−response curves of platelet aggregation induced by (*R*) and (*S*)PNPA. The 100% value is the aggregation induced by 1 μM of palmitoyl LPA. Results are mean ±sd ($n = 5$).

1.3 mM $CaCl_2$, in contrast to our present experiments all performed in a calcium-free medium. As shown in Figure 3C, addition of calcium to platelets had a slightly inhibitory effect on LPA-induced platelet aggregation, whereas it abolished the agonistic effect of NAPS.

In contrast, cPA bearing either a myristoyl, palmitoyl, stearoyl, or oleoyl chain did not trigger any obvious platelet response (not shown), whereas cAGP was revealed to be a poor agonist inducing only reversible platelet aggregation (Figure 4A). In addition, the extent of aggregation did not exceed 41% of the platelet response to LPA (Figure 4B). SPP also appeared as a weak inducer of platelet aggregation, as illustrated in Figure 4. Mass spectrometry allowed us to exclude that the effects of SPP could be due to contamination by 1-*O*-alkenyl-*sn*-glycero-3-phosphate, as recently reported

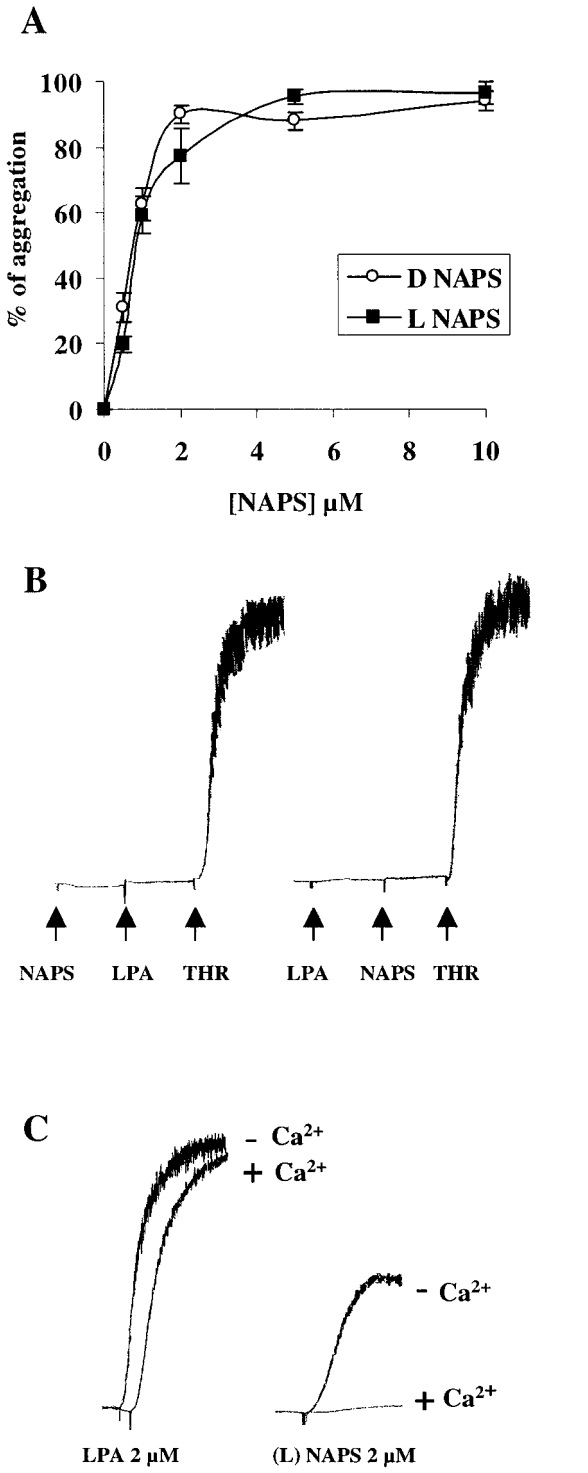

FIGURE 3: Effects of (L) and (D)NAPS on human platelets. (A) Dose−response curve for platelet aggregation induced by (D) and (L)NAPS. The 100% value is the aggregation induced by 1 μM of palmitoyl LPA. Results are means ±sd ($n = 3$). (B) Cross-desensitization experiments between LPA and (L)NAPS. The procedure was the same as that used in Figure 2. Platelets desensitized for 1 min by (L)NAPS (1 μM) did not respond to an additional dose of LPA (1 μM), and the desensitization occurred when the compounds were applied in the reverse order. In contrast, thrombin-induced platelet aggregation (THR) remained unaffected. Identical data were obtained with (D)NAPS (not shown). (C) The effect of calcium on platelet aggregation induced by LPA and (L)-NAPS. Platelets were suspended in a medium lacking calcium (− Ca^{2+}), or containing 1 mM $CaCl_2$ (+ Ca^{2+}) and challenged with LPA or (L)NAPS. Data are representative of two experiments with identical results.

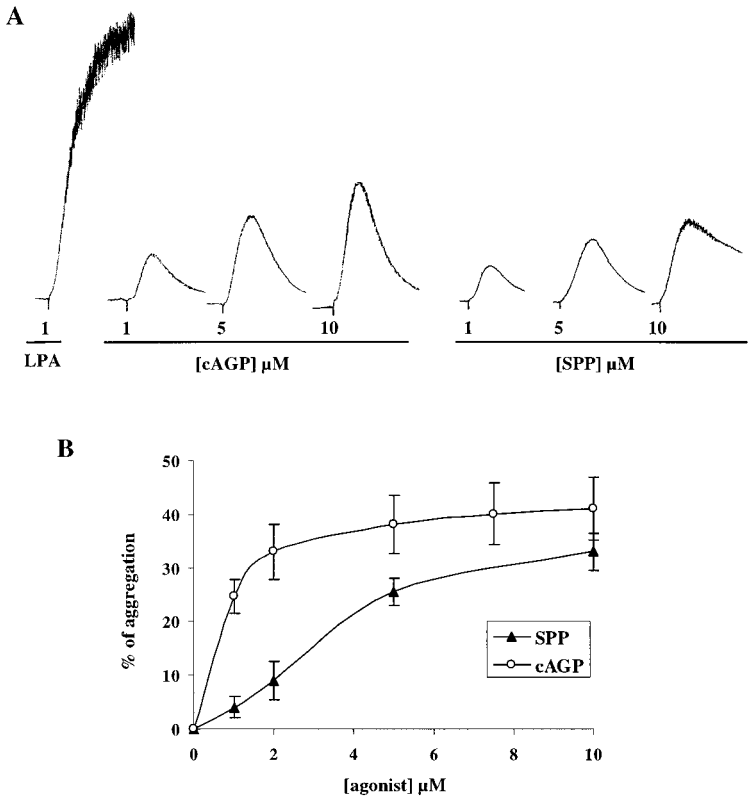

FIGURE 4: Effects of cAGP and SPP on human platelets. (A) Aggregation traces induced by LPA (1 μM), cAGP, and SPP (1 to 10 μM). (B) Dose−response curves of cAGP and SPP-induced platelet aggregation. The 100% value corresponds to platelet aggregation induced by 1 μM palmitoyl LPA. Results are means ±sd ($n = 4$).

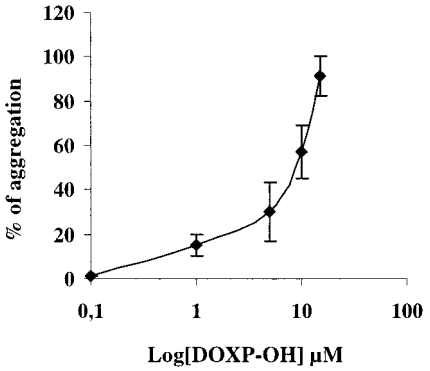

FIGURE 5: Dose−response curve of human platelet aggregation induced by DOXP−OH. The 100% value is the aggregation induced by 1 μM of palmitoyl LPA. Results are means ±sd ($n = 3$).

