Biochimica et Biophysica Acta, 944 (1988) 202–212
Elsevier

BBA 74130

Effects of phosphoinositides on calcium movements in human platelet membrane vesicles

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(Received 18 April 1988)

Key words: Polyphosphoinositide; Inositol trisphosphate; Calcium uptake; Calcium release; Platelet membrane vesicle; (Human)

In a mixed endoplasmic and surface-type membrane vesicle preparation from human platelets the polyphosphoinositides PIP and PIP_2, similarly to IP_3, were found to induce a rapid calcium release reaction. At physiological (resting) cytoplasmic calcium concentrations (0.1–0.3 μM) the PIP_2 and IP_3 concentrations producing half-maximum calcium release were similar (0.7 μM) and both agents could mobilize about 30–40% of the intravesicular calcium. However, the phosphodiesteric degradation of PIP_2 in the membrane vesicles was found to be negligible and the ion- and drug-sensitivities of the calcium release reactions were different. The IP_3-induced calcium release was selectively inhibited by micromolar calcium concentrations and by cinnarizine, while the PIP_2-induced release was blocked by magnesium ions and neomycin. The calcium release evoked by either agent was inhibited by low concentrations of lanthanum but, in contrast to the ATP-dependent calcium pump, it was insensitive to vanadate, quercetin and to the lowering of the incubation temperature. When added simultaneously or in a rapid succession, maximum effective IP_3 and PIP_2 concentrations produced an additive calcium release reaction. Based on these data we suggest that IP_3 and PIP_2, respectively, induce rapid transmembrane calcium movements involving different transport pathways and/or membrane calcium pools, which are not related to the active calcium transport systems.

Introduction

It is now generally accepted that a major trigger for platelet activation is the elevation of cytoplasmic free calcium concentration (see Refs. 1 and 2). The calcium homeostasis in the resting platelets is sustained by the balance between calcium influx, calcium extrusion through the plasma membrane and the sequestration of calcium into internal stores. In a variety of cell types, including human platelets [3,4], agonist stimulation of specific membrane receptors leads to the rapid hydrolysis of phosphoinositides and the formation of diacylglycerol (DAG) and inositol trisphosphate (IP_3). These breakdown products have been identified as intracellular messengers activating protein kinase C, and producing calcium release from intracellular nonmitochondrial stores, respectively [5,6]. The calcium release reaction has a transient nature [6] and is followed by a rapid calcium re-uptake, unless platelet aggregation irre-

Abbreviations: BSA, bovine serum albumin; DAG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; PPI, polyphosphoinositides; PS, phosphatidyl-L-serine; TLC, thin-layer chromatography.

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versibly destroys the functioning membraneous compartments.

The source of the calcium-mobilizing IP_3 is the plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), which is formed by sequential phosphorylations by specific kinases from phosphatidylinositol (PI) and then cleaved by a specific phospholipase C enzyme [7,8]. In the resting platelets PI constitutes of about 90% of the inositol lipids [9] and for inducing a half-maximum calcium release IP_3 concentration has to reach a value of about 1–2 μM [10,11]. All these data indicate that during platelet stimulation the activation of membrane-associated PI kinases may be an equally early event as that of the phospholipase C. There are indications that cAMP-dependent protein kinase [12,13] and protein kinase C [14,15] significantly increase the labelling of polyphosphoinositides in platelet membrane preparations or in intact platelets. In a recent report the possible direct involvement of polyphosphatidyl inositides in the calcium release reaction has been indicated [16].

It is still under discussion, whether after the initial triggering, which requires the liberation of calcium only from internal stores, a sustained calcium influx has a significant role in the aggregation and release reaction of platelets. The possible role of inositol phosphates in this phase has also been suggested [17,18].

In the present paper we describe experiments carried out with mixed endoplasmic and plasma membrane vesicles of human platelets, which further characterize the role of inositol lipids in the regulation of calcium homeostasis in these cells.

