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LYSOPHOSPHATIDIC ACID-INDUCED AGGREGATION OF HUMAN AND FELINE PLATELETS: STRUCTURE-ACTIVITY RELATIONSHIP

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SUMMARY: Lysophosphatidic acid induced aggregation of human and feline platelets, the activity depending on the structure of the *sn*-1-acyl moiety. A hydroxyl group at the *sn*-2-position was not necessary for the activity, but a wedge shaped structure was suitable for induction of aggregation. Almost all the lysophospholipids tested with a head-group attached to the phosphate portion had little or no activity, but the *sn*-2-acetylated analogs of these phospholipids were very active. These phospholipids with activity were suggested to induce aggregation by interaction with the same binding site on the cell surface.

Lysophosphatidic acid(LPA)[1] is known to have some similar biological and pharmacological effects to those of prostaglandins and thromboxanes derived from polyunsaturated fatty acids: It causes contraction of isolated smooth muscle from rabbit duodenum(1,2) and rat uterus(3) and aggregation of human (4,5) and feline(4) platelets and its intravenous injection causes acute hypotension in cats(4,6) and rabbits(6) and hypertension in rats(6,7) and guinea pigs(6). We found that the chain length and degree of unsaturation of the acyl chain at the *sn*-1-position of the glycerol residue of LPA influenced the potencies of the vaso-effects(6). In this communication, the structure-activity relationship of lysophosphatidic acid is evaluated on the basis of its activity for inducing aggregation of human and feline platelets.

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Abbreviations used: LPA, lysophosphatidic acid; PRP, platelet-rich plasma; GP, *sn*-glycero-3-phosphate; GPM, *sn*-glycero-3-phosphorylmethanol; GPE, *sn*-glycero-3-phosphorylethanol; GPP, *sn*-glycero-3-phosphorylpropanol; GPC, *sn*-glycero-3-phosphorylcholine; 12:0, 1-lauroyl; 14:0, 1-myristoyl; 16:0, 1-palmitoyl; 18:0, 1-stearoyl; 18:1, 1-oleoyl; 18:2, 1-linoleoyl; 18:3, 1-linolenoyl; 2:0, 2-acetoyl; 3:0, 2-propionoyl; 4:0, 2-butyroyl; 6:0, 2-caproyl

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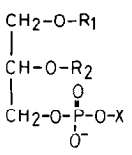

Structure 1
Basic structure of the phospholipids used.
X: H, CH_3, CH_2CH_3, $CH_2CH_2CH_3$, or $CH_2CH_2\overset{+}{N}(CH_3)_3$
R_1: long chain fatty acyl
R_2: H or short chain fatty acyl

MATERIALS AND METHODS

Preparation of phospholipids 1-Palmitoyl(16:0)- and 1-oleoyl(18:1)-*sn*-glycero-3-phosphate(GP) were obtained from Serdary Research Laboratories Inc. (London, Ontario, Canada). 1-Lauroyl(12:0)-, 1-myristoyl(14:0)-, 1-palmitoyl (16:0)- and 1-stearoyl(18:0)-*sn*-glycero-3-phosphorylcholine(GPC) were purchased from Sigma Chemical Co.(St.Louis, Mo. U.S.A.). 1-linoleoyl(18:2)- and 1-linolenoyl(18:3)-GP were kindly provided by Dr. T. Nakajima of Nihon Shoji Kaisha Ltd. 1-Lauroyl(12:0)- and myristoyl(14:0)-GP were prepared by the method of Long et al.(8) from the corresponding 1-acyl-GPC by hydrolysis with phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4: from cabbage) and purified as described previously(6). 1-Palmitoyl-*sn*-glycero-3-phosphorylmethanol(16:0-GPM), 1-palmitoyl-*sn*-glycero-3-phosphorylethanol(16:0-GPE) and 1-palmitoyl-*sn*-glycero-3-phosphorylpropanol(16:0-GPP) were prepared from 16:0-GPC by transphosphatidylation with cabbage phospholipase D in the presence of 4 % methanol, ethanol and propanol, respectively, by a similar procedure to that used for preparation of LPA, but at pH 7.5. The phospholipids were purified by column chromatography on Sephadex LH-20(100 g, 3.5 x 75 cm) with chloroform-methanol mixture(1:1, v/v) as eluting solvent. The hydroxyl group at the *sn*-2-position of various 1-acyl-phospholipids was acetylated as follows: One ml of acetic anhydride and 1 ml of dry pyridine were added to a solution of 25 mg of the phospholipid in chloroform. The mixture was incubated for 2-5 hours at 30°C, and then the reaction was stopped by adding methanol and the 2-acetylated phospholipid was purified by thin layer chromatography(Merck silica gel plate 60) with a developing solvent of chloroform-methanol-water(65:35:5, v/v/v). The *sn*-2-propionoyl-, butyroyl- and caproyl-analogs of 1-palmitoyl-GPC were prepared by the same procedure, but with propionic anhydride, butyric anhydride and caproic anhydride, respectively, instead of acetic anhydride. 1-Palmitoyl-2-acetoyl-*sn*-glycero-3-phosphate(16:0-2:0-GP) and the 2-butyroyl and caproyl analogs were formed by hydrolysis of the corresponding 1-palmitoyl-2-short chain fatty acyl-GPC with phospholipase D by the procedure used for preparing 1-acyl-GP.