Table 1: Comparative Efficiency of Various LPA Analogues Inducing Platelet Aggregation

phospholipid	EC_{50} (μM)	maximal aggregation (%)
AGP[a]	0.0098 ± 0.001	100
LPA (4)	0.265 ± 0.05	100
(*R*)PNPA (5)	0.48 ± 0.02	99 ± 4
(*S*)PNPA (5)	1.05 ± 0.2	87 ± 10
(L)NAPS (3)	0.85 ± 0.1	97 ± 3
(D)NAPS (3)	0.80 ± 0.1	94 ± 2
DOXP−OH (3)	8.3 ± 0.4	96 ± 3
cAGP (4)	0.83 ± 0.09	41 ± 6
SPP (4)	3.33 ± 0.3	33 ± 4

[a] Data are taken from Simon et al. (*34*); other data are means ±sd (number of determinations in parentheses).

(*58*). Apparently, the lack of effect of cPA and the low efficiency of SPP were not due to a decreased solubility, since addition of bovine serum albumin (1%, w/v), which should improve the dispersion of the phospholipid analogues, did not modify the results (not shown). However, both cAGP and SPP desensitized platelets to LPA, without any effect on thrombin-induced platelet aggregation (not shown).

When considering DOXP−OH and DOXP−H, only the former, which contains a single negative charge on the phosphate group, was able to activate platelets. Although the dose−response curve was shifted to the right, compared to previous compounds, platelet aggregation induced by 10−20 μM DOXP−OH was almost equivalent to that induced by 1 μM LPA (Figure 5).

Table 1 reports the various EC_{50} as well as the maximal extent of platelet aggregation determined for nine of these compounds, including AGP. As stated above, these could be divided into two groups (strong or weak inducers) on the basis of maximal extent of platelet aggregation. EC_{50} varied from 9.8 nM to 8.3 μM for the first group and from 0.83 to 3.33 μM for the weaker agonists.

Cross-Desensitization of Platelets to LPA and Its Analogues Displaying Agonistic Activity. Data reported above gave evidence for a cross-desensitization occurring between LPA, (*R*) and (*S*)PNPA, (D) and (L)NAPS, as well as SPP. However, all of these experiments were performed under conditions where the two cross-desensitizing agents were present together in the same suspension. To verify that cell refractory state was still observed upon removal of the first agent, we washed platelets following preincubation with LPA or its analogues. As shown in Figure 6A, nontreated washed platelets retained a full response to LPA provided that fibrinogen was added to the suspension. Under the same conditions, platelets remained fully sensitive to other LPA

