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EFFECT OF 1-O-ALKYL-2-ACETYL-sn-GLYCERYL-3-PHOSPHORYLCHOLINE ON CALCIUM FLUXES BY HUMAN PLATELET MICROSOMES

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Under conditions where optimal concentrations of arachidonic acid, phosphatidic acid, or the calcium ionophore A23187 caused release of 50-95% of calcium from preloaded platelet microsomes, basophil platelet activating factor (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, AGEPC) did not cause the release of calcium at concentrations as high as 2×10^{-5} M. The failure to stimulate calcium release was not due to metabolism or inactivation of AGEPC. These results show that AGEPC is not a calcium ionophore and is unable to directly effect the release of calcium from microsomes by mechanisms other than ionophoric action. The increase in intracellular levels that occurs during AGEPC-induced platelet aggregation must be an indirect effect of the AGEPC.

A major source of the intracellular calcium needed for platelet activation is a membrane organelle called the dense tubular system (1). This system is analogous in many respects to the sarcoplasmic reticulum of muscle. Like sarcoplasmic reticulum, it contains histochemical evidence of a Ca^{++}-ATPase[1] (2) and microsomal fractions containing the dense tubular system accumulate calcium in an ATP-dependent manner (1,3-5). The release of calcium from the dense tubular system provides a mechanism by which intracellular levels of calcium can be increased rapidly. One mechanism of calcium release appears to involve lipids generated during platelet activation. Gerrard and coworkers showed that arachidonic acid stimulated the release of calcium from preloaded microsomes (6). Since this effect was abolished by prior incubation of the microsomes with aspirin, it was suggested that products of arachidonic acid metabolism, thromboxane A_2 and, perhaps, prostaglandins G_2 and H_2, were the active components. Other unsaturated fatty acids of various chain lengths

[1] The abbreviations used are: ATPase, adenosine triphosphatase; AGEPC, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine; PAF, platelet activating factor; ATP, adenosine 3'-triphosphate; HPLC, high pressure liquid chromatography; N_2, nitrogen; arsenazo III, 2,2'-1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bisazo)bis-(benzene arsonic acid).

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were less effective than arachidonate at stimulating calcium release. Phosphatidic acid (or lysophosphatidic acid) was also shown to stimulate calcium release from preloaded microsomes (7). It was suggested that the early generation of these lipid products during platelet activation led to calcium release by the dense tubular system and increased levels of intracellular calcium.

Basophil platelet activating factor (PAF, PAF-acether) is a novel lipid product which has been shown to be a potent stimulator of platelets. This factor was initially described by Benveniste and coworkers (8) and was later identified by Demopoulous and coworkers and by Benveniste and coworkers as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC) (9-10). Platelet activation by this compound is calcium dependent but is independent of prostaglandin formation or secretion of adenosine diphosphate (11-13). The present studies were undertaken to examine the possibility that AGEPC might have ionophore-like actions similar to those of thromboxane and phosphatidic acid and thus affect platelets through the direct release of calcium from the dense tubular system.

MATERIALS AND METHODS

Crystalline disodium adenosine 3'-triphosphate (vanadate free), arachidonic acid, and phosphatidic acid were from Sigma Chemical Co. $^{45}CaCl_2$ (32 Ci/g in aqueous solution) was from Amersham. Micropore filters (HAMK 02412, 0.45 μm) were purchased from the Millipore Corporation. AGEPC was obtained from two sources. For most of the studies described, the AGEPC was obtained from Dr. D. Hanahan (San Antonio, TX). AGEPC was also obtained from Calbiochem. The two preparations were similar structurally and had equivalent platelet activating activity. Lyso-GEPC was also provided by Dr. Hanahan. [^{14}C]arachidonic acid (58.4 mCi/mmole), [^{3}H]AGEPC (45.0 Ci/mmole), [^{14}C]serotonin (50.7 mCi/mmole), and Aquasol 2 were from New England Nuclear.

Platelet aggregation and release: Platelet rich plasma was prepared and aggregation and release of serotonin were determined as previously described (14). The initial rate of aggregation was determined from the slope of the aggregation response. Release of platelet factor 4 was determined using a radioimmunoassay kit (Abbott Laboratories).