Aggregation of platelets Human venous blood was collected from healthy volunteers into 0.1 volume of 3.8 % trisodium citrate or heparin(2 I.U./ml of blood). None of the donors had taken any drugs within the previous week. Feline blood was collected from animals under ether anaesthesia from the carotid artery into a tube containing trisodium citrate or heparin at the concentrations described above. Platelet-rich plasma(PRP, approximately 3 x 10^8 platelets/ml) was prepared by centrifuging whole blood at 150*g* for 10 minutes at room temperature. Platelet aggregation was monitored by continuous recording of light transmission in a platelet aggregation profiler(PAP-3, BIO/DATA). A volume of 50 µl of the test phospholipid in saline was added into 0.5 ml of PRP. The relative effects of different compounds on platelet aggregation were compared by mixing volumes of 0.5 ml of the compounds in saline with 0.5 ml of PRP, beacuse some phospholipids with little activity were poorly soluble in saline at high concentrations.

Arachidonic acid(Sigma) and indomethacin(Sigma) were dissolved in 0.1 M Na_2CO_3 and then diluted with saline for tests. ADP was purchased from Sigma.

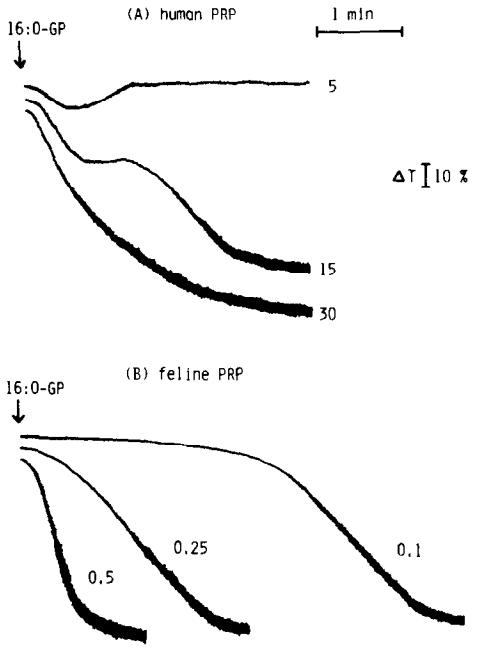

Figure 1 Concentration-dependent aggregation of human(A) and feline(B) citrated platelets produced by 16:0-GP. Numbers represent final concentrations of 16:0-GP in μg/ml. ΔT=change in light transmission.

RESULTS AND DISCUSSION

A low concentration of 16:0-GP caused similar extents of reversible aggregation of platelets in citrated and heparinized human PRP. Higher concentrations of 16:0-GP induced irreversible aggregation of platelets in both PRP preparation. (Figure 1-A) Human platelets gradually became desensitized to 16:0-GP with time, desensitization being most rapid in heparinized PRP. The threshold concentrations of different phospholipids for inducing platelet aggregation were compared using citrated PRP within 60 minutes after collection of the blood.

