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# Lysophosphatidic Acid-Induced Platelet Shape Change Revealed Through $LPA_{1–5}$ Receptor-selective Probes and Albumin

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## Abstract

Lysophosphatidic acid (LPA), a component of mildly-oxidized LDL and the lipid rich core of atherosclerotic plaques, elicits platelet activation. LPA is the ligand of G protein-coupled receptors (GPCR) of the EDG family ($LPA_{1–3}$) and the newly identified LPA $_{4–7}$ subcluster. $LPA_4$, $LPA_5$ and $LPA_7$ increase cellular cAMP levels that would induce platelet inhibition rather than activation. In the present study we quantified the mRNA levels of the $LPA_{1–7}$ GPCR in human platelets and found a rank order $LPA_4$=$LPA_5$>$LPA_7$>$LPA_6$=$LPA_2$>>$LPA_1$>$LPA_3$. We examined platelet shape change using a panel of LPA receptor subtype-selective agonists and antagonists and compared them with their pharmacological profiles obtained in heterologous $LPA_{1–5}$ receptor expression systems. Responses to different natural acyl and alkyl species of LPA, and octyl phosphatidic acid analogs, alpha-substituted phosphonate analogs, N-palmitoyl-tyrosine phosphoric acid, N-palmitoyl-serine phosphoric acid were tested. All of these compounds elicited platelet activation and also inhibited LPA-induced platelet shape change after pre-incubation, suggesting that receptor desensitization is likely responsible for the inhibition of this response. Fatty acid free albumin (10 μM) lacking platelet activity completely inhibited platelet shape change induced by LPA with an $IC_{50}$ of 1.1 μM but had no effect on the activation of $LPA_{1,2,3,\&5}$ expressed in endogenously non-LPA-responsive RH7777 cells. However, albumin reduced $LPA_4$ activation and shifted the dose-response curve to the right. $LPA_5$ transiently expressed in RH7777 cells showed preference to alkyl-LPA over acyl-LPA that is similar to that in platelets. LPA did not increase cAMP levels in platelets. In conclusion, our results with the pharmacological compounds and albumin demonstrate that LPA does not induce platelet shape change simply through activation of $LPA_{1–5,}$ and the receptor(s) mediating LPA-induced platelet activation remains elusive.

### Keywords

lysophosphatidic acid; G protein-coupled receptor; agonist; antagonist; albumin

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## Introduction

Oxidative modifications of LDL and platelet activation are the central events in the pathogenesis of atherosclerosis and cardiovascular disease [1,2]. Oxidatively modified LDL, present in the circulation or exposed after rupture of atherosclerotic plaques to blood cells, is known to stimulate platelets [3,4]. Platelets, once activated, change their shape, aggregate, and become procoagulant, leading to the formation of a platelet- and fibrin-rich intravascular thrombus, which can precipitate acute ischemic syndromes such as unstable angina, myocardial infarction, and stroke [3,5]. Lysophosphatidic acid (LPA) has been identified as a biologically active lipid in mildly-oxidized LDL (mox-LDL), human atherosclerotic plaques, and supernatant of activated platelets [2,6,7,8,9]. It is also known that serum LPA level is significantly elevated in patients with acute myocardial infarction [10]. Importantly, oxidatively modified LDL such as mox-LDL and the lipid-rich core of human atherosclerotic plaques have been shown to stimulate platelets through the activation of LPA receptors [2,6].

LPA is predominantly associated with serum albumin [11] and acts through various sets of specific G protein-coupled receptors (GPCRs) in an autocrine and paracrine fashion [8,12, 13]. The LPA receptor gene products are expressed in most mammalian tissues with spatially and temporally regulated expression patterns. LPA receptors can be divided into two subfamilies. One is composed of three members, $LPA_1$, $LPA_2$, $LPA_3$, belonging to the Endothelial Differentiation Gene (EDG-) subfamily of GPCRs [14]. The second subfamily consists of recently identified LPA receptors: $LPA_4$ (GPR23), $LPA_5$ (GPR92), $LPA_6$ (GPR87), and $LPA_7$ (P2Y5) [15,16,17,18] which are structurally more related to the purinoreceptor (P2Y) cluster of GPCRs.

Platelets express mRNA for the $LPA_{1-5}$ receptors [19,20,21]; the expression of LPA receptors at the protein level is unknown due to the lack of specific antibodies. Although LPA-induced platelet activation is inhibited by the $LPA_1$ and $LPA_3$ receptor antagonist dioctylglycerol pyrophosphate (DGPP) (8:0), as well as either N-palmitoyl-serine phosphoric acid (NPSPA) or N-palmitoyl-tyrosine phosphoric acid (NPTPA), both of which activate several LPA receptors [7,22], the LPA responses in platelets (alkyl-LPA being potent than acyl-LPA) are not consistent with the pharmacological properties of $LPA_1$ and $LPA_3$ receptors [6,23,24]. Therefore, the receptor(s) through which LPA stimulates platelets remain unidentified. Here we applied a comprehensive set of recently identified LPA receptor agonists and antagonists to evaluate their effects in cells individually expressing $LPA_{1-5}$ and compared those with platelet responses to LPA. We have also evaluated the effect of serum albumin that inhibits LPA-induced platelet activation on LPA-GPCR individually expressed in endogenously non-responsive RH7777 cells. The present findings underline the complexity of LPA responsiveness in human platelets revealing a mismatch with the individual pharmacological properties of $LPA_{1/2/3/4/5}$.

## Materials and methods

### Materials

The LPA receptor agonists and antagonists were purchased either from Avanti Polar Lipids (Alabaster, AL) or synthesized as previously described [25,26]. ADP, apyrase (A-6535), fatty acid free bovine serum albumin (FAF-BSA), acetylsalicylic acid were obtained from Fluka (Taufkirchen, Germany). The enzyme immunoassay kit for cyclic adenosine monophosphate (cAMP) measurement was from Assay Designs, distributed by Biotrend (Cologne, Germany). DNase I, ThermoScript RT-PCR System for First-Strand cDNA Synthesis were purchased from Invitrogen (Carlsbad, CA). RT2 Real-Time SYBR Green/ROX kit was from SuperArray (Frederick, MD).

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### Agonist and Antagonist preparation

LPA species were either dissolved and diluted in ethanol, or else after ethanol evaporation reconstituted in FAF-BSA buffer (20 mM HEPES, 138 mM NaCl, FAF-BSA 0.25 mM, at a LPA/BSA ratio of 4:1). The pharmacological compounds were dissolved and used in methanol or in FAF-BSA buffer (compound/BSA ratio of 4:1), as described [26].

### Preparation of Washed Human Platelets for Activation Studies

Blood was obtained by venipuncture from healthy male and female donors using 3.13% natrium citrate as an anticoagulant. Venous blood was drawn and collected into plastic tubes containing natrium citrate in a 1:10 anticoagulant to blood ratio. All studies involving human subjects were conducted in accordance with the Declaration of Helsinki following the protocols approved by the ethical committee of LMU Munich. The whole blood was centrifuge at 180g for 20 min at 24°C. The supernatant (platelet-rich plasma) was aspirated, taking care not to disturb the blood/plasma interface and PRP was incubated in the presence of acetylsalicylic acid (1mM) and apyrase (0.3 U/mL) for 15 min at 37°C. Subsequently, citric acid (9 mM) and EDTA (5 mM) were added and PRP was centrifuged at 800 g for 20 min at 24°C. The platelets were washed in buffer B (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM $NaH_2PO_4$, 0.3 U /mL apyrase; pH 6.2). The platelet pellet was then washed once with buffer B containing apyrase (0.3U/mL). The final platelet pellet was resuspended at a concentration of 400,000/µL in buffer C (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM $NaH_2PO_4$, 5 mM glucose, 0.6 U/mL apyrase; pH 7.4).

### Measurement of Platelet Shape Change

For measurement of shape change, human platelets were isolated and incubated at 37°C with various concentrations of the LPA receptor agonists and antagonists, FAF-BSA or vehicle control before exposure to LPA. Shape change was measured by the decrease in light transmission of the stirred (1100 rpm) platelet suspension as described previously [6]. To analyze agonist effect of the compounds, washed platelets were incubated at 37°C for 2 minutes and stimulated with different concentration of compounds dissolved in methanol or FAF-BSA buffer (at a lipid:BSA molar ratio 4:1). For analysis of the antagonist effect of the compounds, washed platelets were incubated with different concentrations of the compounds at 37°C for 30 minutes or shorter and stimulated with 20nM acyl-LPA 16:0.

### Measurement of platelet cAMP

Platelets were pretreated with aspirin, washed, and resuspended in buffer (400,000/µL) containing apyrase as described above. Platelet suspensions were incubated at 37°C while stirring before exposure to LPA (0.02- 40 µM) or iloprost (50 nM) for 1 minute. Levels of cAMP were determined with an enzyme immunoassay kit from Assay Designs GmBH according to the instructions of the manufacturer.

### Isolation of mRNA from Human Platelets

Two units of platelet rich plasma (~505 ml containing ~57 ml ACD each expired for human use 1 day prior) were put through a Purecell PL high efficiency leukocyte reduction filter for platelets (PL6T from Pall Biomedical Products Company in East Hills, NY). The leukocyte reduced filtrate was then mixed with an equal volume of buffer consisting of 138mM NaCl, 3.3mM $NaH_2PO_4$, 2.9mM KCl, 1mM $MgCl_2$, 20mM HEPES, 1mg/ml glucose, containing 15% of 0.8% citric acid, 2.2% Na-citrate2$H_2O$, 2.45% glucose and 1µM $PGE_1$ (Sigma-Aldrich, St. Louis, MO). The sample was centrifuged at 3000 × rpm for 10 min at room temperature and the supernatant was carefully removed and discarded. The platelet pellet was transferred to 2ml Eppendorf tubes and 1ml of TRIZol reagent (Invitrogen, Carlsbad.CA) was added. RNA

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was extracted according to the protocol provided by the manufacturer. The RNA pellet was dissolved in 15 μl DEPC-treated water yielding ~50 μg per unit of platelets.