Platelet microsomes: An oxalate-loaded microsomal fraction was prepared from normal human platelets as described by Robblee and coworkers (9) and was diluted in "incubation medium" (100 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 20 mM Tris, pH 7.5, with 50 μM $CaCl_2$) to a concentration of 0.5-2.0 mg/ml. Microsomes were used within six hours of venipuncture.

Calcium transport: The uptake of calcium into and the efflux of calcium from platelet microsomes at 21°C was measured by an ultrafiltration technique as previously described (5). In efflux experiments, calcium released was expressed as a percentage of the calcium associated with the microsomes at the time the test agent was added.

Lipid metabolism by microsomes: One hundredth volume of AGEPC, containing tracer (approximately 200,000 CPM) was added to microsomes in "incubation medium". After 60 minutes, the lipids were extracted by the addition of 3.75 volumes of chloroform:methanol (1:2, v:v). After one hour, phase separation was accomplished by the addition of 1.25 volumes chloroform and 1.25 volumes distilled water. The lower phase was aspirated and dried under N_2. For HPLC, the sample was taken up in 100 μl methylene chloride:methanol (60:50, v:v) and chromatographed on a Microporasil column

(Waters Associates) equilibrated with methylene chloride:methanol:water (60:50:5) at 1.7 ml/min.

RESULTS

Effect of AGEPC on Intact Platelets: When tested at concentrations between 10^{-5} and 10^{-11} M, AGEPC stimulated dose-dependent platelet aggregation and secretion in recalcified PRP at concentrations of 10^{-9} M and higher. Half-maximal rate of aggregation was observed at 5.5×10^{-8} M AGEPC. At concentrations of AGEPC that produced maximal aggregation, release of [^{14}C]serotonin averaged 14.0 $\pm$ 4.2% and release of platelet factor 4 averaged 23.5 $\pm$ 8.4%.

Effect of AGEPC on Calcium Transport by Microsomes: Uptake of calcium by freshly prepared platelet microsomes at 21°C averaged 30.6 $\pm$ 10.6 nmol Ca^{++}/mg protein at 60 minutes (n = 30). Calcium uptake by microsomes was not affected by AGEPC at concentrations between 2.0×10^{-5} and 2×10^{-9} M. In order to examine the effect of AGEPC on calcium release, microsomes were loaded with calcium for 60 minutes then divided into six equal portions. AGEPC was added to each portion and the release of calcium determined for an additional 60 minutes as a function of AGEPC concentration. As shown in Fig. 1, AGEPC did not stimulate calcium release from preloaded microsomes at concentrations up to 2.0×10^{-5} M. This concentration of AGEPC induced 60-80% aggregation of human platelets in plasma. The small decrease in calcium associated with the microsomes in the presence of 2×10^{-5} M AGEPC was not progressive and may have been caused by membrane-related effects of AGEPC as this is well above the critical micellar concentration for AGEPC (15).

Recent studies (16-18) have indicated that AGEPC is metabolized in the presence of intact platelets to other products, including, at least transiently, a product that has properties similar to lyso-GEPC, but lyso-GEPC had no effect on calcium uptake or release at concentrations between 2.0×10^{-5} and 2.0×10^{-9} M (results not shown).

Under the same conditions as those used in the above experiments, arachidonic acid, phosphatidic acid (or lysophosphatidic acid), and the calcium ionophore, A23187, caused a rapid release of calcium from preloaded platelet microsomes indicating that the calcium in the microsomes was in a form that could be released (see Fig. 1 for results with A23187). The rate of calcium release was proportional to the concentration of the agent added. Half-maximal release of calcium at 30 minutes was obtained at concentrations of 0.8×10^{-7}, 2.0×10^{-5}, and 2.6×10^{-7} M, respectively (Fig. 2). None of the agents caused release of all of the calcium suggesting that at least a portion of the calcium in the microsomes was in a form that could not be released, perhaps bound to membrane proteins or present as a calcium-oxalate complex.