Feline platelets were irreversibly aggregated by 16:0-GP with a lag time depending on the concentration, as shown in Figure 1-B. Heparinized PRP were about three times more sensitive to 16:0-GP than citrated PRP, but they tended to become desensitized to this phospholipid with time, as mentioned above. The concentrations of different phospholipids causing irreversible aggregation with a lag time of 3-5 minutes after their addition to citrated feline PRP were

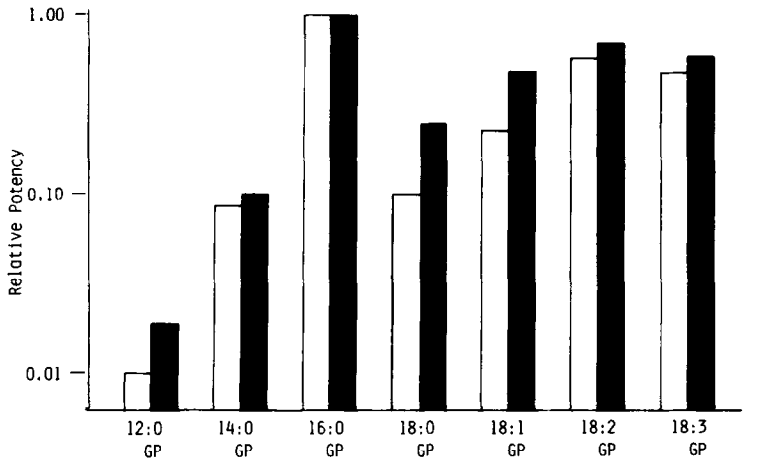

Figure 2 Relative potencies of various 1-acyl-*sn*-glycero-3-phosphates in inducing aggregation of human platelets(black bars) and feline platelets(white bars). The mean threshold concentration of 16:0-GP is 9 μg/ml for human platelets and 0.7 μg/ml for feline platelets. n=5

compared. Figure 2 shows the relative potencies of various 1-acyl-GPs(LPAs) to produce aggregation of human and feline platelets. The chain length of the *sn*-1-acyl moiety greatly influenced the potency, and the optimal length was 16 for both species. On the other hand, increase in the degree of unsaturation of the C_{18}-fatty acyl residue resulted in only slight increase in activity. Thus a long chain hydrophobic region is required for platelet aggregation.

To evaluate the influence of the lyso-form we tested the effects of various 1-palmitoyl-2-short chain fatty acyl-GPs. Their relative potencies in evoking aggregation of feline platelets are shown in Figure 3-A. The activity of 16:0-2:0-GP was about half that of 16:0-GP, and elongation of the acyl chain at the *sn*-2-position led to progressive decrease in activity. Thus the hydroxyl group is not necessary, but a wedge shaped structure is favorable for platelet aggregation

Next, we examined the effect of chemical modification of the phosphate portion of LPA. The activity of 16:0-GPM was about one thirtieth of that of 16:0-GP on feline platelets and one twenty fifith of the latter on human platelets. Other lysophospholipids, such as 16:0-GPE, 16:0-GPP and 16:0-GPC, were all inactive.

