Platelet aggregating activity of lysophosphatidic acids is not related to their calcium ionophore properties

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The calcium ionophore properties of A23187 and of two lysophosphatidic acid (LPA) analogs (1-palmitoyl- and 1-hexadecyl-*sn*-glycero-3-phosphate or P-GPA and H-GPA, respectively) were compared using platelet membrane vesicles loaded with ^{45}Ca. Half maximal effect (HME) was obtained at 5 μM and 10 μM for H-GPA and P-GPA, respectively, against 0.7 μM for A23187, which released 2 times more Ca. The three compounds also induced platelet aggregation with a HME at 0.5 μM, 0.3 μM and 0.01 μM for A23187, P-GPA and H-GPA, respectively. The clear dissociation between the two effects appearing for both LPA raises some doubt about the general idea that (lyso) PA participate in cell activation through their calcium ionophore properties.

Platelet *Aggregation* *Activation* *Calcium* *Lysophosphatidic acid* *Ether phospholipid*

1. INTRODUCTION

An increased turnover of inositol phospholipids upon specific cell stimulation has been recognized as a widespread biochemical event [1–6]. The so called 'phosphatidylinositol effect' follows the activation of a specific phospholipase C, leading to the formation of diglycerides, which are used for phosphatidylinositol resynthesis via phosphatidic acid (PA) and phosphatidyl-CMP (CDP-diglyceride) [1–6]. The transient accumulation of PA occurring under these conditions might be involved in the mechanism of cell activation by promoting an increased level of cytoplasmic calcium, owing to the calcium ionophore properties of this phospholipid [7–11]. Such a role is further supported by the observation that PA is able to evoke physiological responses upon addition to various intact cells [8,12–14].

Lysophosphatidic acid (LPA) was also detected in stimulated platelets, which might involve either a diglyceride lipase coupled to a monoglyceride kinase [15], or a PA-specific phospholipase A_2 [16,17]. This more polar lysophospholipid carries calcium ionophore properties [18] and is able to promote platelet aggregation [18–23], to potentiate arachidonic acid release from thrombin-treated platelets [24] and to enhance neutrophil chemotaxis [25]. As such, it offers a useful model to study the properties of PA on intact cells. In this respect a 1-alkyl derivative of LPA revealed 30 times more potent than its 1-acyl-analog in inducing human platelet aggregation [23]. This was recently confirmed with acetal-PA [26]. We here show that both 1-alkyl-LPA and 1-acyl-LPA display similar potency in releasing calcium from a platelet membrane fraction and that, at a variance with calcium ionophore A23187, no relationship exists between their ability to activate platelets and their calcium gating properties.

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Abbreviations: PA, phosphatidic acid; LPA, lysophosphatidic acid; P-GPA, 1-palmitoyl-*sn*-glycero-3-phosphate; H-GPA, 1-hexadecyl-*sn*-glycero-3-phosphate; HME, half-maximal effect; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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2. MATERIALS AND METHODS

^{45}Calcium chloride (4–50 Ci/g Ca) was purchased from the Radiochemical Centre (Amersham). Calcium ionophore A23187 was obtained from Boehringer (Mannheim), whereas 1-palmitoyl-*sn*-glycero-3-phosphate (P-GPA) was from Serdary (London, Ontario); 1-hexadecyl-*sn*-glycero-3-phosphate (H-GPA) was prepared as in [23].

2.1. *Determination of calcium release from platelet membranes*

Platelet concentrates prepared from healthy human volunteer donors were obtained from the Blood Transfusion Centre and were used within 18 h after blood collection. Platelets were isolated and washed as in [27]. Cells from one concentrate were suspended in 10 ml of ice-cold lysis buffer (pH 7.0) containing 30 mM Hepes, 30 mM KCl, 2.5 mM $MgCl_2$ and 10 mM potassium oxalate. Cells were lysed at 4°C using the nitrogen cavitation procedure after equilibration for 20 min under 70 bars [28]. After a centrifugation at 13 000 × *g* for 10 min, the resulting supernatant was centrifuged at 40000 × *g* for 60 min at 4°C. The pellet was suspended in a minimal volume of lysis buffer and protein concentration was adjusted to 1.5 mg/ml as determined in [29]. One volume of platelet membranes was added to 9 vol. of incubation buffer (pH 7.0) containing 100 mM KCl, 2.5 mM $MgCl_2$, 30 mM Hepes, 2 mM ATP, 1 mM potassium oxalate and 0.01 mM $^{45}CaCl_2$. Incubation was performed at 37°C under shaking for various periods of time. At 30 min various agents were added under a minimal volume of solvent (0.01 ml/ml). Solvents were dimethylsulfoxide for A23187 and ethanol for LPA. The same volume of solvent was added in control samples, with identical results for dimethylsulfoxide and for ethanol.

