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Synergistic activation of human platelets by lysophosphatidic acid and adrenaline

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Background and Objectives. Platelet reactivity is regulated by various important bioactive and physiologic substances. The objective of this study was to characterize lysophosphatidic acid (LPA)-triggered responses in human platelets. In addition, the effect of LPA was compared with that of other activators and possible synergistic interactions were evaluated.

Design and Methods. LPA-triggered cytosolic Ca^{2+} responses were measured using fura-2-loaded platelets in a spectrofluorometer. Furthermore, platelet aggregation and secretion were analyzed in a lumi-aggregometer and protein tyrosine phosphorylation was detected with the Western blot technique.

Results. LPA dose-dependently increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in platelets. This response involved both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores. However, in comparison with other platelet agonists, i.e. thrombin and adenosine 5´-diphosphate (ADP), LPA was a very weak Ca^{2+}-elevating agent. Furthermore, we observed that the LPA-induced rise in $[Ca^{2+}]_i$ was markedly suppressed by cyclic nucleotide-elevating agents. In functional studies, LPA failed to stimulate platelet aggregation and secretion. However, in combination with adrenaline, another weak platelet agonist, LPA could induce an irreversible and complete aggregatory response. There was an individual variation in aggregatory response and tyrosine phosphorylation when LPA and adrenaline were combined. These agents induced a powerful response on platelets from some individuals, but had a weak or no effect on others.

Interpretation and Conclusions. The present study shows, for the first time, that isolated platelets from some healthy blood donors respond synergistically to a combination of LPA and adrenaline. Platelet activation is a key step in distinguishing normal hemostasis from pathologic hemostasis. Increased knowledge about this mechanism might help to predict individual responses and provide new insights into molecular mechanisms responsible for pathologic thrombosis.

Key words: platelets, lysophosphatidic acid, adrenaline, calcium, aggregation, tyrosine phosphorylation

Various important bioactive and physiologic substances such as thrombin, adenine nucleotides, adrenaline, and prostaglandin I_2 as well as other membrane metabolites regulate platelet reactivity. The majority of these agents interact with G-protein-coupled receptors on the plasma membrane of the platelet. Lysophosphatidic acid (LPA) is an intercellular lipid messenger which is produced and released from membrane microvesicles from activated platelets and other cells.[1] However, Alexander *et al.*[2] suggested that platelets can secrete LPA even without marked signs of activation. In microvesicles, phospholipase (PL) C and diacylglycerol kinase or PLD hydrolyze phospholipids to generate high concentrations of phosphatidic acid which is then converted to LPA by PLA2.[3] LPA is thereafter released into the extracellular fluid. LPA activates at least three types of G-protein-coupled receptors called endothelial differentiation gene (Edg). In 1996 Hecht *et al.* isolated mouse cDNA, termed *ventricular zone gene-1* (*vzg-1*), encoding a receptor for LPA.[4] Thereafter the human homolog of *vzg-1*, called Edg-2, was identified and later on another subtype called Edg-4 was discovered.[5, 6] Recently another novel human subtype, Edg-7, was characterized by Bandoh *et al.*[7] However, according to Tigyi a new nomenclature for these receptors has been proposed.[8] The new names are LPA_1 (Edg-2), LPA_2 (Edg-4), and LPA_3 (Edg-7). The cell distribution and intracellular signal transduction mechanisms of these subtypes of receptor proteins differ.[7] In platelets, the interaction of LPA with its receptors is associated with rises in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent platelet activation.[9] The exact mechanisms underlying LPA-induced platelet activation are, however, far from being completely understood. For instance, it has recently been suggested that LPA-stimulated platelet aggregation might be LPA-receptor independent.[10] The objective of this study was to further characterize the LPA-triggered responses in platelets. The response to LPA was compared to the responses to other more well-characterized platelet activators. Furthermore,

adrenaline, which has been shown to potentiate the effect of other agonists, was used together with LPA to evaluate possible synergistic effects.

Design and Methods

Materials

Acetylsalicylic acid, adrenaline, apyrase (grade III), fibrinogen, fura-2-acetoxymethylester and L-α-lysophosphatidic acid (oleoyl-sn-glycero-3-phosphate) were obtained from Sigma Chemicals (St. Louis, MO, USA). The monoclonal anti-phosphotyrosine antibody and the horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Platelet preparation

Platelets were prepared from blood taken from healthy volunteers who had not taken any acetylsalicylic acid in the two weeks before the blood donation. Blood was collected from antecubital veins into siliconized tubes (Becton Dickinson, Oxford, England) containing 1/6 volume of an acid citrate dextrose solution (111 mM dextrose, 85 mM sodium citrate and 71 mM citric acid) and gently mixed. Platelet-rich plasma (PRP) was obtained by centrifuging the solution at 220×g for 20 minutes and aspirating the PRP. Thereafter the PRP was incubated with acetylsalicylic acid (100 μM) and apyrase (0.5 U/mL) for 15 minutes at room temperature to prevent the formation of thromboxane A_2 and the release of adenosine 5´-diphosphate (ADP) and thereby minimize platelet activation during the isolation procedure. The PRP was then centrifuged at 480×g for 20 minutes to form a platelet pellet. After removal of the supernatant, the platelet pellet was gently resuspended in a Hepes buffered solution (145 mM NaCl, 10 mM glucose, 10 mM *N-2*-hydroxyethylpiperazine-*N´*-2-ethanesulfonic acid (Hepes), 5 mM KCl and 1 mM $MgSO_4$, pH 7.4), supplemented with apyrase (1 U/mL). The platelet suspension was stored at room temperature until used. Extracellular Ca^{2+} was adjusted to 1 mM with $CaCl_2$ immediately before each measurement.

