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ACTIVATION OF BOVINE PLATELETS INDUCED BY LONG-CHAIN UNSATURATED FATTY ACIDS AT JUST BELOW THEIR LYTIC CONCENTRATIONS, AND ITS MECHANISM

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The effects of long-chain unsaturated fatty acids such as linoleic acid on bovine platelets were examined. Not only linoleic acid, but also oleic and linolenic acid, at just below the concentrations causing marked cell lysis, induced an absorbance decrease of the platelet suspension in the presence of Ca2+. Since this absorbance decrease was reversed by the addition of EDTA and moreover aggregate formation was found by macroscopic and microscopic observation, it was concluded that unsaturated fatty acids at just below their lytic concentrations caused platelet aggregation. Unsaturated fatty acids also caused release of adenine nucleotides, but there was a lag time between the release and the aggregation, just as with ADP-induced release, suggesting that the aggregation was independent of the release of ADP. It was revealed that this activation of platelets by unsaturated fatty acids was caused by marked Ca2+ uptake into the cytoplasm, resulting from significant membrane perturbation.

Introduction

Various substances that play critical roles in development of thrombosis and hemostasis in vivo, such as ADP, thrombin, collagen and PAF-acether, induce platelet aggregation in vitro [1,2]. Several other substances, such as calcium ionophore and exogenous arachidonic acid, also induce platelet aggregation in vitro [3,4], and have been considered as useful materials to use in studies on the mechanisms of platelet activation. One of these substances, the long-chain unsaturated fatty acid arachidonic acid, is known to trigger platelet activation after its conversion to a potent activator, thromboxane A 2 [4,5]. However, the effect of thromboxane A 2 does not account for the activation by arachidonic acid completely, especially with mammalian platelets other than human ones [6]; nonspecific interaction of arachidonic acid with the platelet membrane may also play some role in the activation.

On the other hand, there are many reports that other long-chain unsaturated fatty acids, such as linoleic acid, at concentrations much below their critical micelle concentrations inhibit platelet aggregation induced by ADP or thrombin [7-9]. They are also known to induce cell lysis at higher concentrations (more than 100/~M [10]). Since it is generally known that marked increase of passive transport of ions occurs in prelytic conditions [11,12], at slightly below their lytic concentrations these fatty acids may have different effects on aggregation. In this work we investigated the activation of platelets by long-chain unsaturated fatty acids such as linoleic acid at just below their lytic concentrations. Our results provide informa-

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tion on fundamental aspects of the mechanism of platelet activation, although the effective fatty acid concentrations are unphysiological.

Materials and Methods

Materials. Oleic, linoleic and linolenic acid (99% purity) were purchased from P-L Biochemical, Inc. (Milwaukee, WI). Their methanolic solutions were prepared just before use. fl-NADH and ADP were purchased from Oriental Yeast Co. (Tokyo, Japan). Bovine fibrinogen, diphenylhexatriene, tetracaine hydrochloride, indomethacine and forskolin were from Sigma Chemical Co. (St. Louis, MO). Highly purified luciferin-luciferase reagent was from LUMAC B.V. (Schaesberg, Netherlands) and all other chemicals were from Wako Pure Chemical Industries (Osaka, Japan). 45Ca was purchased from Amersham International plc. (Buckinghamshire, U.K.).

Measurement of aggregation. Aggregation of bovine (Holstein) blood platelets was measured as described previously [9]. That is, a suspension of platelets separated from plasma protein by centrifugation was mixed with 9 vol. of Na,K-Tris medium (137 mM NaC1/5.4 mM KC1/ll mM dextrose/25 mM Tris-HC1, adjusted to pH 7.4) containing 1 mg/ml fibrinogen; the final platelet concentration was about 9. 104//d. CaC12 was added to the suspension to a final concentration of 0.5 mM. Then unsaturated fatty acids were added and the absorbance change at 600 nm was recorded at 25°C.

Measurement of A TP secretion. Release of ATP was measured simultaneously with aggregation by the luminescence method with luciferin-luciferase as described previously [13]: i.e., aliquots of the platelet suspension were withdrawn at intervals and mixed with an equal volume of a solution containing luciferin-luciferase reagent, and the luminescence was measured in a Biocounter 2010 (LUMAC B.V.).