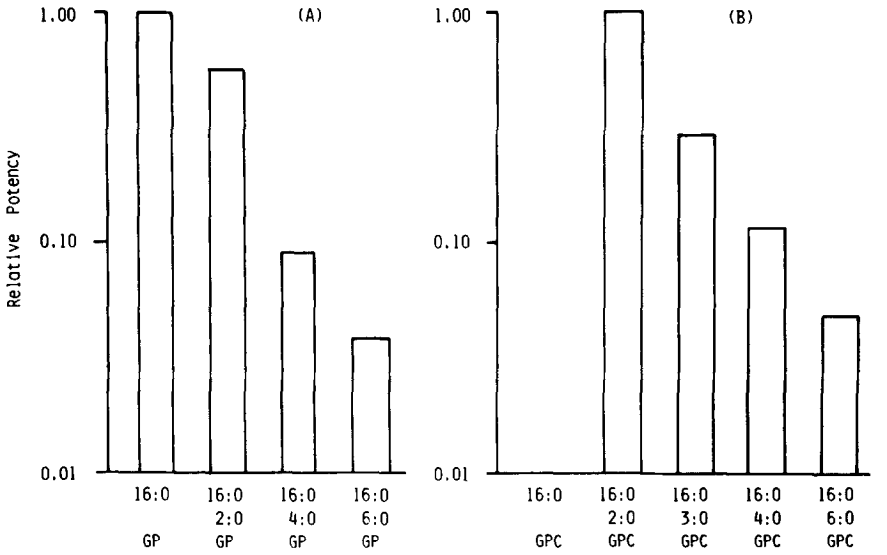

Figure 3 Relative potencies of various 1-palmitoyl-2-acyl-*sn*-glycero-3-phosphates (A) and 1-palmitoyl-2-acyl-*sn*-glycero-3-phosphorylcholines(B) in evoking aggregation of feline platelets. The mean threshold concentration of 16:0-GP is 0.7 μg/ml, and that of 16:0-2:0-GPC is 4 μg/ml. n=5

However, acylation of the hydroxyl group at the *sn*-2-position with a short chain fatty acid anhydride in the series of choline-containing phospholipids resulted in strong aggregatory activity on feline platelets, as shown in Figure 3-B. The *sn*-2-acetoyl analog was the most active. The chain length of the acyl at the *sn*-1-position also influenced the potencies of 1-acyl-2-acetoyl-GPCs in producing aggregation of human and feline platelets, as shown in Figure 4. Figure 5 shows the relative potencies of different 1-palmitoyl-2-acetoyl-phospholipids in inducing aggregation of feline and human platelets. The potency also depended on the size of the chemical group attached to the phosphate portion, and the optimum size for activity was that of ethyl group with feline and human platelets. Possibly, the chemical property of the head-group was not critical factor in determining the potency. Like the structure of *sn*-1-acyl moiety, the balance between the sizes of the chemical group at the *sn*-2-position and head-group is a decisive factor in controlling the potency of activity.

Recently, the platelet activating factor(PAF) released from IgE-sensitized rabbit basophils found to be 1-O-alkyl-2-acetoyl-GPC(9,10). Its activity in

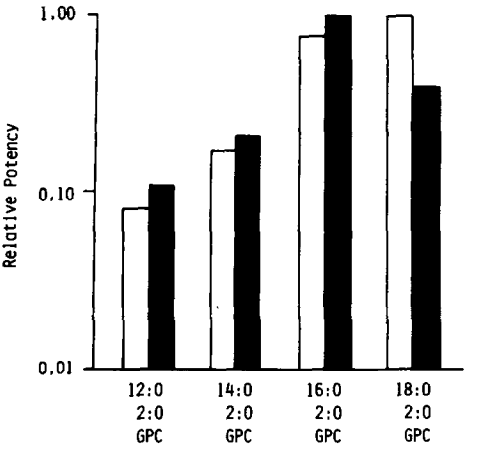

Figure 4 Relative potencies of various 1-acyl-2-acetoyl-*sn*-glycero-3-phosphoryl-cholines in producing platelet aggregation in human PRP(black bars) and feline PRP(white bars). Threshold concentration: 16:0-2:0-GPC, 22 μg/ml(human PRP); 18:0-2:0-GPC, 3.5 μg/ml(feline PRP) n=4

producing aggregation of washed rabbit platelets was about thirty times that of the corresponding 1-acyl analog, indicating that the mode of binding of a long chain hydrocarbon to the glycerol residue influences the potency as well as the structural factors shown in this study.