Analyses of platelet aggregation and secretion

Changes in light transmission and adenosine 5´-triphosphate (ATP)-dependent bioluminescence were simultaneously recorded using a Chronolog Dual Channel lumi-aggregometer (Chrono-Log Corporation, Haverston, PA, USA). Aliquots (0.5 mL) of platelet suspension (2.5×10^8 platelets/mL) were preincubated at 37°C for 5 minutes and then stimulated with different drugs.

A combination of LPA (10 μM) and adrenaline (10 μM) was compared to the drugs used separately. In some experiments, fibrinogen (100 μg/mL) was included in the platelet suspension prior to the addition of other activators. The degree of aggregation and the release of ATP were compared to those induced by either thrombin (0.3 U/mL) or ADP (40 μM). Aggregation was recorded as an increase in light transmission. The concentration of extracellular ATP was registered using a luciferine/luciferace bioluminescent kit (Sigma Chemicals).

Measurement of $[Ca^{2+}]_i$

Platelets were loaded with 4 μM fura-2-acetoxymethylester (fura-2) by incubation for 45 minutes with gentle agitation at room temperature. Thereafter, the platelets were centrifuged at 480×g for 20 minutes and suspended in Hepes buffered solution and stored at room temperature until used. Before each measurement, 2 mL of platelet suspension (1×10^8 platelets/mL) were incubated at 37°C for 5 minutes. Fluorescence signals were recorded on a Hitachi F-2000 spectrofluorometer specially designed for measurement of $[Ca^{2+}]_i$. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated by using the general equation described by Grynkiewicz *et al.*[11] $[Ca^{2+}]_i=K_d(R-R_{min})/(R_{max}-R)(F_o/F_s)$. Maximal and minimal ratios were determined by adding 0.1 % Triton X-100 and 25 mM [ethylenebis(oxy-ethylenenitrilo)]tetraacetic acid (EGTA), respectively.

The platelets were stimulated with thrombin (0.3 U/mL), ADP (40 μM), LPA (0.1-10 μM) and adrenaline (10 μM) in suspensions containing Ca^{2+} (1 mM), EGTA (500 μM), fibrinogen (100 μg/mL), or Mn^{2+} (100 μM). Ca^{2+} ions, fibrinogen, or EGTA were added just before each measurement. By removing external Ca^{2+} with EGTA, internal release of Ca^{2+} could be studied. Forskolin (10 μM) or GEA 3175 (10 μM) was added two minutes prior to LPA. In the experiments in which influx of Mn^{2+} was measured, the ions were added 30 seconds before LPA and fluorescence emission from the fura-2-loaded platelets was recorded at 510 nm during excitation at 360 nm. Mn^{2+}-induced quenching of fura-2 fluorescence has been utilized to study divalent cation entry across the plasma membrane.[12] By using an excitation wavelength of 360 nm, a quench of fura-2 can be followed to report Mn^{2+} entry across the plasma membrane. At this wavelength Ca^{2+} does not alter fura-2 fluorescence.

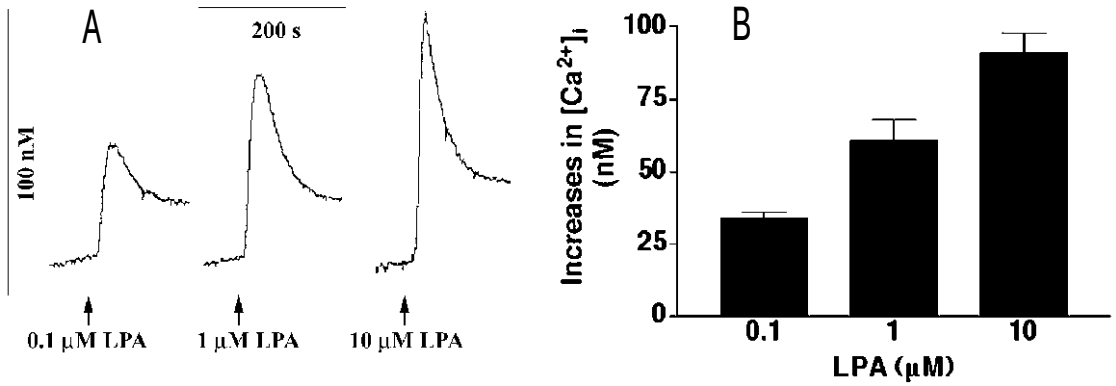

Figure 1. A) Effect of LPA on $[Ca^{2+}]_i$ in fura-2-loaded platelets. LPA was used at a final concentration of 0.1, 1 or 10 μM. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 and 380 nm. The traces shown are from a representative experiment; similar results were obtained from five to seven other platelet preparations and are summarized in Figure 1B. B) Dose-response effects of LPA-induced increases in $[Ca^{2+}]_i$ in platelets. The data represent means ± SEM from five to seven separate experiments with blood obtained from different platelet donors.