Measurement of cell lysis. Platelet lysis was measured by determining lactate dehydrogenase activity liberated into the medium. Lactate dehydrogenase was assayed by the method of Wr6blewski and LaDue [14] by following the decrease in concentration of fl-NADH as change in absorbance at 340 nm. Total lactate dehydrogenase in the suspension was determined by assay of sonicated samples.

Measurement of fluorescence polarization. Fluorescence polarization of diphenylhexatriene-labeled bovine platelets was measured as described previously [9] except that platelet membranes were used instead of intact platelets. Platelets at a concentration of about 9- 104//~1 were lysed with 24 mOsm phosphate buffer (pH 7.4). The membranes were separated by centrifugation at 25000 x g for 20 min, washed and resuspended in the same volume of the above hypotonic phosphate buffer and incubated with 1/~M diphenylhexatriene for 40 min. Diphenylhexatriene fluorescence in platelets was measured in a spectrofluorometer 650-40 (Hitachi Seisakusho Co., Tokyo, Japan) at 25°C. Fluorescence polarization was determined as described previously [9,15].

Measurement of uptake of Ca: +. The uptake of Ca^{2+} was assayed by adding 45Ca to the platelet suspension and measuring the radioactivity of the platelets. For this, 27 ~Ci 45Ca were added to 8 ml of the platelet suspension described above containing 50/~M CaC12 at 25°C. Samples of 0.64 ml of the suspension were transferred at intervals to 1.5 ml polypropylene centrifuge tubes with EDTA at a final concentration of 2 mM. The EDTA prevented spontaneous aggregation of the platelets on centrifugation and removed Ca^{2+} bound to their surface. The platelets were separated from the medium in a microcentrifuge using a mixture of dibutylphthalate and dinonylphthalate as separating oil [16]. The precipitate was solubilized in 0.2 ml of 5% SDS solution and mixed with 10 ml of scintillator. Radioactivity was then determined.

Results

Aggregation of platelets induced by linoleic acid

We found previously that concentrations ot more than 10 /~M linoleic acid inhibited ADP-induced aggregation of bovine platelets [9]. We examined the effects of linoleic acid on the platelets in the absence of any stimulants such as ADP. Experiments were done at 25°C. This temperature is expected to be better than 37°C to investigate the physicochemical interaction of free fatty acids with platelet membranes without enzymatic modification of the acids. As shown in Fig. 1, in the

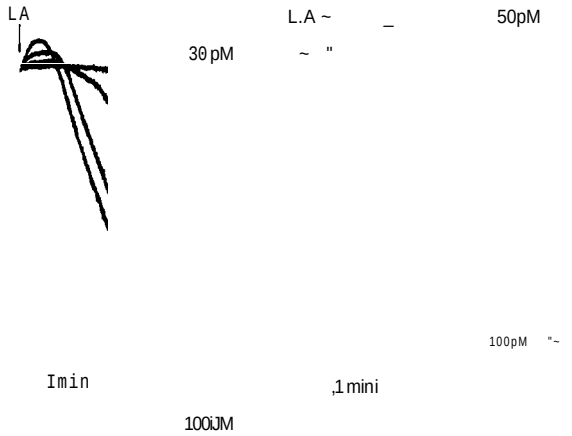

Fig. 1. Absorbance decrease after addition of various concentrations of linoleic acid (L.A.) in the presence (left) and absence (right) of 0.5 mM CaCI 2. The concentrations of linoleic acid are shown in the figure. A platelet suspension obtained as described in Materials and Methods (conc., 9.105//tl) was mixed with 9 vol. of Na,K-Tris medium containing 1 mg/ml fibrinogen. After addition of linoleic acid in the presence or absence of CaC12 the absorbance change at 600 nm was measured at 25°C.

presence of Ca 2+, addition of more than 50 /aM linoleic acid to a suspension of bovine platelets caused a decrease in absorbance after an initial slight increase that was probably due to a change in shape of the platelets. On the other hand, in the absence of Ca2+, addition of 50 /aM linoleic acid caused only an initial increase in absorbance with no subsequent decrease. Addition of more than 100 /aM linoleic acid caused a marked decrease, but the rate of decrease was less than in the presence of Ca2+. These results suggest that the decrease in absorbance shown in Fig. 1 induced by 50 /aM linoleic acid was due to aggregation, not lysis, of the platelets. Formation of macroscopically observed aggregates under these conditions supports this idea strongly. Moreover, it is also improbable that the decreases in absorbance were due to dramatic alteration of cell shape on addition of high concentrations of unsaturated fatty acids [17].