### Quantitative PCR of LPA Receptor mRNA in Human Platelets

One μg of total RNA was digested with DNase I and used for the subsequent synthesis of cDNA using the First Strand Synthesis kit as recommended by the manufacturer. The following primer pairs were used: $LPA_1$: (forward) GTCTTCTGGGCCATTTTCAA and (reverse) TCATAGTCCTCTGGCGAACA; $LPA_2$: (forward) GGGCCAGTGCTACTACAACG and (reverse) ACCAGCAGATTGGTCAGCA ; $LPA_3$: (forward) GAAGCTAATGAAGACGGTGATGA and (reverse) AGCAGGAACCACCTTTTCAC ; $LPA_4$: (forward) TCTGGATCCTAGTCCTCAGTGG and (reverse) CCAGACACGTTTGGAGAAGC ; $LPA_5$: (forward) CGCCATCTTCCAGATGAAC and (reverse) TAGCGGTCCACGTTGATG ; $LPA_6$: (forward) AAATCCAGCAGGCAATTCAT and (reverse) CCCTGATGCTCTGGTTATGTT ; $LPA_7$: (forward) TCTGGCAATTGTCTACCCATT and (reverse) TCAAAGCAGGCTTCTGAGG ; β-actin: (forward) TTCTACAATGAGCTGCGTGTG and (reverse) GGGGTGTTGAAGGTCTCAAA. The primer sets were designed with a melting temperature of 59–61°C. Amplicon size was 50–200 bases. Amplification was performed for 40 cycles at 94ºC/15 sec and 60ºC /60 sec using an ABI Model 7300 Real Time PCR machine (Foster City, CA). Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase of fluorescence is first detected. The transcript number of human β-actin was quantified as an internal RNA control, and each sample was normalized on the basis of its β-actin content. The relative gene expression level of each gene was then normalized to $LPA_1$ gene (calibrator). Final results, expressed as N-fold difference in gene expression relative to β-actin and LPA1, termed N, were calculated as: $N = 2^{(\text{Ct gene - Ct calibrator})}$ (http://dorakmt.tripod.com/genetics/realtime.html), where Ct values of the gene and calibrator were determined by subtracting the average Ct value of a target gene from the corresponding Ct value of the β-actin gene.

### Measurement of LPA-elicited $Ca^{2+}$transients

Wild type McArdle rat hepatoma (RH7777, from ATCC) cells do not respond to LPA with changes in $[Ca^{2+}]_i$. RH7777 cell lines individually expressing either $LPA_1$, $LPA_2$, $LPA_3$ or $LPA_5$ receptors and Chinese hamster ovary (CHO) cell line were used to examine agonism and antagonism of the compounds. CHO cells stably expressing either vector or $LPA_4$ were a kind gift from Dr. Takao Shimizu (University of Tokyo, Tokyo, Japan).

Stable transformants of $LPA_{1/2/3}$ receptors; RH7777 cells stably expressing each receptor were plated onto poly-L-lysine (PLL, 0.1 mg/ml)-coated black-wall clear-bottom 96- well plates (Corning Incorporated Life Sciences, Acton, MA) at a density of $5 \times 10^4$ cells/well and cultured overnight. The following day, the culture medium was replaced with modified Krebs buffer (120 mM NaCl, 5 mM KCl, 0.62 mM MgSO4, 1.8 mM CaCl2, 10 mM HEPES, 6 mM glucose, pH 7.4), and the cells were serum starved for 6 h. Subsequently, cells were loaded with Fura-2 AM (Invitrogen, Carlsbad, CA) for 35 min in modified Krebs buffer containing 2% (v/v) pluronic acid. Stable transformants of $LPA_4$; CHO cells stably expressing either vector or $LPA_4$ were plated non-coated 96 well plates at a density of $4 \times 10^4$ cells/ well and cultured overnight. The following day, cells were loaded with Fura-2 AM for 1 h in modified Krebs buffer containing 2% (v/v) pluronic acid and 2.5mM probenecid.

For transient transfection of $LPA_5$, RH7777 cells in 10 cm dish at a density of $2 \times 10^6$ were transfected with 2 μg of plasmid DNA with Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions for 24 h, then replated onto PLL-coated 96- well plates at a density of $5 \times 10^4$ cells/well and cultured overnight. The following day, the culture medium

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was then replaced with modified Krebs buffer, and the cells were serum starved for 4 h. Subsequently, cells were loaded with Fura-2 AM for 30 min in modified Krebs buffer containing 2% (v/v) pluronic acid, rinsed with Krebs buffer and changes in the intracellular $Ca^{2+}$ concentration were monitored by determining the ratio of emitted light intensities at 520 nm in response to excitation at 340 and 380 nm using FLEXstation II (Molecular Devices, Sunnyvale, CA). Each well was monitored for 80–120s. For testing agonist activity of the compounds, the test compounds were added automatically after 15 s of baseline measurement. To determine antagonist properties, varying concentrations of the compounds were mixed with constant concentration of LPA and responses were monitored. Each test was performed in quadruplicate. CHO cells endogenously express $LPA_1$, therefore, to access the effect of $LPA_4$ in CHO cells, the response in vector-transfected cells were subtracted from the response in $LPA_4$-transfected cells.

### Statistical Analysis

Significant difference was determined by the Student's test at a $P$ value of 0.05. $IC_{50}$ values were calculated by fitting a sigmoid function to data points by using the nonlinear curve-fitting feature of KaleidaGraph (Synergy Software, Essex Junction, VT).

## Results

### Expression of LPA Receptors mRNAs in Human Platelets

We applied real-time PCR to quantify the abundance of $LPA_{1-7}$ mRNAs in purified human platelets. We used platelets isolated and pooled from four healthy human donors. The platelets used for mRNA extraction have been depleted of white blood cells and red blood cells using Purecell PL membranes. The purified platelet preparation was stained with May-Grunwald Giemsa stain and non-platelet cells were counted. The preparation contained less than 0.01% white blood cells and was considered highly pure for platelets. The abundance of LPA receptor RNA arbitrarily normalized to $LPA_1$ is shown in figure 1. The rank order of abundance was $LPA_4 = LPA_5 > LPA_7 > LPA_6 = LPA_2 >> LPA_1 > LPA_3$. These results suggest that LPA receptors of the purinergic cluster represent the most abundant number of transcripts in human platelets.

### The effect of short chain octyl-serinediamide phosphates on platelets and $LPA_{4,5}$ receptors

Durgam and colleagues [26] have synthesized and partially characterized analogs of phosphatidic acid (PA) ***1–7*** (Figure 2). Compounds ***2, 3,*** and ***6, 7*** had no agonist activity, but potent antagonist effects on $LPA_1$ and $LPA_3$ expressed in RH7777 cells; compounds ***4*** and ***5*** had agonistic activity on $LPA_{1-3}$ , but no antagonistic activity; compound ***1*** was a mixed $LPA_2$ agonist/ $LPA_{1,3}$ antagonist (Figure 2; Table I). We expanded the characterization of these compounds to human platelets that express very low copies of $LPA_1$ and $LPA_3$ (Figure1). The compounds were dissolved in either methanol or FAF-BSA buffer and tested for agonist and LPA-antagonist activity on human platelets. Methanol at the highest concentration tested (0.5% V/V) had no effect on LPA-mediated platelet activation (Figure 3A), whereas the FAF-BSA buffer (5μM) inhibited the LPA-response (Figure 3B). Nonetheless, the agonist activity of the compounds was independent of the type of vehicle used and the maximal efficacy of the drugs ($E_{max}$) was not significantly changed (Figure 3A and B; data not shown). Due to the interfering effect of 5 μM FAF-BSA with LPA-induced platelet activation, the compounds were dissolved in methanol for further analysis. In contrast to the results in heterologous expression system, all seven compounds induced platelet activation, and after 30 min incubation, inhibited the LPA-induced platelet shape change in a concentration-dependent manner (Table I). The $EC_{50}$ values of the compounds were much higher than that of LPA and showed no correlation with their potency established at $LPA_{1-3}$. Surprisingly, the $LPA_3$ selective antagonist compound ***7*** was maximally active in inducing platelet shape change with a very low $EC_{50}$

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(~550 nM) even though $LPA_3$ was the least abundant transcript in platelets (Table I, Figure 1–Figure 3). These data reveal a structure-activity relationship (SAR) in platelets that is not consistent with that of $LPA_1$, $LPA_2$, and $LPA_3$.

Since the compound ***4*** an apparent $LPA_1$/$LPA_3$ agonist, was found to be a weak agonist, and had a higher antagonistic than agonistic potency ($IC_{50}$ 0.35±0.1 μM; $EC_{50}$ 2.43±1.1 μM) (Figure 2; Table I), further experiments were carried out with this compound to distinguish whether platelet inhibition by ***4*** was due to LPA receptor desensitization or antagonism. Concentrations of ***4*** that did not induce shape change were chosen, and incubated with platelets for various times (5 sec, 5 min, 15 min) before addition of LPA. No inhibition was found after 5 sec pre-incubation but shape change was inhibited progressively at longer pre-incubation times, consistent with a mechanism of receptor desensitization rather than a LPA receptor antagonistic effect (Figure 4).

As the compounds ***3***, ***5***, ***7*** induced platelet shape change with higher potency, we also determined their action on $LPA_4$ and $LPA_5$ receptors. All compounds had no effect on $LPA_4$ activation and were weak agonists of $LPA_5$ (Table I). These data indicate that $LPA_5$ could be involved in LPA-induced platelet shape change.

## The effect of N-palmitoyl amino phosphoric acids on LPA responses in platelets and heterologously expressed $LPA_{1–5}$

We and others have shown previously that compounds ***10*** NPSPA and ***11*** NPTPA (Figure 2) effectively induce platelet shape change and aggregation; however, after preincubation inhibit platelet responses to LPA [6,27,28]. NPSPA was slightly more potent than NPTPA, the $EC_{50}$ and $IC_{50}$ of these phospholipids were in a similar range (between 0.2 and 0.3 μM) [6]. These compounds were reported not to inhibit the EDG family LPA receptors [22]. Here we tested both compounds for their effects on $LPA_{1–5}$ (Table II). NPSPA was a weak agonist of the EDG family LPA receptors, had no effect on $LPA_4$ and was a partial agonist of $LPA_5$. Also, NPTPA was inactive on $LPA_4$ and in the micromolar range was partial agonist of $LPA_5$. In contrast to NPSPA, NPTPA weakly inhibited $LPA_1$ with a $K_i$ of 533 nM and had no agonist or antagonist effect on $LPA_2$, $LPA_3$. Like in the case of the PA analogs, we could not draw a clear correlation between their pharmacological profile and the expression of the LPA receptor found in platelets.