Gel electrophoresis and Western blot analyses of protein tyrosine phosphorylation

Platelets, at a concentration of 1×10^9/mL, were stimulated with LPA (10 μM), adrenaline (10 μM) or a combination of the two drugs. All incubations were conducted at 37°C with a gentle agitation. The platelet suspensions (100 μL) were incubated for 5 minutes and then challenged with the drugs. As a control, platelets were incubated with Hepes-buffered solution for the same time as with drugs. Stimulation was terminated by adding an equal volume of Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.01% bromophenol blue (Bio-Rad Laboratories, Hercules, CA, USA) with 5% 2-mercaptoethanol. The samples were heated at 97°C for 5 min and the lysates were then stored at –20°C until used. Separation was performed with 12% SDS-polyacrylamide gel electrophoresis (PAGE) using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad). The proteins were transferred from gel to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden), using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Non-specific binding sites on the membranes were blocked by incubation for 1 hour at room temperature in phosphate-buffered saline (PBS; 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4; pH 7.4) containing 5% dry milk and 0.1% Tween-20. The membranes were incubated with a monoclonal anti-phosphotyrosine mouse antibody diluted 1/8,000 in PBS with 0.1% Tween-20 for 1 hour and washed several times in PBS with 0.1 % Tween-20. Thereafter, the membranes were incubated with an HRP-conjugated goat anti-mouse IgG antibody for 1 hour, diluted 1/10,000 in the same buffer as the primary antibody. Immunostained blots were visualized using the ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The samples were also run on a gel that was stained with Coomassie blue to control that the protein levels were the same in the samples. The gels were stained with PhastGel Blue R tablets (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Statistical analyses

Results are expressed as means±standard error of the means (SEM). Data were analyzed using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA).

Results

Cytosolic Ca^{2+} responses

Platelets were loaded with fura-2 to evaluate the effects of LPA on $[Ca^{2+}]_i$. Addition of LPA at concentrations of 0.1, 1 or 10 μM induced rapid and transient rises in $[Ca^{2+}]_i$ in platelets (Figure 1A). The Ca^{2+} response reached a peak a few seconds after stimulation and the magnitude of the response was not further increased with higher concentrations of LPA (*data not shown*). Figure 1B summarizes the dose-dependent effects of LPA on $[Ca^{2+}]_i$ in platelets. LPA at a concentration of 0.1 μM gave a rise in $[Ca^{2+}]_i$ of 33.84±1.84 nM. Furthermore, stimulation with LPA at a concentration of 1 or 10 μM

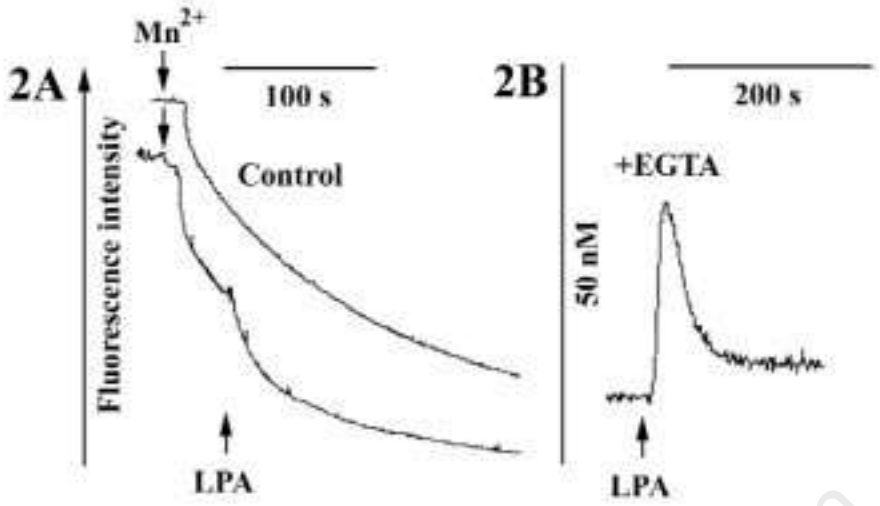

Figure 2. A) LPA-induced influx of Mn^{2+}. The trace shown is obtained from fura-2-loaded platelets stimulated with 1 μM LPA. Mn^{2+} ions (100 μM) were added to the platelet suspension 30 seconds prior to LPA. Quenching of fura-2 fluorescence was compared to that of a control in the absence of LPA. Fluorescence emission was registered at 510 nm during excitation at 360 nm. The trace illustrated is from a representative experiment; similar results were obtained from four other platelet preparations. B) Effect of LPA on $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. The trace shown is obtained from fura-2-loaded platelets stimulated with 1 μM LPA. EGTA (500 μM) was added to the platelet suspension just before each measurement. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 and 380 nm. The trace illustrated is from a representative experiment; similar results were obtained from five other platelet preparations.

gave rise to $[Ca^{2+}]_i$ of 60.98±6.86 nM and 90.83±6.75 nM, respectively. The effect of LPA on cytosolic Ca^{2+} signaling was further examined by analyzing LPA-induced influx of Mn^{2+} and intracellular Ca^{2+} mobilization. The Ca^{2+} signal initiated by LPA included both an intracellular release of Ca^{2+} and an extracellular influx of Ca^{2+}. This is indicated in Figure 2 where the platelets were activated with 1 μM LPA in the presence of either EGTA or Mn^{2+}. The addition of LPA in the presence of 100 μM Mn^{2+} induced quenching of the fura-2 fluorescence, indicative of divalent cation influx (Figure 2A). Figure 2B shows that LPA induced a transient rise in cytosolic Ca^{2+} even in the absence of extracellular Ca^{2+}, i.e. in the presence of 500 μM EGTA. The rise triggered by LPA in Ca^{2+} free solution was 20.86±4.07 nM.