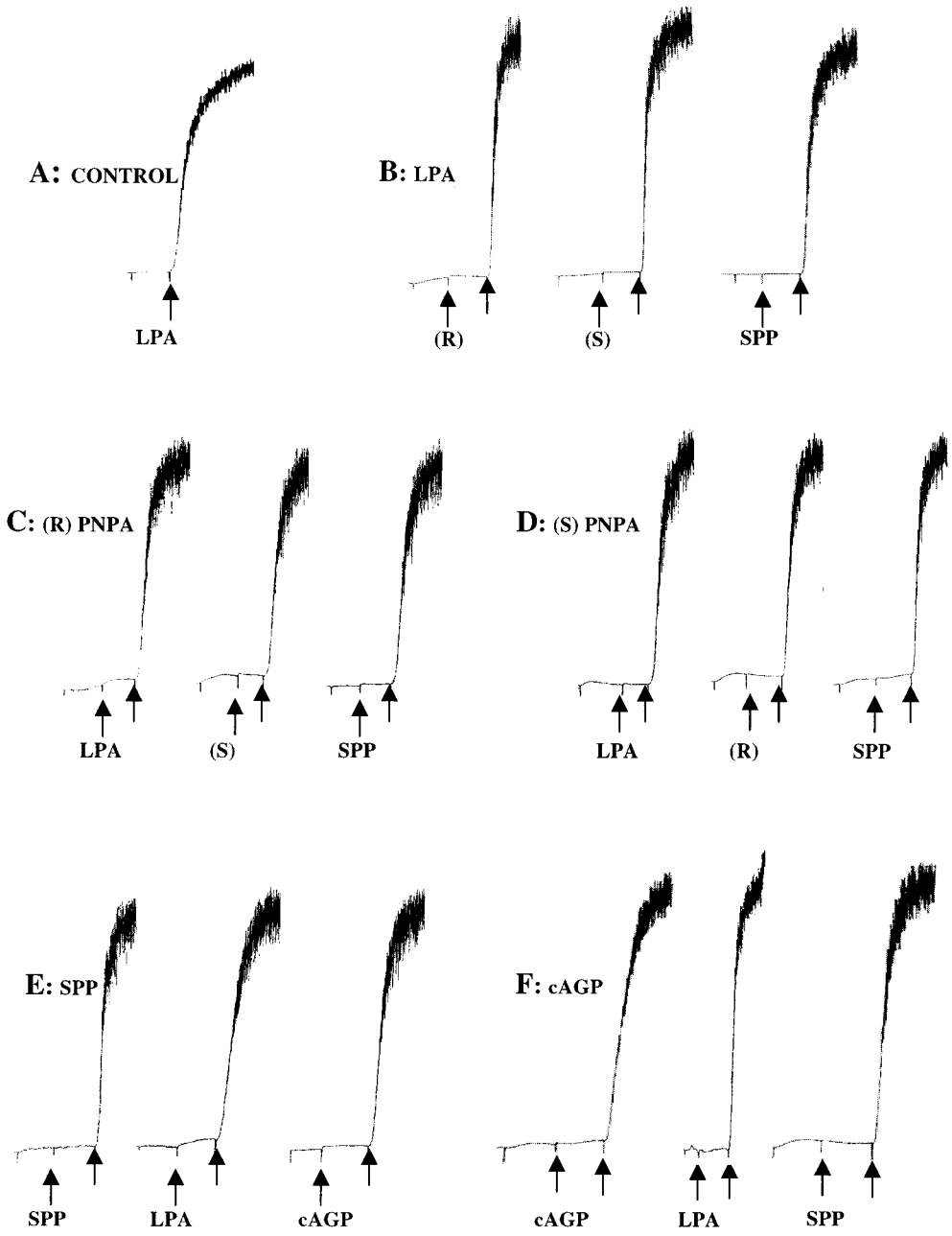

FIGURE 6: Cross-desensitization between LPA, (*R*) and (*S*) PNPA, SPP, and cAGP. In these experiments, platelets were preincubated in the absence or in the presence of a given phospholipid (5 μM) under conditions (lack of stirring) impairing platelet aggregation. Platelets were then washed as described under Materials and Methods, and aliquots were incubated under stirring at 37 °C followed by the addition of fibrinogen (0.5 mg/mL, final concentration), then after 1 min of a given phospholipid (5 μM), and, finally, after 1 min of thrombin (0.2 IU/mL, nonlabeled arrows). Platelets were preincubated in the absence of any phospholipid (A) or in the presence of LPA (B), (*R*)PNPA (C), (*S*)PNPA (D), SPP (E), or cAGP (F).

analogues (not shown). However, platelets pretreated with LPA and then washed did not respond to LPA itself (not shown), (*R*) and (*S*)PNPA, or SPP, whereas they remained fully responsive to thrombin (Figure 6B). In the same manner, upon incubation with either (*R*) or (*S*)PNPA, platelets became insensitive to LPA, (*R*) or (*S*)PNPA, and SPP but still responded to thrombin (Figure 6C,D). Identical data showing cross-desensitization between SPP, LPA, and cAGP are reported in Figure 6E. Finally, Figure 6F shows that preincubation of platelets with cAGP rendered them insensitive to cAGP itself, SPP, and LPA.