At various times of the incubation procedure, 0.5-ml aliquots were removed and filtered through a millipore sampling unit using 0.5 μm pore size filters. Filters were air dried, solubilized in 10 ml aquasol and counted in an Intertechnique liquid spectrometer (model SL 4000) equipped with an automatic quenching collector.

2.2. *Determination of platelet aggregation*

Platelet aggregation was determined as in [23] by the turbidimetric method in [30], using platelets isolated from fresh human blood as in [31].

3. RESULTS

As shown in fig.1, platelet membranes were able to take up actively calcium upon incubation in a medium containing $^{45}CaCl_2$, ATP and potassium oxalate. Under these conditions calcium uptake levelled off at 30 min and the amount of incorporated radioactivity remained stable upon longer incubation. Further addition of A23187 to the incubation mixture 30 min after commencement of the uptake assay was followed by a rapid loss of calcium from the membranes. This was almost complete 20 min later and clearly illustrates the calcium ionophore properties of the drug. Under the same conditions, both kinds of LPA also induced some release of entrapped calcium, as in [18] for 1-acyl-LPA.

More quantitative data about the relative potencies of the 3 compounds toward platelet membranes are given in fig.2. A23187 appeared as the most powerful calcium ionophore, able to release 50% of calcium within 10 min at 5 μM final concentration, whereas 10 μM H-GPA and 50 μM P-GPA released only 25% of calcium under the same conditions. Higher concentrations gave somewhat

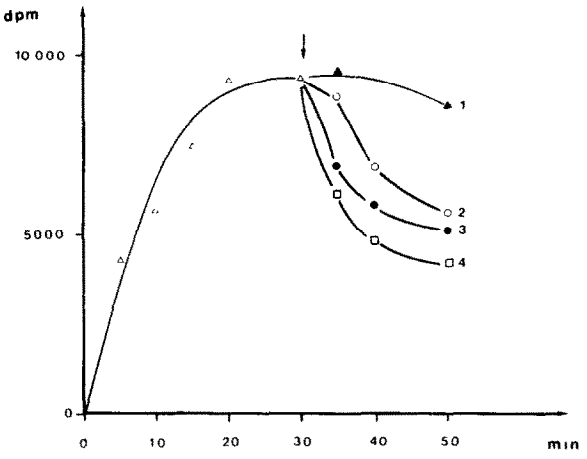

Fig.1. Calcium uptake by platelet membranes and release induced by ionophores. Results indicate the radioactivity remaining on the filters during uptake and after addition at 30 min of dimethylsulfoxide (1), 1-alkyl-LPA (2), 1-acyl-LPA (3) and A23187 (4). This experiment is representative of 3 similar experiments.

lower values, which still remains difficult to explain. From data of fig.2, it was found that half maximal effect (HME) was obtained at 0.7 μM, 5 μM and 10 μM with A23187, H-GPA and P-GPA, respectively.

Fig.2 also compares the platelet aggregating properties of the 3 compounds. A clear dissociation appeared between calcium release and platelet aggregation induced by H-GPA, whereas the two dose–response curves were more closely related in the case of A23187. Under the same conditions, P-GPA displayed an intermediary picture. The concentrations of H-GPA, P-GPA and A23187 giving a full aggregation response were 0.1 μM, 5 μM and 0.5 μM, respectively. The same concentrations added to platelet membranes released 0%, 10% and 10% of calcium within 10 min. Also, relative to platelet aggregation, HME was reached at 0.5 μM, 0.3 μM and 0.01 μM for A23187, P-GPA and H-GPA, respectively.