Increases in cyclic adenosine 3´,5´-monophosphate (cAMP) and cyclic guanosine 3´,5´-monophosphate (cGMP) often suppress agonist-stimulated cytosolic Ca^{2+} responses in platelets.[13] The influence of cyclic nucleotide elevating agents on LPA-induced rises in $[Ca^{2+}]_i$ is shown in Figure 3. Forskolin, a direct activator of adenylyl cyclase, markedly inhibited the LPA-induced rise in $[Ca2^{2+}]_i$ from 75.28±15.04 nM in the absence of forskolin to 14.50±8.62 nM in its presence. Furthermore, GEA 3175, a nitric oxide (NO)-containing compound, inhibited the Ca^{2+} response from 83.17±11.76 nM in the absence of GEA 3175 to 16.61±9.48 nM in its presence.

Almost all activators of platelets provoke rises in $[Ca^{2+}]_i$. Figure 4A shows, as a comparison to the effects of LPA (10 μM), cytosolic Ca^{2+} transients triggered by thrombin (0.3 U/mL), ADP (40 μM) and adrenaline (10 μM). The rises in $[Ca^{2+}]_i$ were 618.10±48.19 nM and 244.70±22.96 nM, respectively, in platelets stimulated with thrombin and ADP. In this experimental design adrenaline did not affect $[Ca^{2+}]_i$.

Platelet aggregation and secretion

To test whether LPA could stimulate platelet aggregation, 10 μM of the drug were added to suspensions of isolated platelets placed in a lumi-aggregometer. Aggregation was recorded as an increase in light transmission. LPA did not induce a detectable increase in light transmission (Figure 4B). However, stimulation with LPA was accompanied by a transient decrease in transmission, indicative of an initial platelet shape change. In comparison, platelets incubated with thrombin (0.3 U/mL) induced a complete, irreversible aggregatory response and ADP (40 μM) stimulated a marked but reversible increase in light transmission (Figure 4B). On the other hand, adrenaline (10 μM) had no impact on light transmission. The combination of LPA and adrenaline resulted in a powerful and synergistic aggregatory response (Figure 5B). It should