## The effect of $LPA_4$ receptor agonist and antagonist compounds on platelets and $LPA_5$

*Jiang et al*. reported the initial characterization of a series of α-substituted phosphonate analogs of LPA and the first two compounds ***8*** and ***9*** (Figure 2) with $LPA_4$ receptor agonist and antagonist properties, respectively [25]. Here we have expanded the characterization of the compounds to include $LPA_5$ and also assessed their actions in platelets. Both compounds ***8*** and ***9*** were weak agonists of $LPA_5$ yielding 59% and 45% activation of the maximum response to LPA 18:1 at 10 μM, respectively (Table III). Both α-substituted phosphonate analogs activated platelets and inhibited LPA-induced platelet shape change after 30 min pre-incubation. Interestingly, these two phosphonate analogs were more active than the PA analogs as shown by their lower $EC_{50}$ values (Table III). These data indicate that these analogs are not specific for $LPA_4$ and also suggest that not $LPA_4$ but $LPA_5$ could be involved in LPA-elicited platelet shape change. We have also examined the agonist properties of alkyl-LPAs relative to acyl-LPAs at $LPA_4$ expressed heterologously in CHO cells and found that the alkyl-ether-analogs were weaker agonists than the acyl-analogs with an equal number of hydrocarbons (Table III).

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### $LPA_5$ Receptor and Platelet Activation Shows Similar SAR

Recently GPR92 has been identified as a new LPA receptor ($LPA_5$) and we have been able to test the SAR of this receptor in RH7777 using the $Ca^{2+}$ mobilization assay [16]. In platelets and in $LPA_5$–transfected RH7777 cells a similar rank order of activation was found: alkyl-LPA 18:1 ≥ alkyl-LPA 16:0 > acyl-LPA 18:1 >> alkyl-LPA 18:0 (Table IV; Figure 5; data not shown). This striking similarity in the SAR argues for the possible involvement of $LPA_5$ receptor in mediating LPA-induced platelet shape change.

### The effect of FAF-BSA on LPA-induced platelet shape change

In serum, LPA is predominantly associated with serum albumin [11]. In order to investigate the effect of FAF-BSA on LPA-induced platelet shape change, we determined the dose-response curves for acyl-LPA 16:0 dissolved in ethanol or FAF-BSA buffer. As shown in Figure 6A, the presence of FAF-BSA (molar LPA: BSA ratio, 4:1) had only a small inhibitory effect on platelet activation induced by LPA, which was not significant ($p>0.05$). However, pre-incubation of platelets with FAF-BSA at a concentration of 2.5 μM shifted the dose-response curve of LPA to the right (Figure 6B). LPA (1 μM) could overcome the inhibition of FAF-BSA. FAF-BSA inhibited platelet shape change regardless of whether acyl- or alkyl-LPA was used (data not shown).

To quantify the inhibitory effect of albumin on LPA-induced platelet shape change, we pre-incubated platelets with increasing concentrations of FAF-BSA (0.1–5μM) before addition of 20 nM acyl-LPA 18:1 (dissolved in FAF-BSA buffer). As shown in Figure 6C, FAF-BSA dose-dependently decreased the LPA-elicited shape change response which was complete at 5μM concentration. The calculated $IC_{50}$ of FAF-BSA was 1.15±0.37 μM (mean SD, n=6). Furthermore, we investigated whether the inhibitory effect of FAF-BSA required pre-incubation with the platelets. As shown in figure 6D, inhibition of LPA-induced shape change by FAF-BSA was independent of the duration of pre-incubation time. This suggests that the inhibitory effect might be due to albumin's avidity for LPA or due to albumin modulation of platelet LPA receptors.

### The effect of FAF-BSA on $LPA_{1-5}$ receptor activation

To evaluate the effect of albumin on the LPA receptors and draw a parallel between the activation of platelet LPA receptors and that of $LPA_{1-5}$, we tested the effect of albumin on these heterologously expressed receptors. In contrast to the FAF-BSA-inhibition of LPA-induced platelet activation, the pre-incubation of FAF-BSA (15μM) for 5 min did not inhibit activation of $LPA_1$, $LPA_2$, $LPA_3$ and $LPA_5$ receptors in the heterologous expression system (Figure 7). However, $LPA_4$ activation was inhibited by 40% in the presence of 15μM FAF-BSA and the dose-response curve of LPA was shifted to the right (Figure 7).

### The effect of LPA on cAMP levels in platelet

It has been shown that $LPA_4$ and $LPA_5$ activation in addition to eliciting $Ca^{2+}$ transients induces an increase of intracellular cAMP [16,29,30]. In the present study we investigated whether LPA might also induce the activation of $G_s$ that leads to an increase of cAMP levels in platelets. Levels of cAMP in unstimulated platelets were 5.83±1.26 pmol/$10^8$ platelets (Figure 8). In platelets treated with a large concentration range of acyl-LPA 18:1 (0.02 – 40 μM) and for different times (30 s, 1 min, 5 min, 15 min) no significant change in cAMP was found ($p>0.05$) (Figure 8; data not shown). The prostacyclin-analog, iloprost (50 nM), elicited a robust increase in cAMP (Figure 8). Also acyl-LPA 16:0 and alkyl-LPA 16:0 did not affect platelet cAMP levels (data not shown). Previously we have found that LPA added subsequently to iloprost did not change the cAMP levels stimulated by this prostaglandin analog [31]. Therefore, platelet LPA receptors are unlikely to couple to $G_i$ and $G_s$.

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## Discussion

LPA accumulating in atherosclerotic plaque and oxidatively modified LDL activates human platelets [2,6,7]. Although, alkyl glycerophosphate, commonly known as alkyl-LPA, is active at subnanomolar concentrations making it a very potent platelet activating mediator, the receptor(s) mediating this effect remain unknown [6]. The LPA response in platelets shows some distinct features – preference to alkyl-LPA over acyl-LPA, sensitivity to albumin, SAR of inhibitors – that could not be reconciled with the EDG family of LPA receptors. The EDG family shows clear preference for acyl forms of LPA and little inhibition by albumin, although $LPA_3$ shows some sensitivity to this protein carrier [32]. In light of the recent discovery of a new subcluster of LPA receptors with high degree of sequence homology to the purinergic GPCR, in the present paper we extended a comprehensive analysis of SAR to $LPA_{1-5}$. The expression of some receptors of the purinergic cluster has been recently reported in human platelets [20]. While this work was nearing completion, $LPA_6$ and $LPA_7$ were reported [17, 18]. Our initial attempts to obtain $Ca^{2+}$ transients from $LPA_6$ and $LPA_7$ transfected RH7777 cells were unsuccessful so we have omitted it from our functional assays. Nevertheless, we found $LPA_6$ and $LPA_7$ transcripts in human platelets at a level similar to that of $LPA_2$. The role of $LPA_6$ or $LPA_7$ and other not yet reported LPA receptors will have to be addressed in future studies.

The main finding of this study is that the antagonists that have been developed to the EDG family $LPA_{1-3}$ receptors and to $LPA_4$ act as agonists for platelet activation. Unexpectedly, some of them, for example the selective $LPA_3$ receptor antagonist octyl serine diamide compound ***7***, were more effective in inducing platelet shape change than inhibiting $LPA_3$. This is in sharp contrast with our earlier analysis of the SAR of platelet responses in which we proposed that $LPA_1$ and $LPA_3$ activation triggers LPA-induced platelet activation [6]. The present observations supported by the low abundance of $LPA_1$ and $LPA_3$ transcripts in human platelets necessitates the revision of this working hypothesis and argues against that the $LPA_1$ or $LPA_3$ receptors mediate LPA-induced platelet shape change. Although knockout mice are available to these two receptor subtypes, direct analysis of their platelet responses is not possible because rodent platelets do not respond to LPA [33].

By comparing the biological effect of three molecular LPA species, it has been previously shown that alkyl-LPA species are more potent at inducing platelet activation than acyl-LPA [6,34]. The present study confirms in part these observations. Our experiments demonstrated that the $LPA_5$ receptor had pharmacological properties similar to LPA-induced platelet activation arguing for the possible involvement of $LPA_5$ receptor in mediating platelet shape change. However, it has been shown that $LPA_5$ activation induces an increase of intracellular cAMP in $LPA_5$ transfected HeLa HF1 and B103 cells [16,29], and an increase of cAMP mediates platelet inhibition. Similarly, $LPA_7$ has been shown to couple to the elevation of cAMP [18]. We did not observe an increase of cAMP upon LPA stimulation of platelets. Very recently, *Pamuklar* and colleagues have noted that platelets of ~20% of individuals failed to aggregate to LPA and showed an increase of platelet cAMP to LPA (1μM), which correlated with a higher expression of $LPA_4$ transcripts as compared to the LPA responsive platelet donors. These authors suggested that LPA stimulation of a receptor coupled to cAMP increase could prevent LPA induced platelet aggregation in the non-responsive donors [21]. We also observed previously donor-dependent variations of LPA-induced platelet aggregation in blood and PRP. However, LPA-induced platelet shape change in blood and washed cell suspensions was donor-independent [31]. Our results showing no measurable changes in cAMP and platelet shape change upon LPA-stimulation do not support this hypothesis.

*Tokumura et al*. have shown that albumin can dose-dependently inhibit LPA-induced platelet aggregation [34]. However, its effect on LPA-induced platelet shape change has not been

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examined systematically. In our study, we found that FAF-BSA inhibited platelet shape change induced by LPA bound to BSA in a competitive manner and independently of incubation time. It is unlikely that this inhibitory effect can be explained by the property of BSA to bind LPA, since platelet shape change was induced by LPA in complex with BSA at a molar ratio of 4:1. Albumin binds LPA with stoichiometry of 3 moles LPA to 1 mole of BSA [11,35] and we showed that LPA in complex with albumin (ratio LPA:BSA 4:1) activated platelets with similar potency as LPA dissolved in 70 % ethanol (final concentration < 0.07%). Therefore, it seems more plausible that albumin modulates LPA receptors. Indeed, *Hama et al*. showed that the inhibitory effect of albumin occurs in a receptor specific manner, and Sf9 cells expressing the $LPA_3$ receptor are more sensitive to BSA than $LPA_1$ and $LPA_2$ expressing cells [32]. However, we found that albumin had no effect on $LPA_3$ receptor activation in transfected RH7777 cells, which might be explained by the differences in the heterologous expression systems used. The lack of effect of albumin on $LPA_1$, $LPA_2$, $LPA_3$ and $LPA_5$ receptor activation in heterologous expression system further support our conclusion that these receptors cannot simply account for eliciting LPA-induced platelet shape change.