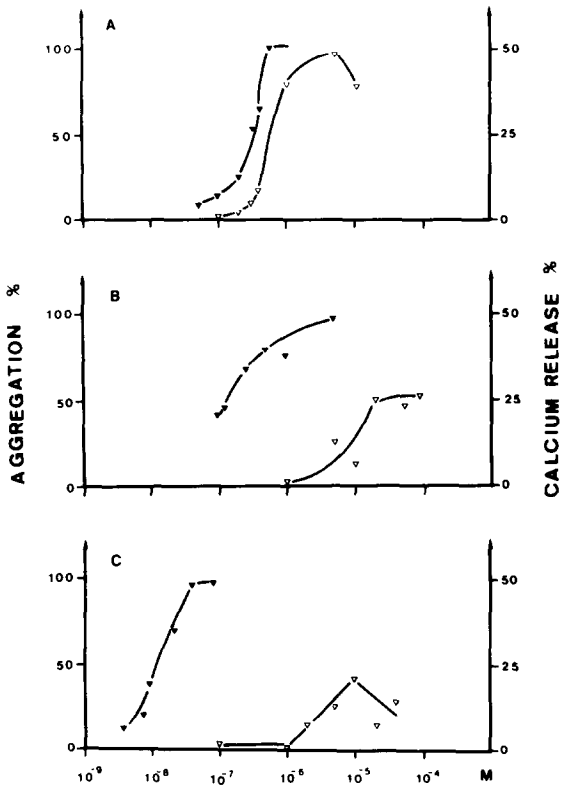

Fig.2. Comparative potency of A23187 and LPA towards platelet aggregation and calcium release from platelet membranes. Platelet aggregation induced by various concentrations of A23187 (A), 1-acyl-LPA (B) and 1-alkyl-LPA (C) is expressed as the percentage of maximal response. For calcium release, the 3 compounds were added 30 min after loading the membranes with ^{45}Ca and the incubation was carried out for 10 min more. Percentage of release was calculated by plotting the decrease of membrane radioactivity compared to control membranes *vs* the total radioactivity remaining in control membranes. (▲) Aggregation; (△) calcium release. Each value is the mean of 2–6 different determinations.

4. DISCUSSION

The '40000 × *g* fraction' used here represents a mixed membrane fraction containing both surface membrane and intracellular membrane elements [32–35]. As shown in [36], the membranes taking up calcium might correspond to vesicular structures originating from the dense tubular system, although the contribution of inside-out resealed vesicles from the surface membrane cannot be definitely ruled out (Levy-Toledano, S. et al., personal communication).

Our results indicate that no clear relationship exists between calcium gating properties and physiological effects of LPA. This is specially evident for H-GPA. A generally accepted hypothesis is that calcium ionophores like LPA trigger platelet activation by mobilizing calcium from intracellular stores, presumably the dense tubular system [18]. One could thus expect that the more stable 1-alkyl-LPA would be able to reach its intracellular target more easily than LPA carrying a 1-acyl-group susceptible to hydrolysis by intracellular lysophospholipase [37,38]. However, we did not find evidence for a significant breakdown of 1-[^{3}H]stearoyl-LPA upon incubation with human platelets (unpublished).

Another explanation might be that platelet aggregation induced by LPA does not involve their calcium ionophore properties. In this respect, authors in [39] observed that platelet shape change and secretion induced by thrombin can occur in the absence of any increase of cytoplasmic free calcium level and they suggested the existence of calcium-independent pathways leading to platelet activation.

It is now well established that platelet stimulation by thrombin leads to the phosphorylation of two proteins displaying M_r values in the range of 20000

and 40000 [40,41]. The so called 20-kDa protein corresponds to myosin light chain and is phosphorylated through a calcium-calmodulin-dependent process [42], whereas the 40-kDa protein is the substrate of the calcium-activated phospholipid-dependent protein kinase C [43]. Authors in [44] could recently dissociate these two events, since 20-kDa protein phosphorylation was specifically triggered by A23187, whereas the synthetic diglyceride 1-oleoyl-2-acetyl-glycerol promoted the selective phosphorylation of the 40-kDa protein in intact human platelets. So in the latter case, no calcium mobilization occurred despite a large conversion of the diglyceride into its corresponding phosphatidate.

In conclusion, our results fit rather well with the scheme proposed by Nishizuka's group and call for another explanation concerning the role of phosphatidic acid in the mechanism of stimulus-activation coupling.

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