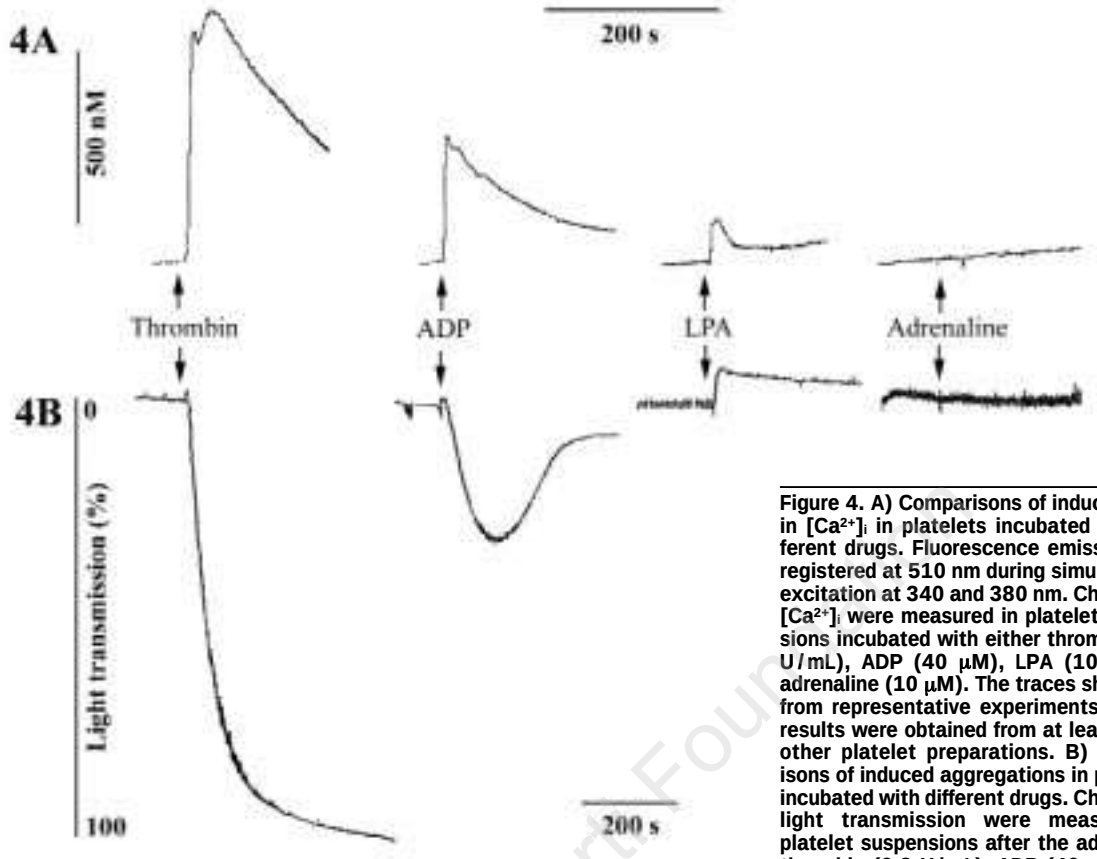

Figure 4. A) Comparisons of induced rises in $[Ca^{2+}]_i$ in platelets incubated with different drugs. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 and 380 nm. Changes in $[Ca^{2+}]_i$ were measured in platelet suspensions incubated with either thrombin (0.3 U/mL), ADP (40 μM), LPA (10 μM) or adrenaline (10 μM). The traces shown are from representative experiments; similar results were obtained from at least seven other platelet preparations. B) Comparisons of induced aggregations in platelets incubated with different drugs. Changes in light transmission were measured in platelet suspensions after the addition of thrombin (0.3 U/mL), ADP (40 μM), LPA (10 μM) or adrenaline (10 μM). The traces shown are from representative experiments; similar results were obtained from at least seven other platelet preparations.

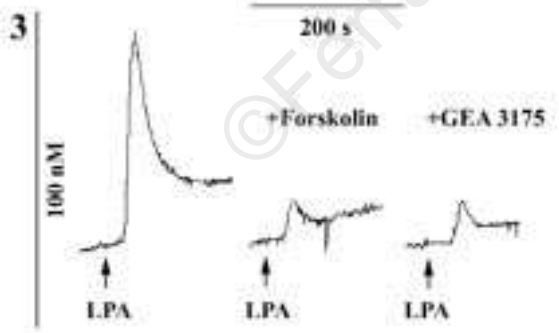

Figure 3. Effects of cyclic nucleotide-elevating agents on LPA-induced rises in $[Ca^{2+}]_i$ in fura-2-loaded platelets. LPA was used at a final concentration of 10 μM. Both forskolin (10 μM), a direct activator of adenylyl cyclase, and GEA 3175 (10 μM), an NO-containing compound, were added to the platelet suspensions two minutes prior to LPA. Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 and 380 nm. The traces shown are from representative experiments; similar results were obtained from three and two other platelet preparations, respectively.

be pointed out that, in the absence of external fibrinogen, the addition of LPA together with adrenaline failed to induce aggregation. To elucidate the role played by $[Ca^{2+}]_i$, fura-2 measurements were performed when LPA and adrenaline were combined. These experiments were performed both in the absence and the presence of fibrinogen. However, the synergistic aggregatory response was not associated with a more powerful cytosolic Ca^{2+} response (141.50±7.75 nM in the absence of fibrinogen, 90.07±8.69 nM in the presence of fibrinogen)(Figure 5A). There was a distinct individual variation in response to the combination of LPA and adrenaline on aggregation. The aggregatory response was analyzed in platelets isolated from eight different blood donors and the results varied from 0 % up to 100 % aggregation (Figure 6). As can be seen in Figure 6, almost no individual variation in responses was detected when LPA or thrombin was used alone.

Furthermore, a combination of LPA and adrenaline resulted in almost complete aggregation in four different platelet preparations. Three of these

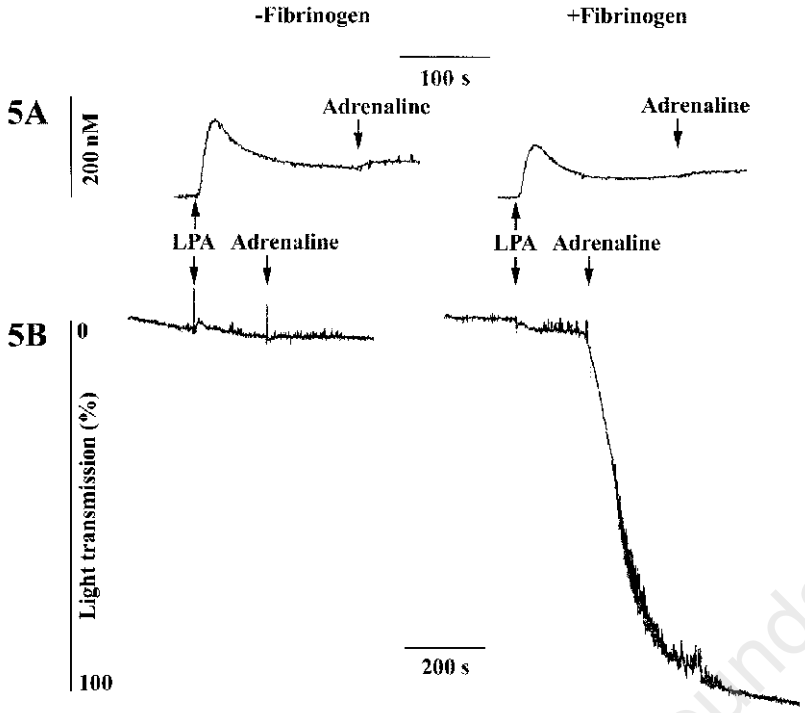

Figure 5. A) Effect of LPA and adrenaline on $[Ca^{2+}]_i$ in fura-2-loaded platelets. Changes in $[Ca^{2+}]_i$ were measured in platelet suspensions incubated with a combination of LPA (10 μM) and adrenaline (10 μM), in the absence or the presence of fibrinogen (100 μg/mL). Fluorescence emission was registered at 510 nm during simultaneous excitation at 340 and 380 nm. The traces shown are from a representative experiment; similar results were obtained from two other platelet preparations. B) LPA and adrenaline-triggered platelet aggregation. Changes in light transmission were measured in platelet suspensions incubated with a combination of LPA (10 μM) and adrenaline (10 μM) in the absence or the presence of fibrinogen (100 μg/mL). The traces shown are from representative experiments; similar results were obtained from seven other platelet preparations.

platelet preparations also released ATP whereas one did not (0.073±0.025 nmol, n=4). The amount of released ATP was, however, ten times lower than the amount of ATP released by thrombin-stimulation (0.770±0.054 nmol, n=4). Platelets stimulated with LPA alone did not secrete detectable amounts of ATP.