We also found that activation of $LPA_4$ receptor was significantly inhibited by a low concentration of albumin (15μM), raising the possibility that $LPA_4$ could play a role in LPA-mediated platelet activation. This would be supported by our Q-PCR data and those by others showing that $LPA_4$ and $LPA_5$ transcripts are much more abundant in human platelets than $LPA_1$ and $LPA_3$ transcripts [20,21]. However, our experiments with the compound ***9***, a $LPA_4$ antagonist, which stimulated platelets, argue against an involvement of $LPA_4$ in LPA-induced platelet activation.

In summary, the present results provide a comprehensive analysis of the pharmacological properties of the LPA-induced platelet shape changes, and indicate that LPA activates platelets not simply by either one of the $LPA_{1-5}$ receptors, but perhaps through a new LPA receptor such as $LPA_6$, $LPA_7$ or a yet unidentified GPCR. They point to the need of further studies on this pathophysiologically important question.

## Acknowledgments

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## References

1. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868–874. [PubMed: 12490960]
2. Siess W, Tigyi G. Thrombogenic and atherogenic activities of lysophosphatidic acid. J Cell Biochem 2004;92:1086–1094. [PubMed: 15258894]
3. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. Circulation 1998;98:1487–1494. [PubMed: 9769301]
4. Weidtmann A, Scheithe R, Hrboticky N, Pietsch A, Lorenz R, Siess W. Mildly oxidized LDL induces platelet aggregation through activation of phospholipase A2. Arterioscler Thromb Vasc Biol 1995;15:1131–1138. [PubMed: 7627706]
5. Sevanian A, Hwang J, Hodis H, Cazzolato G, Avogaro P, Bittolo-Bon G. Contribution of an in vivo oxidized LDL to LDL oxidation and its association with dense LDL subpopulations. Arterioscler Thromb Vasc Biol 1996;16:784–793. [PubMed: 8640406]

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6. Rother E, Brandl R, Baker DL, Goyal P, Gebhard H, Tigyi G, Siess W. Subtype-selective antagonists of lysophosphatidic Acid receptors inhibit platelet activation triggered by the lipid core of atherosclerotic plaques. Circulation 2003;108:741–747. [PubMed: 12885756]
7. Siess W, Zangl KJ, Essler M, Bauer M, Brandl R, Corrinth C, Bittman R, Tigyi G, Aepfelbacher M. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc Natl Acad Sci U S A 1999;96:6931–6936. [PubMed: 10359816]
8. Gardell SE, Dubin AE, Chun J. Emerging medicinal roles for lysophospholipid signaling. Trends Mol Med 2006;12:65–75. [PubMed: 16406843]
9. Sano T, Baker D, Virag T, Wada A, Yatomi Y, Kobayashi T, Igarashi Y, Tigyi G. Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. J Biol Chem 2002;277:21197–21206. [PubMed: 11929870]
10. Chen X, Yang XY, Wang ND, Ding C, Yang YJ, You ZJ, Su Q, Chen JH. Serum lysophosphatidic acid concentrations measured by dot immunogold filtration assay in patients with acute myocardial infarction. Scand J Clin Lab Invest 2003;63:497–503. [PubMed: 14743959]
11. Tigyi G, Miledi R. Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. J Biol Chem 1992;267:21360–21367. [PubMed: 1383223]
12. Siess W. Athero- and thrombogenic actions of lysophosphatidic acid and sphingosine-1-phosphate. Biochim Biophys Acta 2002;1582:204–215. [PubMed: 12069830]
13. Meyer zu Heringdorf D, Jakobs KH. Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. Biochim Biophys Acta 2007;1768:923–940. [PubMed: 17078925]
14. Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S, Tigyi G. International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. Pharmacol Rev 2002;54:265–269. [PubMed: 12037142]
15. Noguchi K, Ishii S, Shimizu T. Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. J Biol Chem 2003;278:25600–25606. [PubMed: 12724320]
16. Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. J Biol Chem 2006;281:23589–23597. [PubMed: 16774927]
17. Tabata K, Baba K, Shiraishi A, Ito M, Fujita N. The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor. Biochem Biophys Res Commun 2007;363:861–866. [PubMed: 17905198]
18. Pasternack SM, von Kugelgen I, Aboud KA, Lee YA, Ruschendorf F, Voss K, Hillmer AM, Molderings GJ, Franz T, Ramirez A, Nurnberg P, Nothen MM, Betz RC. G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. Nat Genet 2008;40:329–334. [PubMed: 18297070]
19. Motohashi K, Shibata S, Ozaki Y, Yatomi Y, Igarashi Y. Identification of lysophospholipid receptors in human platelets: the relation of two agonists, lysophosphatidic acid and sphingosine 1-phosphate. FEBS Lett 2000;468:189–193. [PubMed: 10692584]
20. Amisten S, Braun OO, Bengtsson A, Erlinge D. Gene expression profiling for the identification of G-protein coupled receptors in human platelets. Thromb Res. 2007
21. Pamuklar Z, Lee JS, Cheng HY, Panchatcharam M, Steinhubl S, Morris AJ, Charnigo R, Smyth SS. Individual Heterogeneity in Platelet Response to Lysophosphatidic Acid. Evidence for a Novel Inhibitory Pathway. Arterioscler Thromb Vasc Biol. 2008
22. An S, Bleu T, Zheng Y, Goetzl EJ. Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol Pharmacol 1998;54:881–888. [PubMed: 9804623]
23. Fujiwara Y, Sardar V, Tokumura A, Baker D, Murakami-Murofushi K, Parrill A, Tigyi G. Identification of residues responsible for ligand recognition and regioisomeric selectivity of lysophosphatidic acid receptors expressed in mammalian cells. J Biol Chem 2005;280:35038–35050. [PubMed: 16115890]

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24. Bandoh K, Aoki J, Taira A, Tsujimoto M, Arai H, Inoue K. Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors. FEBS Lett 2000;478:159–165. [PubMed: 10922489]
25. Jiang G, Xu Y, Fujiwara Y, Tsukahara T, Tsukahara R, Gajewiak J, Tigyi G, Prestwich GD. alpha-Substituted Phosphonate Analogues of Lysophosphatidic Acid (LPA) Selectively Inhibit Production and Action of LPA. ChemMedChem 2007;2:679–690. [PubMed: 17443831]
26. Durgam GG, Tsukahara R, Makarova N, Walker MD, Fujiwara Y, Pigg KR, Baker DL, Sardar VM, Parrill AL, Tigyi G, Miller DD. Synthesis and pharmacological evaluation of second-generation phosphatidic acid derivatives as lysophosphatidic acid receptor ligands. Bioorg Med Chem Lett 2006;16:633–640. [PubMed: 16263282]
27. Liliom K, Bittman R, Swords B, Tigyi G. N-palmitoyl-serine and N-palmitoyl-tyrosine phosphoric acids are selective competitive antagonists of the lysophosphatidic acid receptors. Mol Pharmacol 1996;50:616–623. [PubMed: 8794902]
28. Sugiura T, Nakane S, Kishimoto S, Waku K, Yoshioka Y, Tokumura A, Hanahan DJ. Occurrence of lysophosphatidic acid and its alkyl ether-linked analog in rat brain and comparison of their biological activities toward cultured neural cells. Biochim Biophys Acta 1999;1440:194–204. [PubMed: 10521703]
29. Kotarsky K, Boketoft A, Bristulf J, Nilsson NE, Norberg A, Hansson S, Owman C, Sillard R, Leeb-Lundberg LM, Olde B. Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. J Pharmacol Exp Ther 2006;318:619–628. [PubMed: 16651401]
30. Lee CW, Rivera R, Dubin AE, Chun J. LPA(4)/GPR23 is a lysophosphatidic acid (LPA) receptor utilizing G(s)-, G(q)/G(i)-mediated calcium signaling and G(12/13)-mediated Rho activation. J Biol Chem 2007;282:4310–4317. [PubMed: 17166850]
31. Haseruck N, Erl W, Pandey D, Tigyi G, Ohlmann P, Ravanat C, Gachet C, Siess W. The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: involvement of P2Y1 and P2Y12 receptors. Blood 2004;103:2585–2592. [PubMed: 14645014]
32. Hama K, Bandoh K, Kakehi Y, Aoki J, Arai H. Lysophosphatidic acid (LPA) receptors are activated differentially by biological fluids: possible role of LPA-binding proteins in activation of LPA receptors. FEBS Lett 2002;523:187–192. [PubMed: 12123830]
33. Schumacher KA, Classen HG, Spath M. Platelet aggregation evoked in vitro and in vivo by phosphatidic acids and lysoderivatives: identity with substances in aged serum (DAS). Thromb Haemost 1979;42:631–640. [PubMed: 116384]
34. Tokumura A, Sinomiya J, Kishimoto S, Tanaka T, Kogure K, Sugiura T, Satouchi K, Waku K, Fukuzawa K. Human platelets respond differentially to lysophosphatidic acids having a highly unsaturated fatty acyl group and alkyl ether-linked lysophosphatidic acids. Biochem J 2002;365:617–628. [PubMed: 11982483]
35. Thumser AE, Voysey JE, Wilton DC. The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. Biochem J 1994;301(Pt 3):801–806. [PubMed: 8053904]