Materials and Methods

All the reagents used were of analytical grade. The phospholipids PA, PS, and PE, as well as arachidonic acid were obtained from Calbiochem; PI, PIP and PIP_2, IP_3, neomycin sulphate, quinacrine, and phospholipase C from Sigma. The proteinase inhibitor aprotinin was purchased from Serva and the HPTLC plates precoated with Silicagel H 60 from Merck. [γ-^{32}P]ATP (1–2 TBq/mmol) and phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate (376 GBq/mmol) were obtained from RCC, Amersham.

Preparation of membrane vesicles

For platelet membrane preparations blood from healthy volunteers was collected into bags containing acid citrate dextrose (ACD) solution. For obtaining platelet membrane vesicles we followed the modified version [19] of the method of Käser-Glanzmann et al. [20]. Briefly: the platelets were washed in a Tyrode solution, resuspended in KCl-Hepes (10^{10} cells/ml) containing proteinase inhibitors, and sonicated three times for 10 s with an MSE sonicator. The lysate was centrifugated for 20 min at 40000 × *g* and the supernatant was sedimented for 60 min at 160000 × *g*. The pellet was resuspended in KCl-Tris buffer with 0.1 mM dithiothreitol and stored at −70 °C. For a further purification of the endoplasmic reticulum we applied the Percoll gradient separation (pH 9.6) suggested in Ref. 21.

Inside-out human red cell membrane vesicles and white ghosts were prepared as described in Ref. 22.

Membrane enzyme assays

NADPH–cytochrome *C* reductase (EC 1.6.2.4) activity was measured by the method described in Ref. 23, the determination of glucose-6-phosphatase (EC 3.1.3.9) activity was based on Ref. 24, and 5′-nucleotidase (EC 3.1.3.5) was assayed both in the presence and absence of Triton X-100, as described in Ref. 25. Alkaline phosphodiesterase (EC 3.1.4.1) activity was determined by measuring the hydrolysis of thymidine *p*-nitrophenylphosphate [26] Phospholipase C activity was measured as described in Ref. 27.

Preparation of polyphosphoinositide liposomes

0.5 ml of 1 mg/ml PIP or PIP_2 solution in chloroform/methanol (1 : 1, v/v) was dried under nitrogene stream then the lipids were suspended in Tris-HCl buffered (20 mM, pH 7.0) 160 mM KCl solution, vortexed for 5 min, and ultrasonicated under nitrogene atmosphere for 5 min. Aqueous solutions of the polyphosphoinositides could be stored without loss of calcium release activity for one week at 4 °C, but had to be sonicated again before each use.

Calcium transport studies

For measuring calcium uptake into membrane vesicles the incubation media contained 120 mM

KCl, 20 mM Hepes-K (pH 7.0), 1 mM $MgCl_2$, with or without 0.5 mM ATP, 5 mM potassium oxalate, 100 μM $CaCl_2$ (including tracer ^{45}Ca) and 120 μM EGTA (free calcium concentration 1.7 μM). The membranes (150–300 μg/ml) were incubated at 37°C for the time indicated in the figures, and than the vesicles were separated by rapid filtration through Sartorius membrane filters [19]. Tracer calcium activity on the filters was determined in a liquid scintillation counter (Beckman LS 7500).

Calcium release from the vesicles was followed in the same incubation media but in the absence of oxalate. Membrane vesicles were preloaded with calcium in the presence of ATP at 20°C or at 37°C for 4 min. Reagents inducing calcium mobilization or the same volume of the incubation buffer as a control, were added to the media at the fifth min and thereafter samples were taken at 15, 30, 120 and 240 s. The loss of tracer calcium from the vesicles was determined with rapid filtration as in the uptake experiments.