Inhibition of LPA-Induced Platelet Aggregation by DOXP−H and cPA. Both DOXP−H and cPA, which did not evoke platelet aggregation, as mentioned above, still inhibited LPA-induced platelet aggregation in a dose-dependent manner. The two inhibitors were also active against other LPA analogues such as cAGP and SPP (Figure 7A,B). This effect was specific since the platelet response to thrombin (0.2 IU/mL) or to platelet-activating factor (0.5 μM) remained unaltered in the presence of 5 μM of either DOXP−H or cPA (not shown). The inhibitory potency of the two compounds is compared in Figure 7C,D, showing that palmitoyl-cPA and DOXP−H displayed IC_{50} values of 0.94 $\pm$ 0.2 and 0.8 $\pm$ 0.1 μM, respectively, when using 1 μM LPA. Finally, the nature of the fatty acyl chain did not influence significantly the biological activity of cPA, which

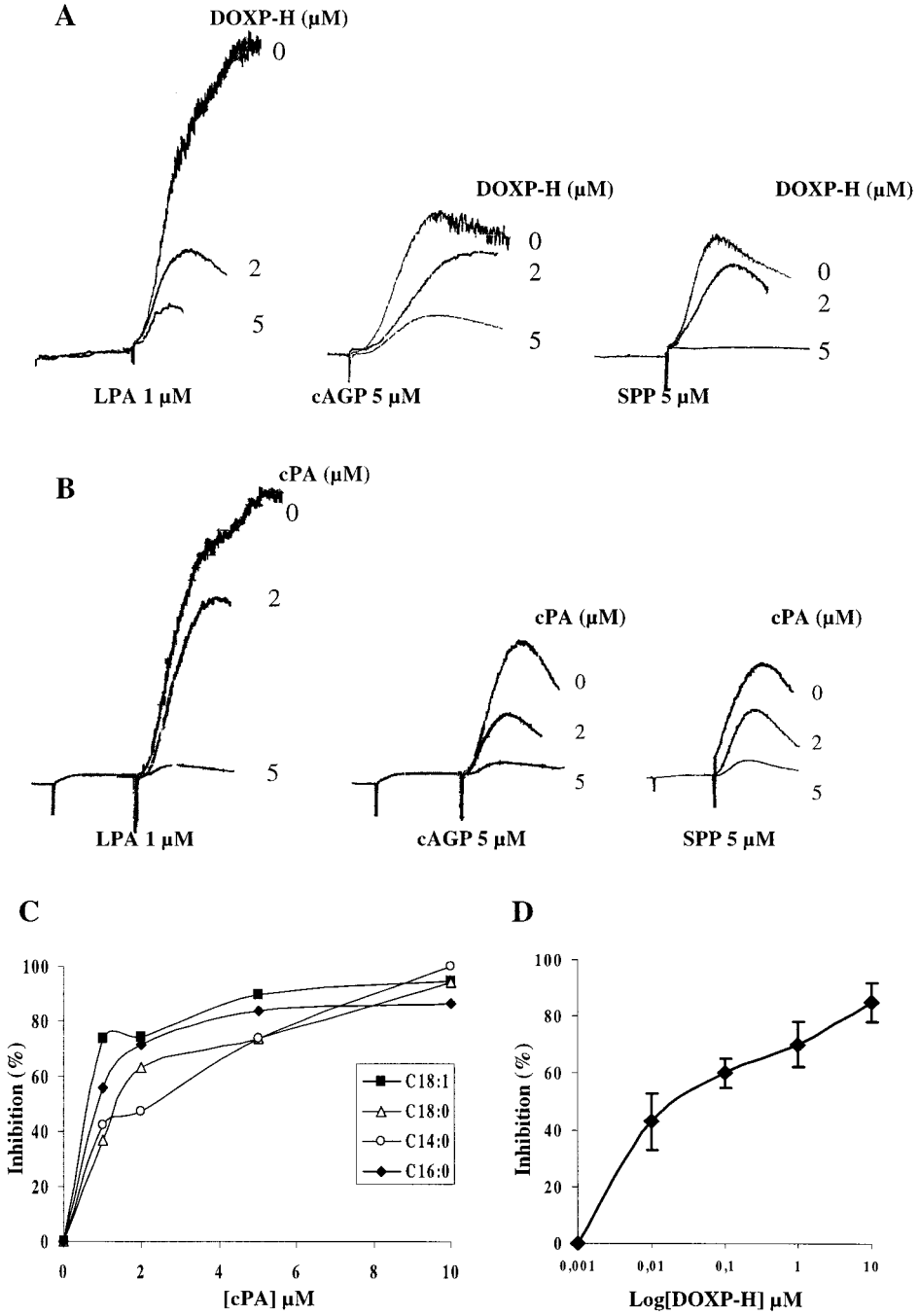

FIGURE 7: Inhibition by DOXP−H and cPA of platelet aggregation induced by LPA, cAGP, and SPP. (A) Typical aggregation traces showing the inhibitory effect of DOXP−H. Platelets were incubated for 1 min in the absence (0 μM) or in the presence (2 and 5 μM) of DOXP−H, followed by the addition of LPA (1 μM), cAGP (5 μM), or SPP (5 μM) as indicated. (B) Same procedure as in part A except that preincubations were performed in the presence of palmitoyl-cPA. Stirring was maintained over all of the experiments. (C) Dose−response curves of the inhibition of LPA-induced platelet aggregation by various cPA: C18:1, oleoyl-cPA; C18:0, stearoyl-cPA; C14:0, myristoyl-cPA; C16:0, palmitoyl-cPA. Results are means ±sd ($n = 3$) for