Protein tyrosine phosphorylation

Activation of platelets is accompanied by tyrosine-specific protein phosphorylation. To examine the influence of LPA, adrenaline and the combination of the two agonists on tyrosine phosphorylation, platelets were incubated for 3 minutes with 10 μM of the drugs.

The proteins were separated by SDS-PAGE and analyzed with Western blotting with an anti-phosphotyrosine antibody. Similarly to the results presented in the section about platelet aggregation and secretion, a combination of LPA and adrenaline enhanced the degree of tyrosine-specific phosphorylation of multiple platelet proteins in platelets obtained from some blood donors. Figure 7 shows two immunoblots in which (A) represents platelets from a blood donor that responded to a combination of LPA and adrenaline (lane 2) whereas (B) represents platelets that did not respond.

The samples were also run on a gel stained with Coomassie blue to control that the protein levels were the same in the samples. This is shown as a protein band below the respective lane.

Discussion

LPA interacts with G-protein-coupled receptors which can be divided into the three subtypes LPA_1, LPA_2, and LPA_3.[5,6,7] Motohashi *et al.* recently used RT-PCR with mRNA to identify the LPA-receptors in platelet preparations.[9] They found that mRNA from all three of these receptor subtypes were expressed in human platelets. In addition, it has been suggested that LPA interacts with platelets independently of LPA-receptors.[10]

In this study we characterized LPA-induced cytosolic Ca^{2+} responses and the capacity of LPA to initiate platelet activation compared to that of other well-known platelet agonists. We noted that LPA was able to increase $[Ca^{2+}]_i$, in a dose-dependent manner, in fura-2-loaded platelets. The LPA-induced cytosolic Ca^{2+} response was detectable around 0.01 to 0.1 μM and reached a maximum at 10 μM. The complex mechanisms underlying receptor-mediated increases in $[Ca^{2+}]_i$ are not completely understood. However, in almost all cell types it is due to a rapid release of Ca^{2+} from intracellular stores with a concomitant influx of Ca^{2+} across the plasma membrane, followed by a more sustained influx of Ca^{2+}. Based on the results obtained with the Mn^{2+} quenching technique and the response

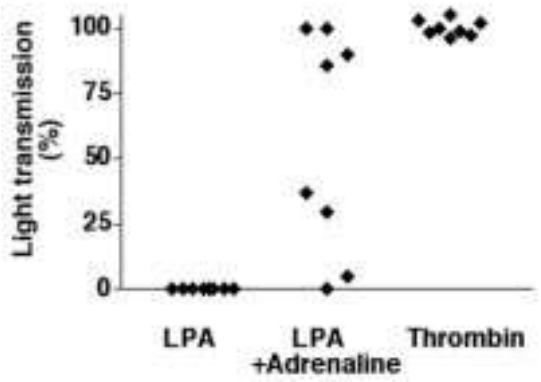

Figure 6. Summary of effects on platelet aggregation. Platelets were stimulated with LPA (10 μM), a combination of LPA (10 μM) and adrenaline (10 μM), or thrombin (0.3 U/mL). Thrombin-induced aggregations were measured in the absence, whereas the other experiments were performed in the presence of external fibrinogen (100 μg/mL). Changes in light transmission were analyzed in blood from eight different blood donors for each drug.

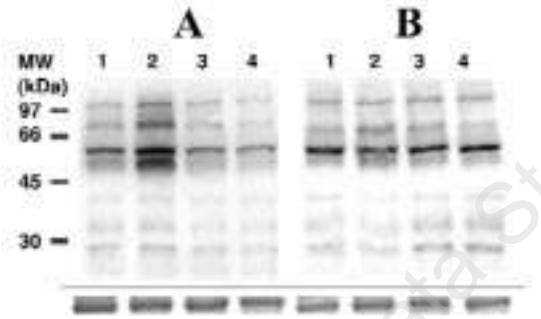

Figure 7. Western blot analyses of platelet proteins on 12% SDS-PAGE, transferred and immunoblotted with anti-phosphotyrosine antibody. A) represents platelets from a blood donor that responded to a combination of adrenaline and LPA whereas B) represents platelets that did not respond. Platelets were stimulated as follows; lane 1: adrenaline (10 μM), lane 2: adrenaline (10 μM) and LPA (10 μM), lane 3: LPA (10 μM), lane 4: Hepes-buffered solution, used as a control. A) is representative of eight separate experiments and B) is representative of three separate experiments. Molecular weights are indicated on the left in kilo Dalton (kDa). Protein levels in samples were controlled by Coomassie blue staining and are shown as protein bands below the respective lane.

detected in the presence of EGTA, we suggest that LPA-induced rises in $[Ca^{2+}]_i$ involve both an intracellular release of Ca^{2+} from stores and an extracellular influx of Ca^{2+}. We also found that LPA, in comparison to thrombin and ADP, was a weak Ca^{2+}-elevating compound. Taken together, these results show that LPA stimulated cytosolic Ca^{2+} signaling at a relatively low concentration, but had a low capacity to elevate the $[Ca^{2+}]_i$.