Preparation and determination of ^{32}P-labelled polyphosphoinositides

In order to obtain labelled PIP and PIP_2, inside-out human red cell vesicles were phosphorylated by [γ-^{32}P]ATP at 37°C for 5–10 min in media containing 65 mM KCl, 35 mM Hepes-K (pH 7.0), 10 mM $MgCl_2$, 1 mM EGTA and 5–50 μM [γ-^{32}P]ATP, supplemented with 5 mM KF to block phosphatase activities as suggested in Ref. 12. The reaction was stopped with 10 volumes of chloroform/methanol/HCl (40:20:0.5, v/v) and the lipids were extracted after the addition of 2 vol. of 1 M HCl/ 1.7 M KCl (1:0.2, v/v). TLC separation of the lipids was carried out in preparative silicagel plates in the solvent system containing chloroform/methanol/2 M NH_4OH in the ratios of 45/35/10 (v/v). Before use, the plates were pretreated with 1% potassium oxalate solution and activated for 30 min at 110°C. The lipids were visualized by iodine vapour and by autoradiography. The labelled PIP and PIP_2 (these are the only ^{32}P-labelled phospholipids in the red cell membrane) were extracted by a Hepes-buffered methanol/water (2:1, v/v), pH 7.4 from the silicagel. After centrifugation for 10 min at 1500 × *g*, the lipids from the supernatants were extracted again by acidic chloroform/methanol, as described above. The recovery for labelled PIP and PIP_2 was more than 90%. The organic phase was evaporated under N_2 and the labelled lipids were stored at −20°C and used within 48 h. Thin-layer chromatography of the lipids was performed on F-254 HPLC plates (Merck) in the above described solvent system.

Determination of protein and free calcium concentrations

The protein content of the membranes was measured by the method of Lowry et al. [28] using BSA as a standard. The free calcium concentrations in the media with different ligand concentrations were calculated by a computer program as described in Ref. 29.

Results

Calcium uptake by platelet membrane vesicles

The platelet membrane vesicles used in these experiments were obtained by differential centrifugation as described in Refs. 19 and 20. Enzyme assays showed that this is a mixed preparation of endoplasmic reticulum and plasma membranes with practically no contamination of mitochondrial membranes (Table I). There was no measurable azide-sensitive calcium uptake, characteristic for the mitochondria, in the vesicles (data not shown). Further purification on Percoll at pH 9.6 [21] produced vesicles with greater calcium transport capacity but still with a mixed membrane enzyme characteristics. Thus in the following experiments the non-purified membranes were used.

Fig. 1 shows the time course of active calcium uptake by platelet membrane vesicles in the presence of 5 mM oxalate. This was found to be linear up to 15 min and, as demonstrated, the addition of 5 μM IP_3, 10 μM PIP, or 5 μM PIP_2 significantly reduced the rate of calcium uptake (Fig. 1). The addition of PI to the medium also decreased the calcium uptake, but this effect was delayed and became significant only in concentrations more than 4-times greater than with the phosphorylated compounds. Preincubation of the vesicles with PI in the presence of ATP but without calcium, did not significantly shortened the delay

TABLE I

ACTIVITY OF MARKER ENZYMES IN PLATELET MEMBRANE PREPARATIONS

Membranes were prepared and various markers were determined as described in Materials and Methods. Results are given as means of three independent experiments. $^{45}Ca^{2+}$ uptake was measured in the presence of 5 mM potassium oxalate and 10 mM sodium azide, at 37°C pH 7.4 with 1.7 μM free Ca^{2+} concentration, as described in Materials and Methods. n.d., not detected.

Fractions	Alkaline phosphodiesterase (nmol *p*-nitrophenol ·(mg protein)$^{-1}$ ·min^{-1})	5′-Nucleotidase (ng P_i· (mg protein)$^{-1}$ ·min^{-1})	Glucose-6-phosphatase (ng P_i· (mg protein)$^{-1}$ ·min^{-1})	NADPH-cyt. *c* reductase (nmol cyt. *c*· (mg protein)$^{-1}$ ·min^{-1})	$^{45}Ca^{2+}$ uptake (nmol Ca^{2+}· (mg protein)$^{-1}$ ·min^{-1})
Sonicated cells	0.8	8.7	10	11.2	n.d.
100000 × *g* pellet	2.5	7.8	7.6	18.4	23.2
Separated on Percoll pH 9.6	3.5	12.6	9.8	22.2	71.8

in the inhibition of calcium uptake (data not shown).