palmitoyl-cPA and means of 2 experiments for other cPA species (standard deviation bars were omitted for the sake of clarity). (D) Dose−response curve of the inhibition of LPA-induced platelet aggregation by DOXP−H. Results are means ±sd ($n = 3$). In parts C and D, the LPA concentration was 1 μM.

was essentially the same as with myristoyl, palmitoyl, stearoyl, and oleoyl-cPA (Figure 7C).

Since cPA was recently shown to stimulate cAMP accumulation in NIH3T3 fibroblasts (*59*), a similar action was investigated in platelets. As shown in Figure 8, both cPA and cAGP had only marginal nonsignificant effects on platelet cAMP levels, in contrast to prostaglandin I_2. This indicated that cPA did not inhibit platelet aggregation by stimulating adenylate cyclase.

DISCUSSION

As a first conclusion of this study, we fully confirm the lack of stereospecificity of platelet LPA receptor(s). This was initially demonstrated by using two enantiomers of AGP (*34*) and brought some doubt about the possible existence of specific membrane receptors, as discussed by Durieux (*13*). In the present study, the lack of stereospecificity of platelet LPA receptor was observed by using two couples of

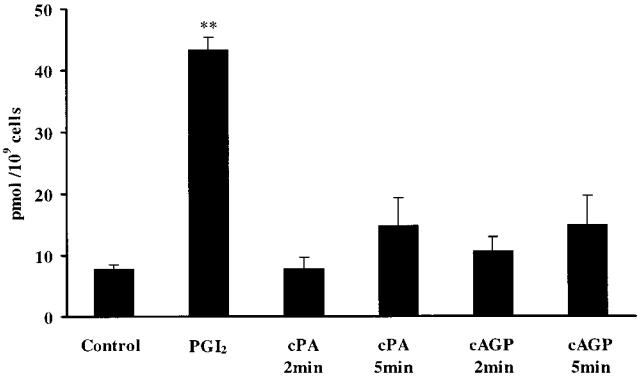

FIGURE 8: Lack of effect of cPA and cAGP on cAMP levels in human platelets. Platelet suspensions were incubated at 37 °C with prostaglandin I_2 (PGI_2, 0.5 μM, 2 min), cPA, or cAGP (10 μM, 2 or 5 min, as indicated); then cAMP level was determined using a cAMP enzyme immunoassay kit from Amersham. Data (means ± sem) are from 3 determinations: **, $P < 0.01$ according to t Student test.