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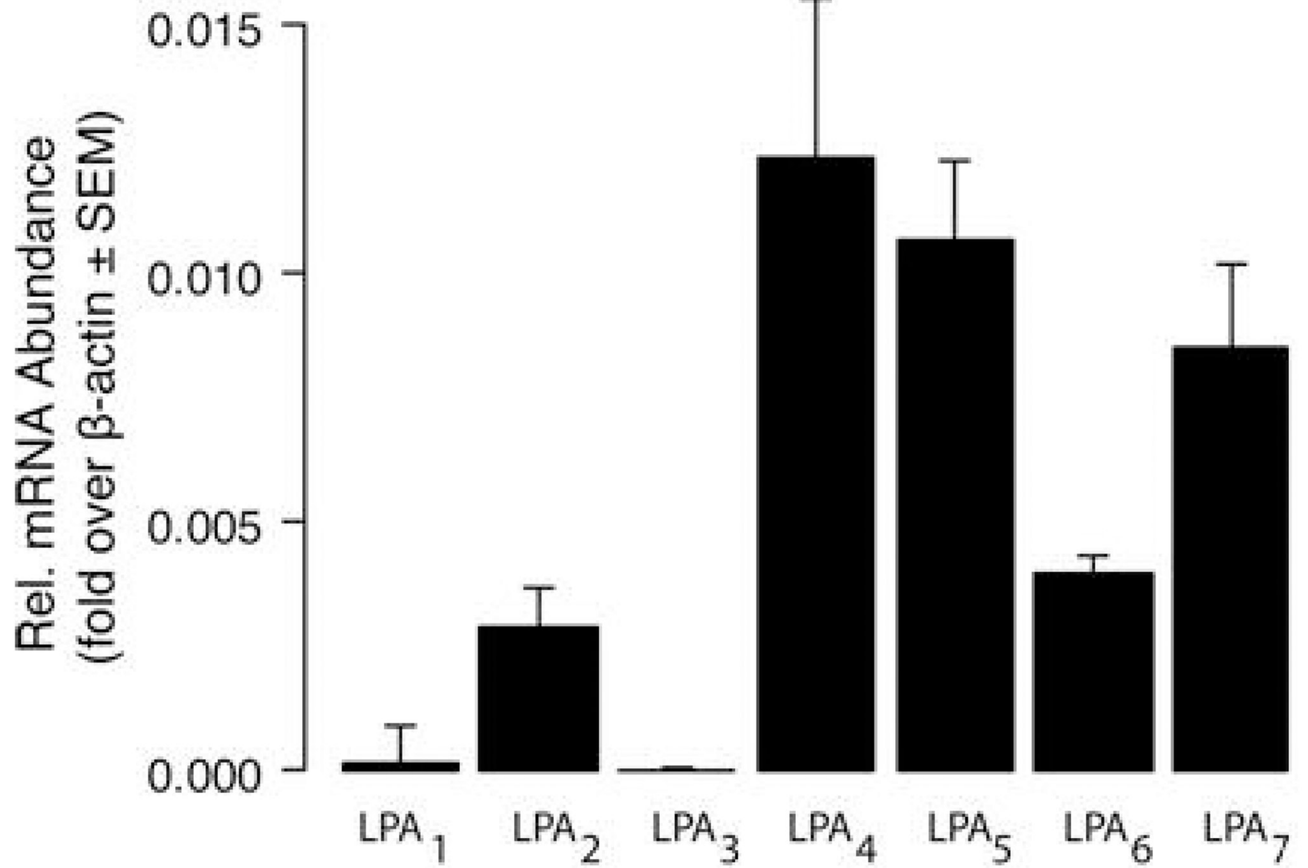


**Figure 1.**
Relative abundance of LPA receptor transcripts in purified human platelets determined by quantitative real-time PCR.

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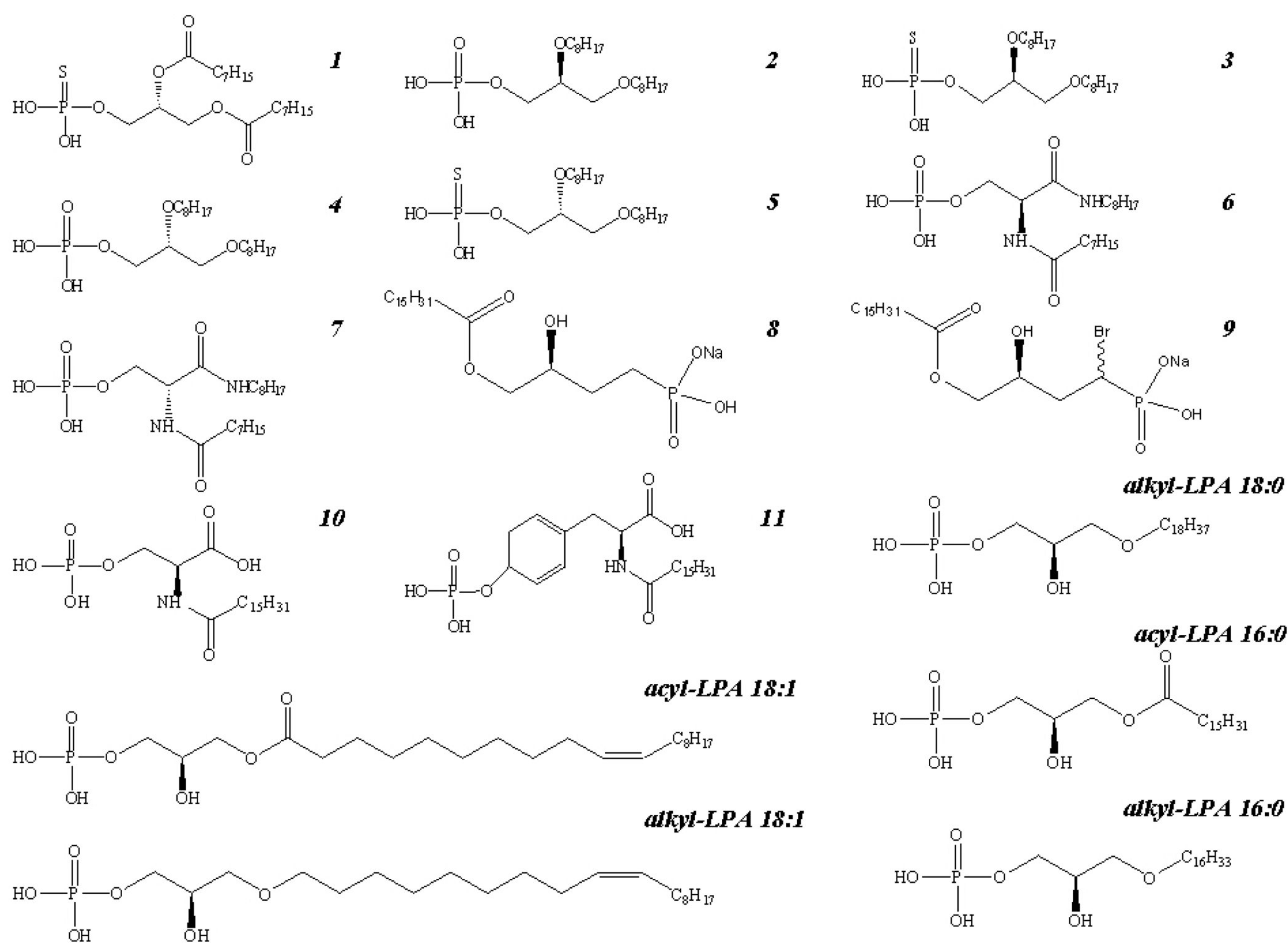


**Figure 2.**
Chemical structure of the compounds used in the present study.

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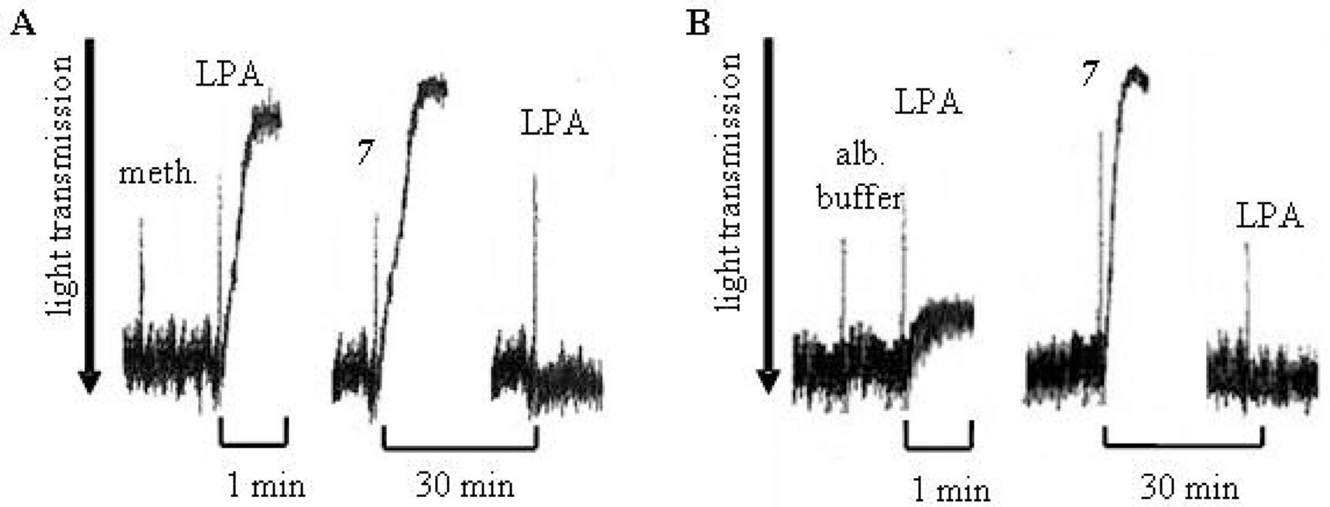


**Figure 3. Effect of $LPA_3$ antagonist (7) on platelet**

Suspensions of washed human platelets were incubated with solvent or 20 μM ***7*** dissolved **A,** in methanol or **B**, in albumin buffer (lipid:BSA 4:1) for 30 min before exposure to acyl-LPA 16:0 (20 nM). Shape change was recorded as decrease of light transmission. Tracings shown are representative for 3 experiments.