Increases in cAMP or cGMP are associated with inhibition of agonist-induced rises in $[Ca^{2+}]_i$ in platelets.[13] To study whether this was the case also in LPA-induced rises in $[Ca^{2+}]_i$ we used forskolin, an adenylyl cyclase stimulator which raises the cAMP concentration, or GEA 3175, an NO-donor which raises the cGMP concentration via stimulation of guanylyl cyclase. GEA 3175 belongs to a rather novel class of oxatrizole derivatives with slow NO-releasing properties.[14] These drugs, added separately, markedly inhibited rises in $[Ca^{2+}]_i$ induced by LPA. Consequently, we suggest that LPA-induced increases in $[Ca^{2+}]_i$ are sensitive to both cAMP and cGMP. It is tempting to speculate that inhibition of both adenylyl and guanylyl cyclase might be involved in stimulation of platelets by LPA. Our results are in agreement with those of Torti *et al*.[15] These authors found, by measuring the intracellular concentration of cAMP, that LPA caused a rapid decrease in the level of cAMP. It has been proposed that LPA affects platelets independently of LPA-receptor proteins and that it disturbs biological membranes.[10] Consequently, LPA may act as an ionophore and facilitate Ca^{2+} translocation independently of G-protein-coupled receptors. Our data do not support a role for receptor-independent mechanisms. This conclusion is based on the following findings. The kinetics of LPA-induced rises in $[Ca^{2+}]_i$ closely resembles that of other receptor-coupled activators, and the LPA-induced Ca^{2+} responses were extremely sensitive to elevation of both cAMP and cGMP. These findings are contradictory to Ca^{2+} responses triggered by drugs acting independently of receptor proteins. For instance, the Ca^{2+} ionophore-induced increase in $[Ca^{2+}]_i$ is insensitive to cAMP- as well as cGMP-elevating compounds.[16]

Holmsen has classified platelet agonists based on their ability to stimulate aggregation.[17] Adrenaline and ADP were classified as weak, and thrombin as strong agonists. We wanted to characterize in which of these two groups LPA belongs and therefore used these drugs for comparison with LPA. Both platelet aggregometry and measurement of $[Ca^{2+}]_i$ were used. In our experimental design adrenaline did not induce any detectable responses. In contrast, both ADP and thrombin provoked aggregation and raised $[Ca^{2+}]_i$. As could be expected thrombin was a much stronger agonist than ADP. Taken together we found that LPA was a weak activator of isolated human platelets.

The first functional response to platelet activation by different agonists is often a shape change, i.e. the discoid platelet becomes sphere-shaped, pseudopodia forms and the surface membrane folds.[18] We noted that LPA induced a transient decrease in light transmission, indicative of an initial shape change. Previous studies have shown that LPA stimulates platelet shape change, secretion and aggregation.[19, 20] In contrast, we found that LPA alone had no impact on platelet aggregation and secretion. The reason for differences in response to the same agonist is probably variations in experimental conditions. More specifically, the discrepancy between different studies could be due to different techniques to measure the aggregatory response, use of different anticoagulants, and use of different kinds of platelet suspensions (i.e. isolated platelets, platelet-rich plasma or whole blood). For example, it has been shown that the concentration of extracellular Ca^{2+} could be critical. Addition of millimolar Ca^{2+} concentrations reduced platelet aggregation,[21] and induction of platelet aggregation, by lipid phosphoric acids, in Ca^{2+}-containing medium required the addition of ADP.[22] In the present study we used platelets suspended in a physiologic buffer supplemented with 1 mM Ca^{2+}.

The platelets were treated with acetylsalicylic acid and apyrase to minimize the influence of two important positive feedback loops, i.e. thromboxane A_2 and ADP. Thus platelet aggregation depended on release of adhesive proteins, such as fibrinogen, from the α-granules. Under such experimental conditions, we found that LPA induced platelet shape change, but did not induce platelet aggregation and secretion. It should be noted that even in the presence of extracellular fibrinogen, LPA alone did not trigger platelet aggregation. Therefore, it is likely that the LPA-induced signal transduction did not affect the current low-affinity state of the fibrinogen binding glycoprotein IIb/IIIa (GPIIb/IIIa).

The GPIIb/IIIa complex is present on the surface of resting platelets but it serves as a receptor for fibrinogen only on activated platelets.[23] Recently, Retzer *et al.*[20] showed that LPA induced platelet shape change in the absence of an increase in $[Ca^{2+}]_i$ and they suggested that this was mediated via Rho/Rho kinase-induced myosin light-chain and moesin phosphorylation. In that study LPA, at a concentration between 0.01 and 0.1 μM, did not elevate $[Ca^{2+}]_i$. However, it was reported in another study that LPA, at concentrations from 1 μM, induced rises in $[Ca^{2+}]_i$.[19] We found that 0.1 μM of LPA triggered rises in $[Ca^{2+}]_i$ and it is likely that the Ca^{2+} response contributes to platelet shape change.

Several papers have observed synergistic effects when two activators are combined. We showed that a combination of two very weak activators, LPA and adrenaline, induced irreversible aggregation in platelet suspensions from some blood donors. In a previous study Crouch and Lapetina[24] showed that adrenaline, added after thrombin, re-coupled thrombin-receptor triggered rises in cytosolic Ca^{2+}. Our results show that adrenaline did not indirectly amplify LPA-induced Ca^{2+}-responses.