analogues, PNPA and NAPS. The former compounds are derived from a family of synthetic amidophospholipids described as potent and stereospecific inhibitors of secretory phospholipases A_2 (*51*, *60*−*62*). They are easily prepared by phospholipase D degradation of parent phospholipids and display an evident structural homology with LPA, so it was not surprising that they acted as agonists of LPA receptors. However, a main difference with LPA is the presence of the fatty acyl chain on the asymmetric carbon, which was expected to create a greater difference between the two enantiomers compared to (*R*) and (*S*)AGP. This was actually not the case and was also observed with NAPS, which differs from PNPA only by the nature of one substituent on the asymmetric carbon, that is, a carboxyl instead of a *n*-butyl group. Another interpretation could be that platelets possess two different receptors displaying opposite stereospecificity. This might be suggested by some biphasic dose−response curves obtained with some of the agonists. If this were the case, the equal potency of each couple of enantiomers would be highly coincidental. In addition, the various compounds cross-desensitized each other, which is a good argument for their action on the same receptor(s), although one can never exclude that cross-desensitization simply reflects that downstream signaling pathways converge or interact.

Such a lack of stereospecificity was also reported for the LPA receptor inducing calcium mobilization in A431 cells (*49*); however this was not the case for a subtype of LPA receptor recently described in the human breast cancer cell line MDA MB231 (*48*) or for the SPP receptor identified in guinea pig atrial myocytes (*31*). Since A431 cells did not display the same selectivity for AGP versus LPA as we observed in platelets (*34*, *49*), we can thus conclude that platelets contain (a) pharmacologically distinct receptor(s).

We have attempted to identify the LPA receptor(s) possibly expressed in platelets by performing reverse transcription of mRNA coupled to polymerase chain reaction, using oligonucleotides specific for known recently cloned cDNA. These included edg-1 to edg-4 (*22*−*27*), as well as the human homologue of the cDNA cloned in *Xenopus laevis* (*28*). No specific amplification could be detected, suggesting that platelet LPA receptor differs from previously identified receptors. Although we cannot exclude a technical failure due to the low abundance of mRNA in these nonnucleated cells, the fact that recombinant LPA receptors Edg-2 and Edg-4 could be activated by cPA (*63*) further suggests that the platelet receptor(s) differ(s) from Edg-2 and Edg-4.

A surprising finding of this study was the observation that NAPS behaved as an agonist of LPA receptor, whereas it was initially described as an antagonist (*44*), as later confirmed in *Xenopus laevis* oocytes, where it also displayed a lack of stereospecificity (*46*, *47*). However, NAPS was recently described to act as an agonist or a partial agonist for Edg-2 and Edg-4, respectively (*63*). The apparent discrepancy between platelet studies is probably due to the different platelet preparations used in our laboratory compared to those of Sugiura et al. (*44*). In the latter case, platelet suspensions contained 1.3 mM $CaCl_2$, which we found to reduce LPA-induced platelet aggregation (*34*). Actually, in calcium-containing medium, induction of platelet aggregation by LPA required the addition of 10 μM ADP, another platelet-activating agent, thus demonstrating the different sensitivity to LPA of the two platelet preparations (*44*). This was fully confirmed in experiments depicted in Figure 3C, showing that NAPS became inactive in the presence of calcium. Jalink et al. (*64*) clearly illustrated the ability of LPA to form insoluble complexes with calcium, with a subsequent decrease of available LPA. From our present data, we suggest that NAPS might be still more susceptible to calcium complexation, owing to an additional carboxyl group close to the phosphate group, resulting in a loss of its agonistic activity. However, the low suboptimal concentration of NAPS still available in the medium might be sufficient to desensitize platelets, resulting in an inhibitory effect against LPA.

Similar differences in platelet sensitivity could be noted for SPP, which was found either virtually inactive (*44*) or acting as a poor agonist (ref *45*, and this study). This is also clearly illustrated by the comparison of cPA and cAGP. Indeed, the former appeared as an inhibitor, whereas the alkyl analogue had some agonistic activity and displayed cross-densensitization with LPA. Obviously, cyclization of the phosphate group greatly decreases the biological activity of the two compounds. However, since AGP was previously found to act on platelets at around 30-fold lower concentrations, the same difference is observed between the two cyclic analogues, cAGP appearing as a poor agonist and cPA as a specific inhibitor. Here again, our data differ from those obtained in *Xenopus laevis* oocytes, where cPA was described as a selective agonist of a novel subtype of LPA receptor (*30*). Apparently, the *Xenopus* cPA-selective receptor is absent from platelets, since in addition cPA completely inhibited platelet aggregation induced by LPA.