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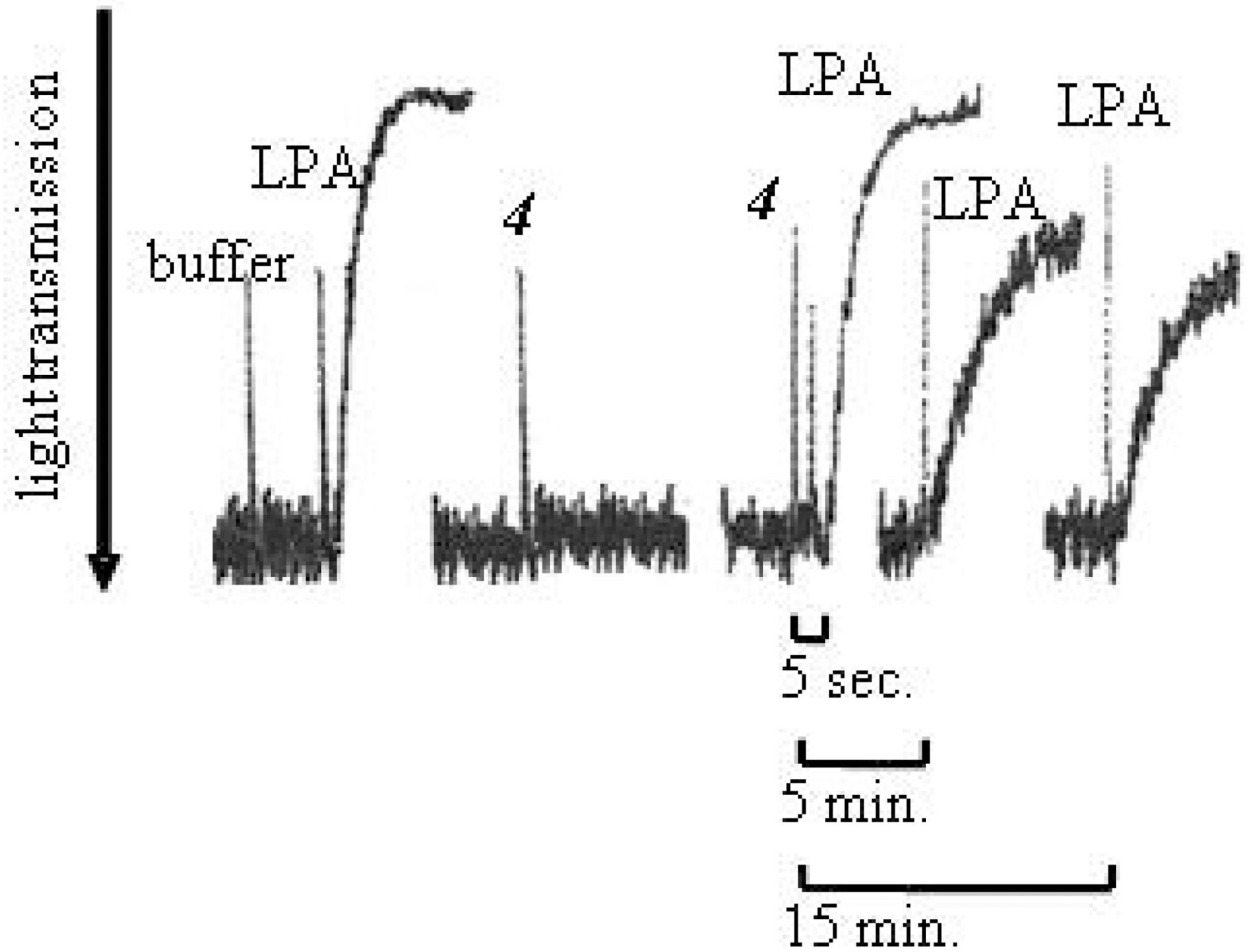


**Figure 4. The inhibitory effect of a $LPA_{1/3}$ agonist (compound 4) on platelets is pre-incubation time dependent**

A concentration of 0.2 μM compound ***4*** that itself did not induce shape change was incubated for different time periods before exposure to acyl-LPA 16:0 (20nM).

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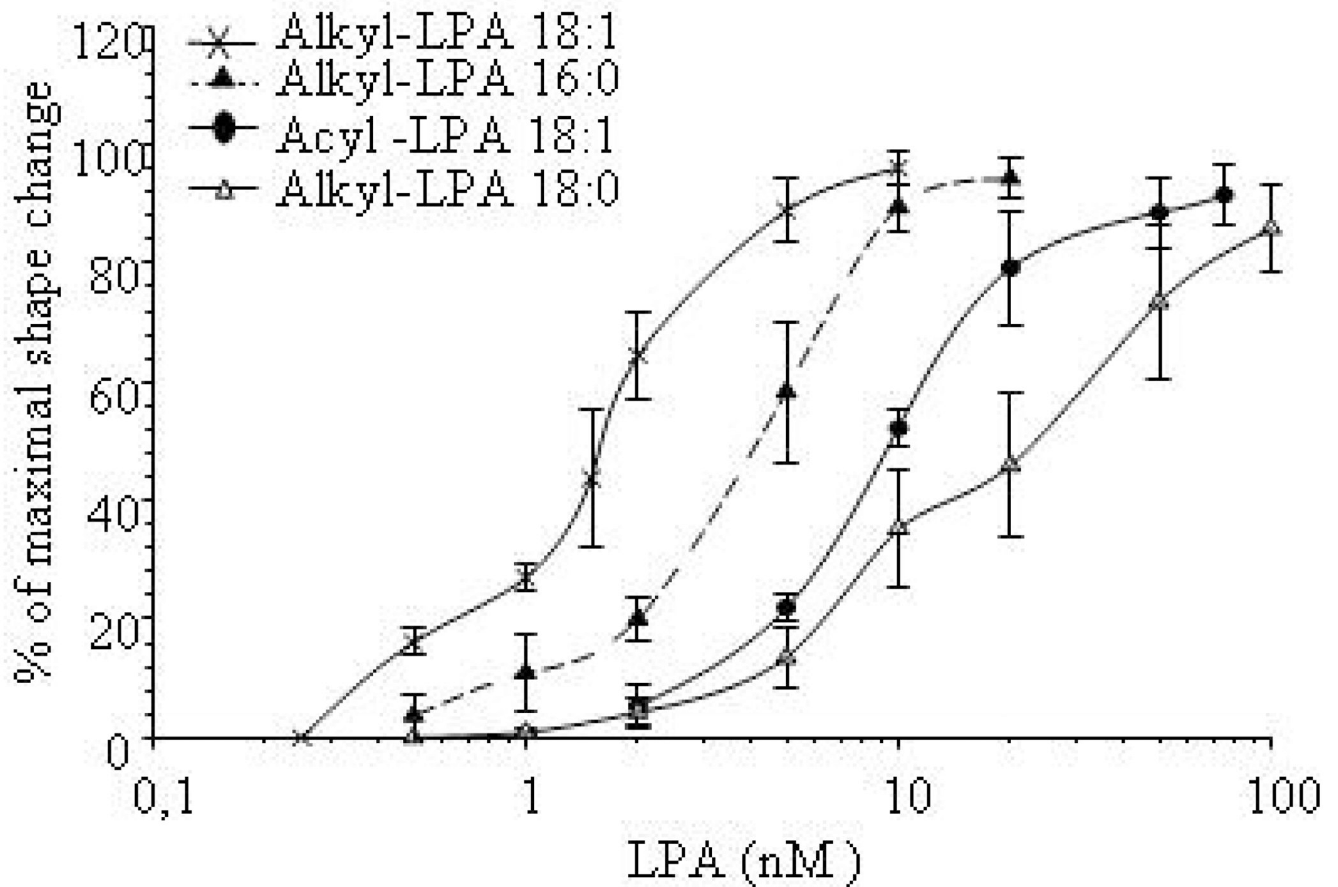


**Figure 5. Dose-response curves induced by various LPA species**

Suspensions of washed human platelets were stirred at 37°C for 2 minutes and then exposed to increasing concentrations of LPA species. Shape change was measured by the decrease in light transmission in a LABOR aggregometer®. Values represent the mean ± SD from 4 different experiments with different platelet donors.

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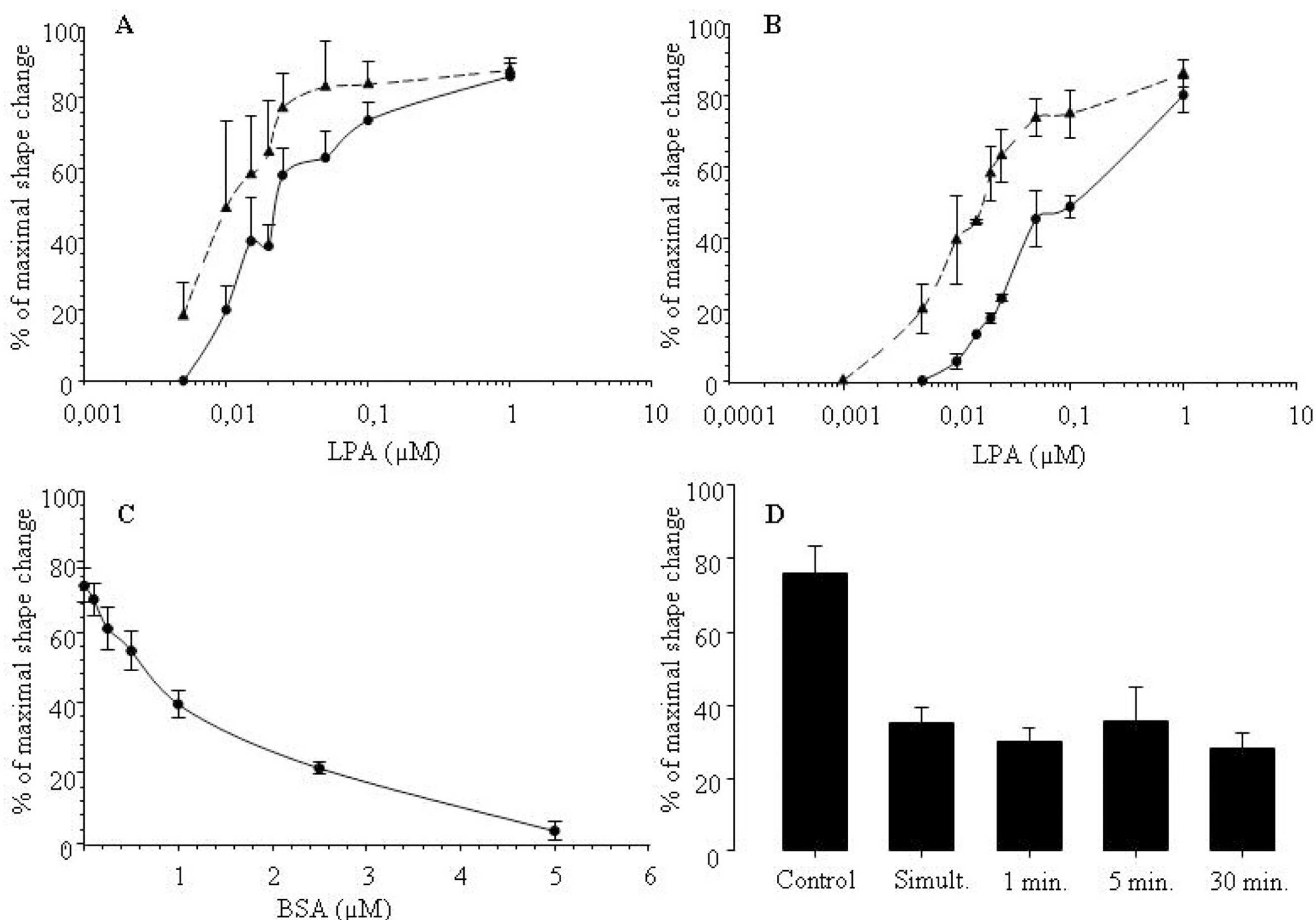