Then we measured lysis induced by linoleic acid by determining liberation of lactate dehydrogenase activity into the medium. As shown in Fig. 2, in the presence of concentrations of linoleic acid of less than 75 /aM lysis was not marked compared

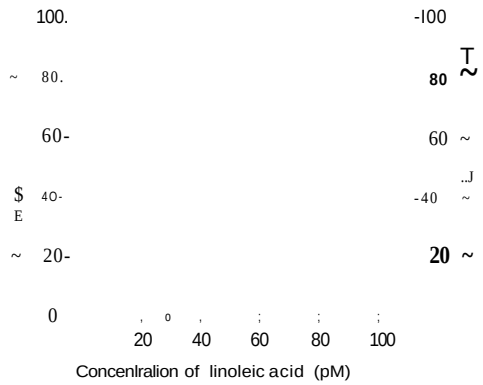

Fig. 2. Concentration-dependent absorbance decrease and cell lysis of platelet suspensions mixed with linoleic acid for 4 min with stirring. Platelet lysis was determined by assay of lactate dehydrogenase (LDH) in the extracellular fluid. Values are means + S.D. for three experiments.

with that of control platelets, at least within 4 min after addition of linoleic acid. Aggregation of platelets was finally demonstrated by microscopic observation after addition of 50 /aM linoleic acid. Moreover, normal aggregation of the platelets by linoleic acid was also demonstrated by showing that the decrease in absorbance by linoleic acid was reversed by addition of the Ca2+-chelating agent EDTA. The results in Fig. 3 show that addition of EDTA reversed the decrease in absorbance induced by 50 /aM linoleic acid, but not that by 100/aM linoleic acid. The latter finding is consistent with the fact that the higher concentration causes cell lysis, as described above. Macroscopically observable aggregates formed by the

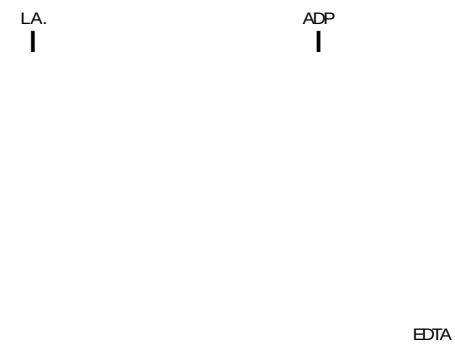

Fig. 3. Effects of 1 mM EDTA on the absorbance decrease induced by 50/,M and 100/*M linoleic acid (L.A.) (left) and 2 #M ADP (fight).

addition of 50 #M linoleic acid also disappeared on addition of EDTA. Reversal of ADP- and collagen-induced aggregation of human and rabbit platelets by EDTA has been reported [18], and reversal of ADP-induced aggregation of bovine platelets by EDTA is confirmed in Fig. 3. From these results, it was concluded that addition of linoleic acid at just below its lytic concentration caused aggregation of platelets. Similar aggregation of platelets induced by 50 /~M linoleic acid was also observed at 37°C (data not shown).

We next examined the effects of platelet-aggregation inhibitors, inhibitors of thromboxane A2 synthesis, reagents increasing intracellular cAMP and Ca2+-blockers on the aggregation induced by linoleic acid. Of these reagents, only Ca2+-blockers inhibited the aggregation. Fig. 4 shows the effects of the Ca2+-blocker tetracaine and a reagent increasing the cAMP level, forskolin.

A TP secretion induced by linoleic acid

On activation of platelets by stimulants, the contents of dense secretory granules, adenine nucleotides, serotonin and Ca2+ are released [19] simultaneously with or after irreversible aggregation [13,20,21]. We examined the release of ATP from platelets induced by linoleic acid. Fig. 5 shows that ATP secretion occurred with a lag after aggregation, just like ADP-induced release of ATP from bovine and human platelets [13,20], although the release was less than that induced by ADP [13]. These results suggest that the aggregation

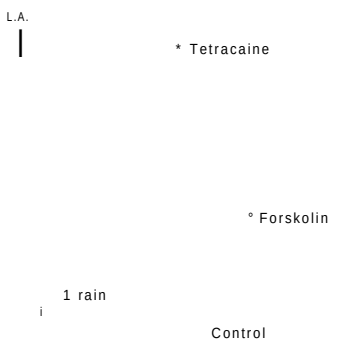

Fig. 4. Effects of 10 I~M forskolin and 1 mM tetracaine on the aggregation induced by 50 ~M linoleic acid (L.A.). Forskolin and tetracaine were added to the platelet suspension just before linoleic acid.