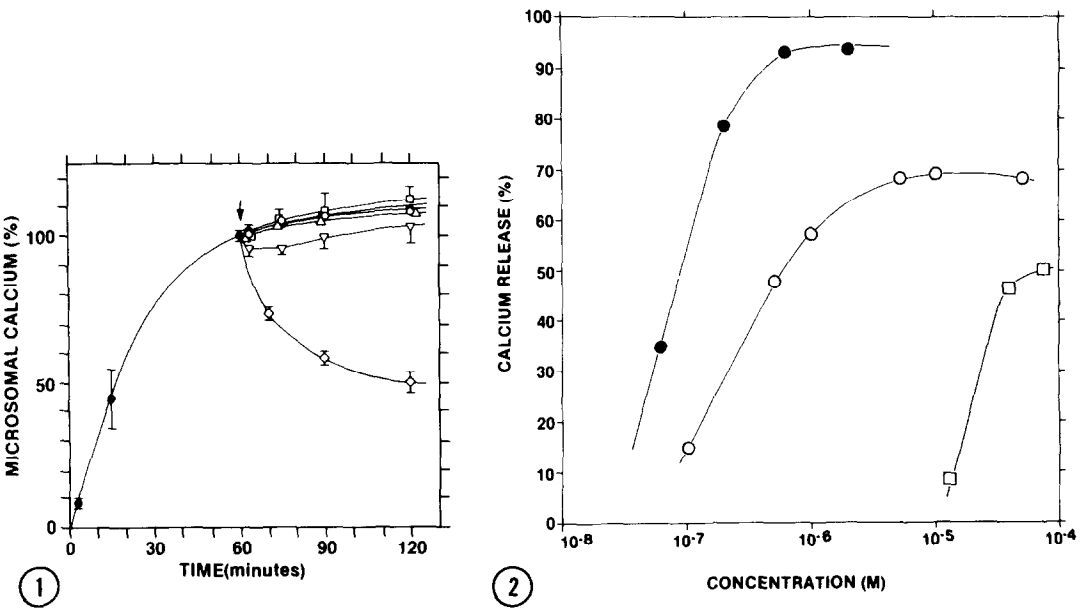

Fig. 1: **Effect of AGEPC on calcium release from platelet microsomes.** Microsomes at a concentration of 0.24-0.28 mg/ml in 10.0 ml of incubation medium were loaded with ^{45}Ca for 60 minutes. At 3, 15, and 60 minutes, duplicate 125 µl samples were removed and filtered through 0.45 µm filters for determination of calcium uptake. After the 60 minute sample, the microsomes were divided into six equal portions and one hundredth volume of AGEPC was added to give the concentration indicated. Calcium associated with the microsomes at each time point is expressed as a percentage of the calcium associated with the microsomes at 60 minutes. ●, buffer control. O, 2.5×10^{-6} M AGEPC. □ , 5.0×10^{-6} M AGEPC. △, 10^{-5} M AGEPC. ▽, 2×10^{-5} M AGEPC. ◇, 10^{-6} M A23187.

Fig. 2: **Effect of agents on calcium release from platelet microsomes.** These studies were performed as described in Fig. 1, but the results show the percent calcium released at 30 minutes after the addition of the agent to be tested. O, A32187. ●, arachidonic acid. □ , phosphatidic acid.

The failure of AGEPC to effect release of calcium from preloaded microsomes was not due to metabolism of AGEPC to an inactive product during incubation with the microsomes as more than 80% of [^{3}H]AGEPC incubated with microsomes for 60 minutes retained an elution profile typical of AGEPC by HPLC (Fig. 3). We also examined the possibility that an inhibitor of AGEPC was generated by incubation with the microsomes and found that the AGEPC retained full platelet stimulating activity during incubation with the microsomes (Table I).

DISCUSSION

Several experimental observations led us to explore the role of AGEPC as a calcium ionophore using platelet microsomes. First, other products of membrane phospholipid metabolism, like thromboxane and phosphatidic acid, have ionophore-like actions and are able to release calcium from platelet microsomes (6-7). Second, the effect of AGEPC on platelets is at least partly