Calcium release from platelet membrane vesicles

When membrane vesicles were incubated in the presence of tracer calcium (0.12–50 μM free calcium concentration) and ATP (550 μM) a steady-state vesicular calcium level was reached within 3–4 min at 20°C. As shown in Fig. 2, the addition of IP_3 (Panel A) or PIP_2 (Panel B) induced a dose-dependent, reversible calcium release. When the free calcium concentration in the incubation media was buffered to 1.7 μM, the maximum value of calcium release was greater in the case of PIP_2 than with IP_3 and the concentration producing half-maximum calcium release was also much smaller in the case of the former compound ($K_{0.5}$ $PIP_2 = 0.7$ μM) than in the latter one ($K_{0.5}$ $IP_3 = 1.5$ μM). PIP (Panel C) could also produce similar calcium release than PIP_2 but only at much greater concentrations ($K_{0.5}$ for PIP = 10 μM). The rate of calcium re-uptake depended on the concentration of the mobilizing agent but it was also variable between the membrane preparations examined.

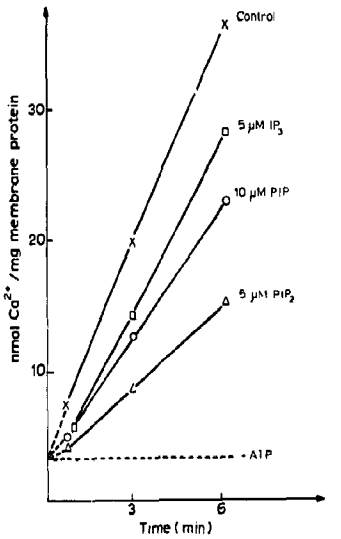

Fig. 1. Active calcium uptake by platelet membrane vesicles: effects of inositol lipids and inositol trisphosphate. Calcium uptake was measured at 37°C at 1.7 μM free calcium concentration (buffered with EGTA) and in the presence of 5 mM potassium oxalate, 0.55 mM ATP, as described in Materials and Methods. Additions: ×——×, control; □——□, 5 μM IP_3; ○——○, 10 μM PIP; △——△, 5 μM PIP_2. Each data point represents the mean values of duplicates from at least three separate experiments.

Various phospholipids, such as PI, PS, PC and PE (up to concentrations of 100 μM) proved to be ineffective in inducing calcium release from the platelet membrane vesicles. Since breakdown products of membrane lipids may act as calcium ionophores [30,31], we have also examined the effects of DAG, PA and arachidonic acid on the calcium release from the vesicles. While the former compounds proved to be ineffective (maximum concentrations tested 100 μM), arachidonic acid (AA) above 30 μM caused a rapid and irreversible calcium release (Fig. 2, Panel C), probably by inducing a non-specific calcium leakage.

In the following experiments we examined the possible interactions between PIP_2 and IP_3 in the