Based on this, it is unlikely that cytosolic Ca^{2+} is involved in the LPA- and adrenaline-induced synergistic aggregation. In platelets, the effects of adrenaline can be mediated by its interaction with α_2-adrenergic receptors (α_2-ARs) as evidenced by their sensitivity to the antagonist yohimbine.[25] The α_2-AR was cloned and sequenced in human platelets in 1987.[26] Furthermore, it has been shown that platelets also express β-ARs.[27] Variations in AR expression may explain the variability in responsiveness of human platelets towards the combination of LPA and adrenaline. There are conflicting reports on the ability of adrenaline to induce activation of platelets by itself. It has been reported that adrenaline primarily serves to increase platelet aggregation induced by other autocoids.[28] As an example, adrenaline can potentiate ADP-induced aggregation.[29] According to Freeman *et al.*,[30] genetic variation and heritability might result in increased adrenaline-mediated platelet aggregation.

Furthermore, in another study Nakamura *et al.*[31] compared adrenaline-sensitive and insensitive platelets. While studying the variability in aggregatory response in Japanese subjects, they found that 16% of the population had a reduced number of α_2-ARs. The number of receptors was decreased to about 50% that of normal individuals, but synergistic effects of adrenaline were still observed. They also found that fibrinogen binding to GPIIb/IIIa had a crucial role in activating PLA2 which is required for a full aggregatory response of adrenaline. We found that LPA and adrenaline interact synergistically to induce platelet aggregation. Furthermore, exposure to the drug combination revealed sensitive and insensitive platelet preparations. We also found that the combination of LPA and adrenaline could induce an increase in tyrosine phosphorylation in samples from some blood donors.

Activation of tyrosine kinases is coupled to fibrinogen binding to GPIIb/IIIa and to thrombin and collagen interactions with their respective receptor proteins.[32] Wang *et al.*[33] have been studying adrenaline-potentiated thrombin-induced aggregation

and tyrosine phosphorylation. Adrenaline itself did not cause detectable tyrosine phosphorylation or platelet aggregation, but could potentiate the effects of thrombin through interaction with α_2-ARs. In our experimental design, the synergistic activation of platelet aggregation was dependent on external fibrinogen included in the samples. Thus the drug combination did not induce a sufficient secretory response of proteins from the α-granules. One plausible explanation could be that the combination of LPA and adrenaline, but not separate treatment, induces a conformational change of GPIIb/IIIa to its high-affinity state. Additionally, Smyth *et al.*[34] have suggested that LPA is a potent activator of fibrinogen binding to GPIIb/IIIa.

Consequently, it could not be excluded that LPA indirectly facilitates adrenaline-induced platelet aggregation. Such an action would presumably lead to aggregation-dependent (i.e. integrin-dependent) tyrosine phosphorylation. Tyrosine kinases in platelets are regulated both by classical receptor proteins and by integrins.[32] We have not distinguished between different phosphorylated proteins. However, aggregation-dependent tyrosine phosphorylation of focal adhesion kinase, p95/97, Src, and Syk has previously been reported.[32] It is likely that some of these proteins are phosphorylated after the addition of LPA and adrenaline and subsequent aggregation.

Siess *et al.*[35] found that the lipid-rich core in carotid atherosclerotic lesions had the highest content of LPA and that LPA was the main activating lipid of platelets in the plaques. This region is very thrombogenic and most prone to rupture and thereby LPA could trigger platelet aggregation in the circulation. This might lead to arterial thrombus, myocardial infarction and stroke. The concentration of LPA in serum has been estimated to be in the range of 1-5 μM,[36] suggesting that LPA is one of the mitogens in serum.

It is also well-known that LPA can exert such diverse effects as smooth muscle contraction,[37] and cellular growth of both fibroblasts[38] and smooth muscle cells,[39] all effects known to be significant for the pathogenesis of atherosclerosis and cardiovascular diseases. Our findings reveal that isolated platelets from some healthy blood donors could synergistically respond to a combination of two weak activators, namely LPA and adrenaline. Increased knowledge about this might be used in the prevention and treatment of some severe diseases and, therefore, warrants further investigation.

Contributions and Acknowledgments

UN, SS and MG designed the study. UN and MG performed all the experimental work. UN wrote the manuscript, which was reviewed by MG and SS. We wish to thank the staff at the Department of Transfusion Medicine and Clinical Immunology for skillful help with the blood sampling. GEA 3175 was a generous gift from GEA Company (Copenhagen, Denmark).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Carlo Balduini, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Prof. Balduini and the Editors. Manuscript received November 26, 2001; accepted May 6, 2002.

What is already known on this topic

Lysophosphatidic acid (LPA) is considered an agonist of platelet activation, but its mechanism of action and its role in physiologic and pathologic conditions are largely unknown.

What this study adds

Although LPA belongs to the category of weak platelet activator, its combination with adrenaline can induce an irreversible platelet aggregation *in vitro*. However, this synergistic effect is evident only in some healthy subjects.

Potential implications for clinical practice

Since atherosclerotic lesions have a high content of LPA, the synergistic effect of LPA and adrenaline might be a new risk factor for arterial thrombosis.

Carlo Balduini, Associate Editor