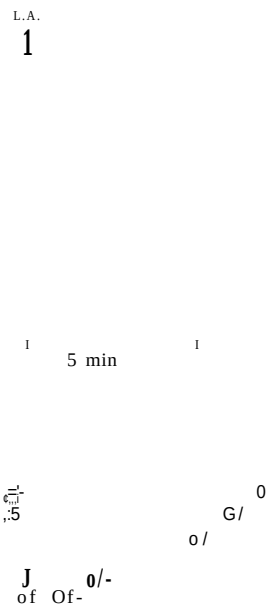

Fig. 5. Simultaneous measurements of aggregation and ATP secretion induced by 50 y.M linoleic acid (L.A.). Upper and lower curves show aggregation and release of ATP, respectively. ATP release was measured at intervals by luminescence assay, on aliquots of the platelet suspension. Concentrations of ATP released into the medium are shown as means + S.D. for three experiments.

induced by linoleic acid was independent of secreted ADP. This idea is supported by the fact that aggregation by linoleic acid was not affected by the creatine phosphate/creatine phosphokinase enzyme system that converts released ADP to ATP (data not shown).

Effects of other long-chain unsaturated fatty acids

We also examined platelet aggregation by the long-chain *cis-unsaturated* fatty acids oleic and linolenic acid. The absorbance of a platelet suspension decreased markedly on addition of 50/~M oleic acid or linolenic acid in the presence of Ca2+, and aggregate formation was observed macroscopically (data not shown). Moreover, the absorbance decrease was also reversed by addition of EDTA and then aggregates disappeared. In the presence of oleic and linolenic acid, the percentage cell lyses, determined by LDH assay, were 3.4 and 11.0%, respectively, which were similar to the control values. Marked cell lysis occurred with these

fatty acids at concentrations of more than 100 #M. These results show that the aggregation occurred on addition of long-chain unsaturated fatty acids in general at just below their lytic concentrations. These results are consistent with the results of Zentner et al. [22] on the aggregation induced by long-chain fatty acids, in suspension or taurocholate-solubilized, involving oleic acid as unsaturated fatty acid, although the concentrations of acids that they used were mostly too high to compare with ours.

Membrane fluidity change by unsaturated fatty acids

In studies on the mechanism of activation of platelets induced by unsaturated fatty acids, we examined the effects of the acids on the membrane fluidity by measuring fluorescence polarization of diphenylhexatriene-labeled platelets. We have already reported [9] that linoleic acid at concentrations of up to 30 /~M caused an increase in membrane fluidity of platelets. In this work we investigated the effects of higher concentrations of the acids. We used platelet membranes prepared by hypotonic lysis to avoid the effects of the change in light-scattering properties on fluorescence polarization induced by lytic concentrations of the acids.

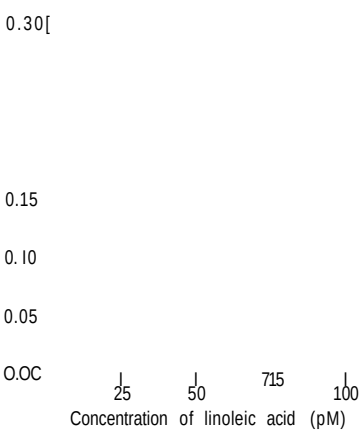

Fig. 6. Effects of linoleic acid on fluorescence polarization of diphenylhexatriene-labeled platelet membranes. Fluorescence intensities of 1 /~M diphenylhexatriene-labeled platelet membranes were detected through a polarizer oriented parallel and perpendicular to the direction of the polarized excitation beam in the presence or absence of linoleic acid. Fluorescence polarization was calculated as described previously [9,15]. Data are means + S.D. for 7-10 experiments.