**Figure 6. Effect of albumin on LPA-mediated shape change**

**A**, Dose-response curves comparing the effect of LPA dissolved in ethanol (dotted line) and in albumin buffer (LPA:BSA 4:1) (solid line) on platelet shape change (mean SD, n=3). **B**, BSA inhibits platelet shape change in a competitive manner. Platelets were incubated with BSA (2.5 µM) (solid line) for 1 min before exposure to increasing concentration of LPA (in albumin buffer), n=3. **C**, BSA inhibits LPA-induced platelet shape change in a dose-dependent manner. Washed platelets were incubated with different concentrations of BSA for 1 min before exposure to 20nM of LPA (in albumin buffer), n=6. **D**, BSA reduced LPA-initiated platelet shape change in a time-independent manner. Incubation of platelets was performed with BSA (2.5 µM) for different time periods before exposure to 20nM of LPA (in albumin buffer), n=3. Values represent the mean ± SD. *<0.05 vs. control.

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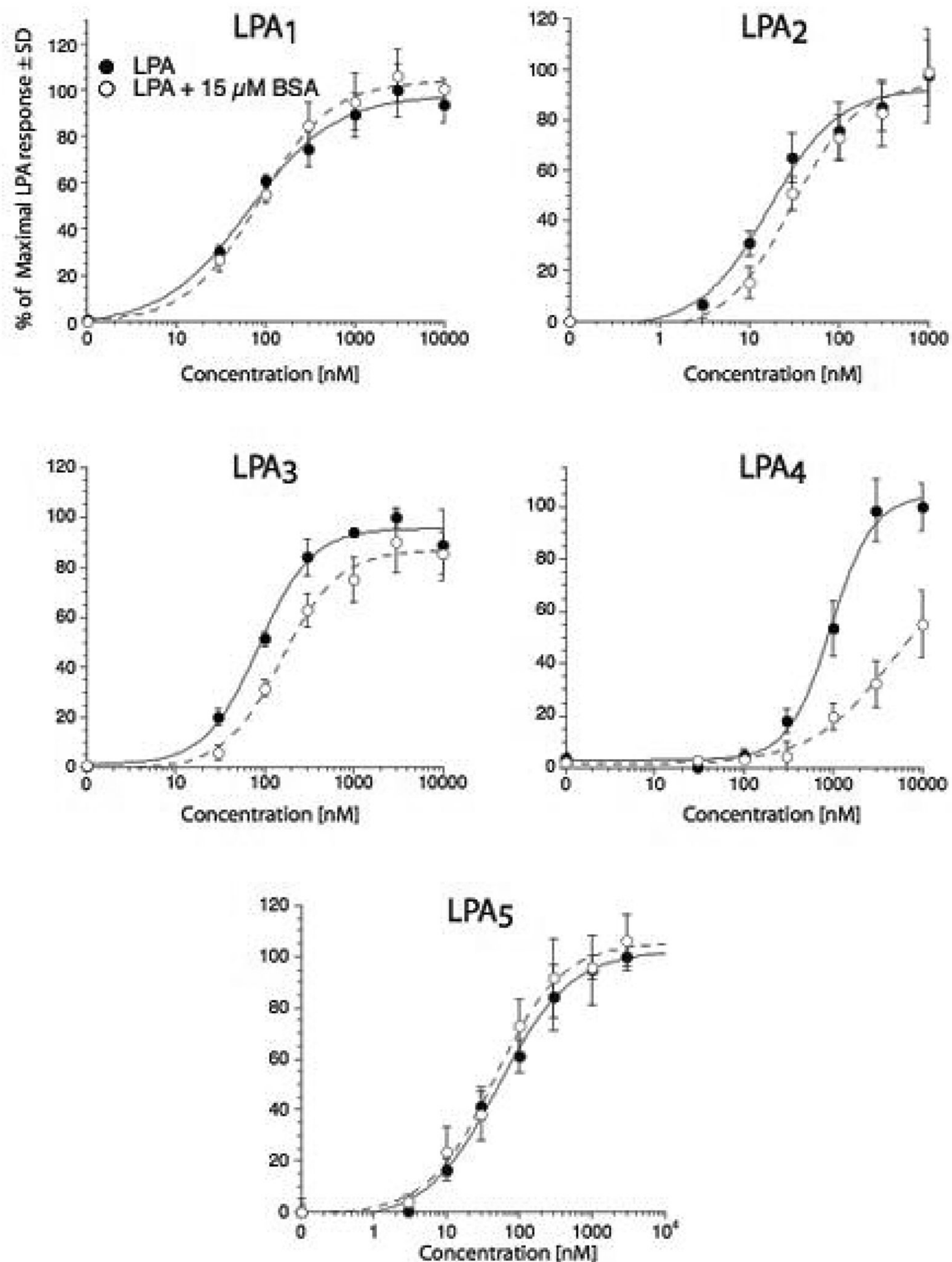


**Figure 7. Effect of albumin on $LPA_{1-5}$ activation in heterologous expression systems**

The effect of BSA (15μM, 5min pre-incubation) on LPA receptor activation induced by LPA was examined by measuring the transient increase of intracellular [$Ca^{2+}$] in Fura-2-loaded RH7777 cells expressing human $LPA_{1-3}$ and $LPA_5$, or CHO cells expressing $LPA_4$ (mean ± SD).

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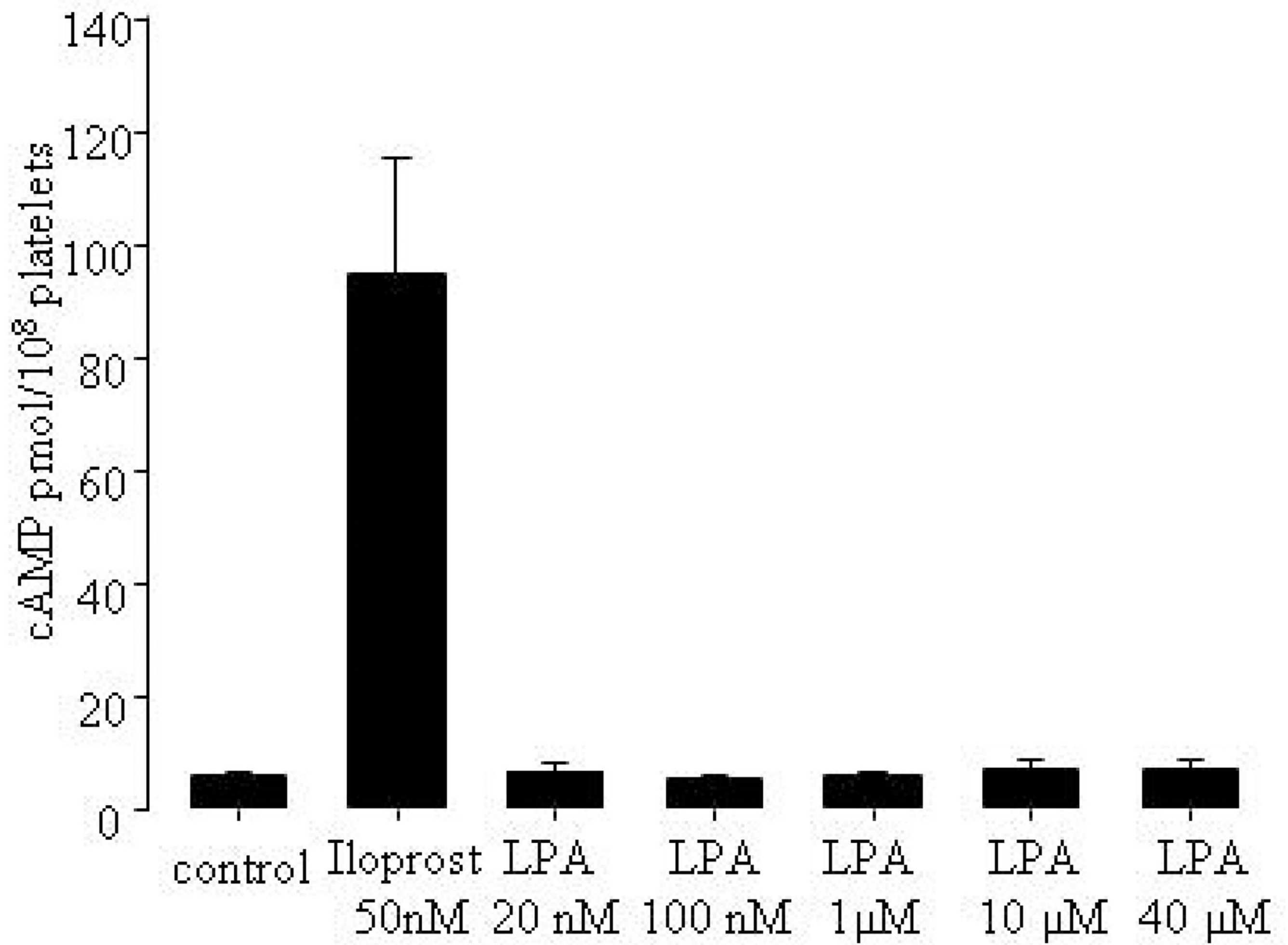


**Figure 8. Effect of LPA on cAMP levels in platelet**

Suspensions of washed human platelets were at 37°C for 2 minutes and were exposed to increasing concentration of LPA (0.02 µM – 40 µM) or iloprost (50nM) for 1 minute. Levels of cAMP were determined with an enzyme immunoassay kit. Values represent the mean ± SD from different experiments with different platelet donors (n=3).