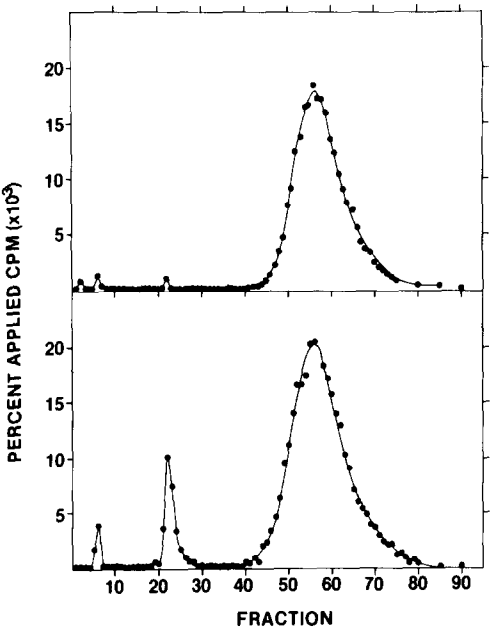

Fig. 3: Metabolism of AGEPC by platelet microsomes. Microsomes were suspended at a concentration of 1 mg/ml in incubation medium. To 1 ml of microsomes or buffer was added 0.01 ml of AGEPC at a concentration of 1 mg/ml containing approximately 200,000 CPM of labeled AGEPC. After 60 minutes at room temperature, the samples were extracted as described in Methods and prepared for HPLC. Top panel, AGEPC + buffer. Bottom panel, AGEPC + microsomes.

independent of thromboxane generation and adenosine diphosphate secretion and so must involve mechanisms other than these. One mechanism could be through calcium release from microsomes. Finally, there are somewhat conflicting reports on the ionophoretic action of AGEPC. Lee and coworkers have reported that AGEPC, but not AGEPC analogs, produced an ionophore-like increase in calcium uptake by intact rabbit platelets (19). Conversely, Serhan and coworkers were unable to demonstrate ionophoretic activity for AGEPC using phospholipid liposomes and arsenazo III (20). In the present study, we show

TABLE I

EFFECT OF MICROSOMES ON PLATELET ACTIVATING ACTIVITY OF AGEPC

	slope aggregation	percent release
buffer + AGEPC[a]	28.5 ± 3.0	38.8 ± 46.0
microsomes + AGEPC[a]	33.0 ± 3.9	32.3 ± 29.1

[a]Buffer or microsomes at a final concentration of 0.7 mg/ml in 1 ml of incubation medium were incubated with 2×10^{-5} M AGEPC for 60 minutes at room temperature. The samples were then centrifuged at 100,000 x g for 60 minutes at 4°C. The resulting supernatants were aspirated and 0.1 ml added to 0.9 ml of platelet-rich plasma. The rate of aggregation and percent release of platelet factor 4 were determined as described in Methods.

that AGEPC is unable to induce calcium release from microsomes. The failure of AGEPC to release calcium was not due to metabolism of AGEPC to inactive products or to inactivation of the AGEPC. We were further able to show that the calcium accumulated by platelet microsomes was released by A23187 and by other lipids indicating that calcium in the microsomes was in a form that could be released. The results therefore support the contention that AGEPC is not a calcium ionophore. More importantly, the results show that AGEPC is unable to directly release calcium from microsomes by either ionophoretic or other methods and indicate that AGEPC differs in its mechanism of action from thromboxane and lysophosphatidic acid, both of which are able to release calcium directly.

The observed difference between AGEPC, on the one hand, and thromboxane and lysophosphatidic acid, on the other, in terms of their effect on calcium release from platelet microsomes can be analyzed further by a comparison of the structures of these compounds. As pointed out by Gerrard and White (21), the structure of thromboxane A_2 is such that calcium can be efficiently bound and surrounded by hydrophobic portions of the molecule and thus be transported across a membrane. Lysophosphatidic acid can also bind calcium by virtue of its phosphate moiety and is lipid soluble. The spatial relationship between the bound calcium and the fatty acid is uncertain so it is not clear whether or not the calcium is surrounded by hydrophobic portions of the molecule as has been postulated for thromboxane A_2. Although AGEPC and lysophosphatidic acid are similar structurally, one significant difference between the two compounds is the choline group of AGEPC. The polar nature of the choline group might be expected to keep AGEPC from crossing the membrane and thus prevent it from acting as an ionophore.

Saturable binding of AGEPC to isolated platelet plasma membranes has been demonstrated (22). It has also been shown that AGEPC stimulates metabolism of labeled phosphoinositides in platelets with a decrease in phosphatidylinositol (PI), an increase in phosphatidic acid, and a transient decrease followed by an increase in PI-4-phosphate (DPI) and PI-4,5-phosphate (TPI) (23-24). This stimulation of the "PI cycle" is characteristic of other receptor-mediated agonists. These observations and the present results showing that AGEPC itself is incapable of transporting calcium suggest that exogenous AGEPC affects platelets from the platelet surface through a receptor-mediated stimulation of phospholipase C. The metabolites of PI which are generated by phospholipase C are likely to be responsible for the increase in intracellular levels of calcium that occurs during stimulation with AGEPC (19).

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