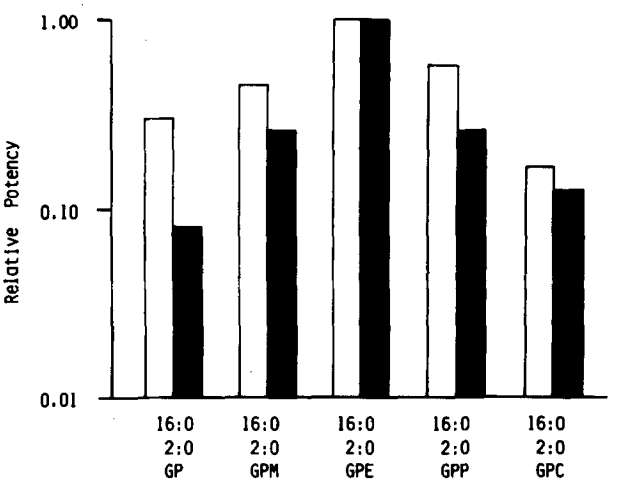

Figure 5 Relative potencies of various 1-palmitoyl-2-acetoyl-phospholipids in producing aggregation of human platelets(black bars) and feline platelets(white bars). Threshold concentration: 16:0-2:0-GPE, 2.5 μg/ml(human platelets) and 0.4 μg/ml(feline platelets) n=5

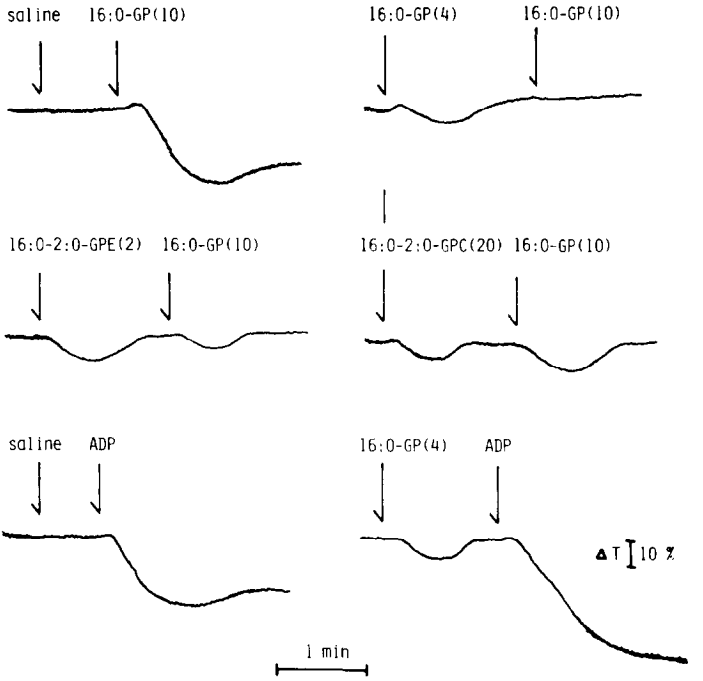

Figure 6 Tachyphylaxis of human platelets treated with different phospholipids on subsequent addition of 16:0-GP. Numbers represent final concentrations in μg/ml of added phospholipids. The final concentration of ADP is 5 mM. Arrows indicate the times of additions of the phospholipids and ADP in 50 μl saline. ΔT=change in light transmission.

Feline platelets were more sensitive to the phospholipids tested in this study than human platelets. The effects of the phospholipids on platelet aggregation did not involve endogenous prostaglandin synthesis because 5 mM indomethacin blocked the irreversible aggregation of human and feline platelets induced by arachidonic acid, but not by these phospholipids at any concentration tested.

Human platelets exposed to the threshold concentration of 16:0-GP or concentration inducing reversible aggregation became desensitized when this phospholipid was subsequently added, as reported by Schumacher et al.(4). This tachyphylactic effects was observed with all the active phospholipids, and the platelets exposed to 1-acyl-GP were the most tachyphylactic. Cross-tachyphylaxis was observed not only between various molecular species of LPA but also 16:0-GP and the phospholipids such as 16:0-2:0-GP, 16:0-2:0-GPM, 16:0-2:0-GPE, 16:0-2:0-GPP,

and 16:0-2:0-GPC, respectively. However, exposure to active phospholipids potentiated the aggregation of human platelets produced by ADP. Figure 6 shows representative tracings of results on the tachyphylactic properties of human platelets. Feline platelets exposed to threshold concentrations of active phospholipids also exhibited similar tachyphylactic effects. This cross-tachyphylaxis among the active phospholipids, together with the structural similarities of these phospholipids suggests that in both species these compounds cause aggregation by interaction with the same binding sites on the platelet surface.

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