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**Table I**

Effect of PA 8:0 analogs on intracellular $Ca^{2+}$ transients of $LPA_{1-5}$ transfected cells [25] and on platelet shape change

| | | *HETEROLOGOUS EXPRESSION SYSTEMS* | | | *PLATELETS* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **PA(8:0) Analogs** | **LPA receptor** | **Agonist activity** | | **Antagonist activity** | **Agonist activity** | | **Antagonist activity** | | |
| | | **$^{a}E_{max}$ %** | **$EC_{50}$ µM** | **$IC_{50}$ µM** | **$^{b}E_{max}$ %** | **$EC_{50}$ µM** | **$^{c}I_{max}$ %** | **$IC_{50}$ µM** | **n** |
| ***1*** | | | | | | | | | |
| $LPA_1/LPA_3$ | $LPA_1$ | NE | | 0.68 | | | | | |
| Antagonist | $LPA_2$ | 58 | 6.33 | NE | 69±12 | 1.77±0.22 | 76±18 | 2.92±1.73 | 3 |
| $LPA_2$ | $LPA_3$ | NE | | 0.011 | | | | | |
| Agonist | | | | | | | | | |
| ***2*** | $LPA_1$ | NE | | 1.58 | | | | | |
| $LPA_1/LPA_3$ | $LPA_2$ | NE | | NE | 38±18 | 5.91±2.62 | 72±9 | 1.7±1.5 | 3 |
| Antagonist | $LPA_3$ | NE | | 0.14 | | | | | |
| ***3*** | $LPA_1$ | NE | | 0.14 | | | | | |
| $LPA_1/LPA_3$ | $LPA_2$ | NE | | NE | | | | | |
| Antagonist | $LPA_3$ | NE | | 0.184 | 69±13 | 1.79±0.95 | 91±9 | 0.55±0.37 | 3 |
| $LPA_5$ | $LPA_4$ | NE | | NE | | | | | |
| Agonist | $LPA_5$ | 92 | NS | NE | | | | | |
| ***4*** | $LPA_1$ | 57 | 3.26 | NE | | | | | |
| $LPA_1/LPA_3$ | $LPA_2$ | NE | | NE | 57±4 | 2.43±1.1 | 93±4 | 0.35±0.1 | 3 |
| Agonist | $LPA_3$ | 109 | 0.16 | NE | | | | | |
| ***5*** | $LPA_1$ | 30.6 | 0.69 | NE | | | | | |
| $LPA_{1-3}$ | $LPA_2$ | 27 | 5.72 | NE | | | | | |

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| PA(8:0) Analogs | LPA receptor | HETEROLOGOUS EXPRESSION SYSTEMS: Agonist activity | | Antagonist activity | PLATELETS: Agonist activity | | Antagonist activity | | |
|---|---|---|---|---|---|---|---|---|---|
| | | [a]$E_{max}$ % | $EC_{50}$ μM | $IC_{50}$ μM | [b]$E_{max}$ % | $EC_{50}$ μM | [c]$I_{max}$ % | $IC_{50}$ μM | n |
| Agonist | $LPA_3$ | 109 | 0.003 | NE | 48±10 | 0.39±0.11 | 87±13 | 0.21±0.16 | 4 |
| $LPA_5$ | $LPA_4$ | NE | | NE | | | | | |
| Agonist | $LPA_5$ | 97 | NS | NE | | | | | |
| ***6*** | $LPA_1$ | NE | | NE | | | | | |
| select.$LPA_3$ | $LPA_2$ | NE | | NE | 68±12 | 8.4±0.74 | 80±2 | 8.2±0.96 | 3 |
| Antagonist | $LPA_3$ | NE | | 0.414 | | | | | |
| ***7*** | $LPA_1$ | NE | | NE | | | | | |
| select.$LPA_3$ | $LPA_2$ | NE | | NE | | | | | |
| Antagonist | $LPA_3$ | NE | | 0.935 | 110±9 | 0.55±0.18 | 95±5 | 2.6±0.4 | 3 |
| $LPA_5$ | $LPA_4$ | NE | | NE | | | | | |
| Agonist | $LPA_5$ | 36 | NS | NE | | | | | |

[a]Note: $E_{max}$ = maximal efficacy of the drug/maximal efficacy of LPA 18:1; NE= no effect; NS = the response did not saturate at the highest concentration tested (10 μM).

[b]$E_{max}$= (maximal shape change induced by drug/ shape change induced by 20 nM acyl-LPA 16:0)x100. Shape change induced by 20–25 nM LPA was 89±11 % (mean SD, n=22) of maximal, and set to 100 %. Values are mean ± SD from different experiments with different platelet donors. Inhibitory activity was tested 30 min after addition of the phospholipids, when platelets had reached again their discoid shape (Figure 3A, right tracings).

[c]$I_{max}$= maximal inhibition of LPA-induced shape change tested by 20 μM of PA analogs.

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## Table II

Effect of NPSPA NPTPA on LPA responses of heterologously expressed $LPA_{1-5}$

| Compound | $LPA_1$ | $LPA_2$ | $LPA_3$ | $LPA_4$ | $LPA_5$ |
|---|---|---|---|---|---|
| NPSPA | Partial agonist ($E_{max}$= 63%) $EC_{50}$= 0.74 µM | Partial agonist ($E_{max}$= 46%) $EC_{50}$= 11.9 µM | Partial agonist ($E_{max}$= 81%) $EC_{50}$= 0.73 µM | No effect | Partial agonist ($E_{max}$= 23%) $EC_{50}$= 0.42 µM |
| NPTPA | Antagonist $K_i$= 0.53 µM | No effect | No effect | No effect | Partial agonist ($E_{max}$= 72.0%) $EC_{50}$ - NS |

Note: $E_{max}$ = (maximal efficacy of compound/maximal efficacy of LPA 18:1)×100

$EC_{50}$ = concentration eliciting half maximal activation

$K_i$ = inhibitory binding constant

NS= the response did not saturate at the highest concentration tested

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**Table III**

Effect of acyl and alkyl LPAs and α-substituted phosphonate analogs of LPA on intracellular $Ca^{2+}$ transients of $LPA_{1-5}$ transfected cells and platelet shape change

| *HETEROLOGOUS EXPRESSION SYSTEMS* | | | | | *PLATELET* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Analogs | LPA receptor | Agonist activity | | Antagonist activity | Agonist activity | | Antagonist activity | | |
| | | $^{a}E_{max}$ % | $EC_{50}$ µM | $IC_{50}$ µM | $^{b}E_{max}$ % | $EC_{50}$ nM | $^{c}I_{max}$ % | $IC_{50}$ nM | n |
| | $LPA_1$ | 100 | 0.130 | | | | | | |
| | $LPA_2$ | 100 | 0.003 | | | | | | |
| Acyl LPA 18:1 | $LPA_3$ | 100 | 0.081 | | 100 | 9±2.9 | | | 4 |
| | $LPA_4$ | 100 | 0.245 | | | | | | |
| | $LPA_5$ | 100 | 0.015 | | | | | | |
| | $LPA_1$ | 100 | 1.445 | | | | | | |
| | $LPA_2$ | 108 | 0.100 | | | | | | |
| Alkyl LPA 18:1 | $LPA_3$ | 63 | $^{d}NS$ | | 100 | 1.85±0.7 | | | 4 |
| | $LPA_4$ | 98 | 0.303 | | | | | | |
| | $LPA_5$ | 97 | 0.002 | | | | | | |
| ***8*** | $LPA_1$ | NE | | PA | | | | | |
| $LPA_{4,5}$ | $LPA_2$ | NE | | 2.59 | | | | | |
| Agonist | $LPA_3$ | NE | | 2.56 | 78±9 | 280±40 | 81±5 | 900±110 | 3 |
| $LPA_{1-3}$ | $LPA_4$ | 29 | 5.4 | NE | | | | | |
| Antagonist | $LPA_5$ | 59 | $^{d}NS$ | | | | | | |
| ***9*** | $LPA_1$ | NE | | 1.5 | | | | | |
| $LPA_{1-4}$ | $LPA_2$ | NE | | 1.42 | | | | | |
| Antagonist | $LPA_3$ | NE | | 1.16 | 73±9 | 810±370 | 80±9 | 300±150 | 3 |

| Analogs | LPA receptor | HETEROLOGOUS EXPRESSION SYSTEMS | | | PLATELET | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Agonist activity | | Antagonist activity | Agonist activity | | Antagonist activity | | |
| | | [a]$E_{max}$ % | $EC_{50}$ µM | $IC_{50}$ µM | [b]$E_{max}$ % | $EC_{50}$ nM | [c]$I_{max}$ % | $IC_{50}$ nM | n |
| $LPA_5$ | $LPA_4$ | NE | | 0.27 | | | | | |
| Agonist | $LPA_5$ | 45 | [d]NS | | | | | | |

[a] Note: $E_{max}$ = (maximal efficacy of compound/maximal efficacy of LPA 18:1)x100; NE= no effect; PA=partial antagonist with 39±3.2% inhibition of 200 nM LPA response at the highest concentration (30µM) tested.

[b] $E_{max}$=(maximal shape change induced by drug/ shape change induced by 20 nM acyl-LPA 16:0)×100. Values are mean ± SD from different experiments with different platelet donors. Inhibitory activity was tested 30 min after addition of the phospholipids.

[c] $I_{max}$= maximal inhibition of LPA-induced shape change tested by 10 µM of analogs.

[d] NS = the response did not saturate at 10 µM the highest concentration tested preventing the determination of $EC_{50}$



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**Table IV**

Activities of different molecular species of LPA

| LPA species | Platelets | $LPA_5$ transiently transfected RH7777 cells |
|---|---|---|
| | $EC_{50}$ nM | $EC_{50}$ nM |
| acyl-LPA 18:1 | 9 ±2.9 | 15.2±5.4 |
| alkyl-LPA 16:0 | 4.6±0.98 | 4.1±2.1 |
| alkyl-LPA 18:0 | 20.17±17.5 | 69.9±21.4 |
| alkyl-LPA 18:1 | 1.85±0.7 | 2.1±0.9 |

Note: $EC_{50}$ values were calculated from concentration-response curves induced by various LPA species in platelets (n=4) and $LPA_5$ receptor transiently transfected RH7777 cells (mean ± SD).