Jalink et al. (*49*) underlined the crucial requirement of a free phosphate group to maintain the agonistic activity of various LPA analogues, which disappeared in phosphono derivatives. This probably explains the difference between LPA and cPA observed here, although other steric factors might also be involved. However, this does not seem to be absolute when considering the platelet-aggregating activity of DOXP−OH, which still displays one single negative charge in the cyclic phosphate group. Maybe more interesting is the observation that the nonoxidized derivative DOXP−H behaved as a specific inhibitor of LPA. We have indeed described a novel LPA analogue with specific inhibitory

activity and unique structural features. It can thus be added to the list of other inhibitors of LPA, including NAPS and *N*-acyl-phosphotyrosine (*44*, *46*−*47*), or the anesthetics propofol (*65*). We suggest that comparison of the properties of these structurally different inhibitors toward various cells expressing natural or recombinant LPA receptors might help to define the functional properties of each subtype of LPA receptor.

Finally, this study was restricted to the analysis of the functional consequences resulting from variations in the whole structure of LPA analogues, since most of the phospholipids tested in the present study contained a sixteen-carbon saturated chain. We should keep in mind that the nature of the aliphatic chain present in these compounds might also be crucial to determine their biological activity. When the literature data is considered, it appears that the length as well as the degree of unsaturation of the fatty acid modulates the activity of LPA in a way that does not seem to be identical when comparing various biological responses, including platelet aggregation (*44*), calcium mobilization in A431 cells (*49*), proliferation of rat-1 fibroblasts (*66*), activation of serum response element-regulated genes (*67*), or vasoactive effects of LPA (*68*). The nature of the fatty acyl chain might be particularly crucial in the case of cPA, which was shown to inhibit cell proliferation and eukaryotic DNA polymerase α only when bearing a cyclopropane ring on its 16 carbon acyl chain (*69*−*72*). However, this naturally occurring cPA isolated from *Physarum polycephalum* (also called PHYLPA) was found as potent as palmitoyl-cPA when applied externally to *Xenopus laevis* oocytes (*30*). Thus our data showing essentially identical potencies of four cPA species bearing different acyl chains (Figure 7C) are in agreement with the view that cPA inhibits platelet aggregation by competing with LPA at the same membrane receptor.

In conclusion, although the molecular characterization of LPA receptor(s) expressed in platelets has not yet been achieved, our data allow us to draw a number of conclusions which definitely establish crucial functional differences with other LPA receptors. Among these, the G_q-coupled receptor present in A431 cells (*49*), the cPA-specific receptor identified in *Xenopus laevis* oocytes (*30*), the stereospecific receptor revealed with enantiomeric NAPS in MDA MB231 cells (*48*), the sphingophospholipid-specific receptors, including edg-1 and edg-3 (*24*−*26*, *29*, *31*, *73*, *74*), do not share with platelet receptors the functional features described herein with a unique series of LPA analogues. Also, neither vzg-1/edg-2 (*22*, *23*) nor the *Xenopus laevis* receptor whose cDNA was recently cloned (*28*) displays cross-desensitization between LPA and SPP, in contrast to platelet receptor. To the best of our knowledge, the only other reported case of cross-desensitization between LPA and SPP was in *Xenopus laevis* oocytes (*75*), although opposite conclusions were reached by other authors using either the same model (*30*) or the recently cloned receptor (*28*). However, a very recent study reported that edg-1, which was initially recognized as a SPP receptor (*25*, *26*), could also be stimulated by LPA acting as a low-affinity agonist (*76*). This illustrates the rather large diversity of this novel family of G-protein receptors, whose molecular characterization is still beginning and should not be restricted to sequence and expression data, but would also benefit from functional analysis with a number of structural analogues.

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