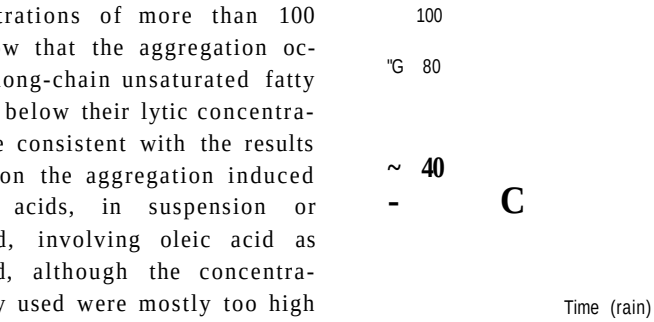

Fig. 7. Ca2+ *uptake* induced by lino]eic acid. The concentrations of linoleic acid used were zero (O), 20/~M (e), 40/~M (D) and 60/tM (m). 27/~Ci 45Ca was added to 8 ml of the platelet suspension containing 50 /~M CaCI2. Platelets were isolated and Ca2+ uptake was determined as described in the methods. Data are means_+S.D. for six experiments.

With increase in the concentration of unsaturated fatty acids, the fluorescence polarization decreased markedly, as shown in Fig. 6 for linoleic acid. Thus these fatty acids increased membrane fluidity more markedly at high concentrations.

Ca2÷ uptake induced by unsaturated fatty acids

Platelet activation is known to be induced by an increase in the concentration of cytoplasmic unbound Ca2÷ due to influx or change in intracellular distribution of Ca2+ after addition of stimulants [23,24]. Therefore, since it is also known that marked increase of passive transport of ions occurs in prelytic conditions [11,12], we examined the influence of unsaturated fatty acids on Ca2+ uptake into the cytoplasm.

Fig. 7 shows that relatively high concentrations of unsaturated fatty acids, which activated the platelets, caused marked uptake of Ca2+, whereas low concentrations, which inhibited the aggregation induced by other stimulants such as ADP [9], caused much less uptake of Ca2÷.

Discussion

Platelet activation induced by various stimulants, such as ADP, epinephrine or collagen, is accompanied by an increase in the concentration of cytoplasmic unbound Ca2+. This increase is due to influx or intracellular redistribution of Ca2+ after addition of stimulants [23,24] and causes

activation of some enzymes, such as phospholipase A 2 and myosin light-chain kinase, which leads to aggregation and release of the contents of dense secretory granules, adenine nucleotides, serotonin and Ca2+ [19]. In the present work we found that platelet aggregation occurred on addition of various long-chain unsaturated fatty acids, as with arachidonic acid. However, the aggregation occurred in a narrower concentration range of these fatty acids than of arachidonic acid, being observed only at concentrations just below their lytic concentrations.

The present results indicate that activation of platelets was induced by marked Ca2+ uptake due to significant membrane perturbation caused by unsaturated fatty acids in this concentration range. Several workers, including us, [7-9] have found that lower concentrations of unsaturated fatty acids inhibit platelet aggregation induced by various stimulants such as ADP and thrombin. Their inhibitory effects, like those of alcohols [15], seem to be related to their effects in causing membrane perturbation [9]. Therefore, their actions are quite different at different concentrations, possibly due to differences in their extents of membrane perturbation, although the exact mechanism of their effects is not clear. As found in this work, relatively high concentrations of unsaturated fatty acids caused much more membrane perturbation than lower concentrations, which tended to inhibit platelet functions. The greater preturbation probably caused a marked increase in passive transport of Ca2÷, resulting in platelet activation. Such a nonspecific interaction of the acids with the platelet membrane may also play some role in the activation by arachidonic acid, especially with mammalian platelets other than human ones.

This phenomenon is probably not specific to long-chain unsaturated fatty acids, but is common to other lytic agents at sublytic concentrations. Results on saturated fatty acids, in suspension or taurocholate-solubilized [22], and on Ca2+-blockers [25,26] support this idea. Relatively high concentrations of Ca2+-blockers such as verapamil and tetracaine, which inhibit platelet functions at lower concentrations [9], have also been found to induce secretion of serotonin [25,26]. The reason why Ca2+-blockers induced secretion but not aggregation is probably that they compete with Ca2+, which is essential for aggregation, at binding sites on the outside of the plasma membrane [9]. Fatty acids and Ca2+-blockers probably induce platelet activation by similar mechanisms.

It is generally known that expansion of cells and marked increase of passive transport of ions occur in prelytic conditions [11,12]. Therefore, all lytic agents at sublytic concentrations probably induce platelet activation by increasing the cytoplasmic concentration of unbound Ca2÷, unless they have specific inhibitory activities on platelet functions.

Although these phenomena may not be physiologically significant, they clearly show that Ca2. is essential in platelet activation.

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