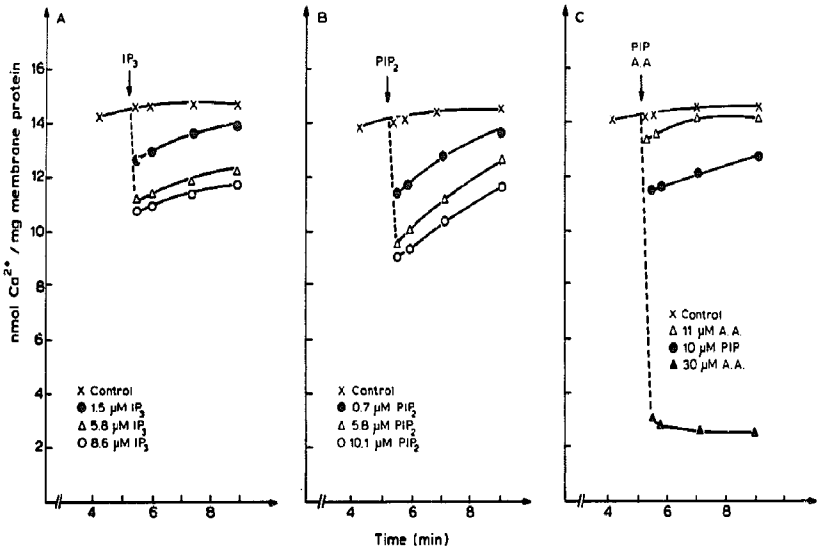

Fig. 2. Calcium release from platelet membrane vesicles evoked by various concentrations of: IP_3 (Panel A), PIP_2 (Panel B), or PIP and arachidonic acid (AA) (Panel C). The membrane vesicles were loaded actively (in the presence of ATP) by calcium at 20 °C and 1.7 μM medium calcium concentration under the standard conditions (see Materials and Methods). The release was initiated by Panel A: ×——×, 0 μM; ●——●, 1.5 μM; Δ——Δ, 5.8 μM; and O——O, 8.6 μM of IP_3; Panel B: ×——×, 0 μM; ●——●, 0.7 μM; Δ——Δ, 5.8 μM; and O——O, 10.1 μM of PIP_2. Panel C: ×——×, no addition; Δ——Δ, 11 μM arachidonic acid; ●——●, 10 μM PIP; ▲——▲, 30 μM arachidonic acid. The release reaction was measured by following the loss of tracer calcium from the vesicle fraction by rapid filtration as described in Materials and Methods.

TABLE II

EFFECTS OF MODIFIERS OF THE PIP_2 AND IP_3 INDUCED CALCIUM RELEASE IN PLATELET MEMBRANE VESICLES

n.m., not measured.

Addition	Free Ca^{2+} concn. (μM)	% Ca^{2+} release induced by		% reduction in ATP dependent Ca^{2+} uptake
		2 μM PIP_2	2 μM IP_3	
–	0.35	35±2	33±3	–
–	50.0	34±3	0	–
–	3.0 [a]	35±3	20±2	–
10 mM $MgCl_2$	0.35	0–5	33±2	10±2
1 mM $MnCl_2$	50.0	0–5	n.m.	22±3
1 mM $NiSO_4$	50.0	32±3	n.m.	11±2
15 μM $LaCl_3$	3.0 [a]	0	0	8±5
50 μM sodium orthovanadate	0.35	33±4	32±3	15±5
50 μM quercetin	0.35	31±2	33±2	35±4
100 μM verapamil	0.35	35±2	30±2	0
50 μM sensit	0.35	33±3	27±2	35±4
20 μM cinnarizine	0.35	33±4	0–5	0–5

[a] Ca^{2+} initially adjusted to 3 μM with ^{45}Ca, without EGTA buffering.

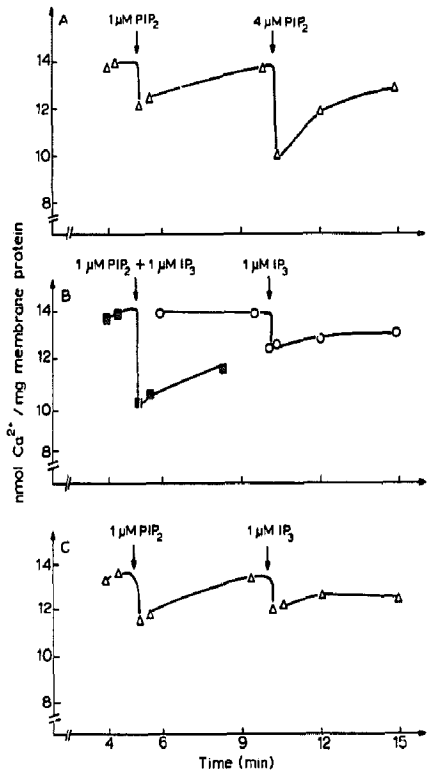

Fig. 3. Interactions between IP_3 and PIP_2 in evoking calcium release from platelet membrane vesicles. Panel A: Sequential calcium release evoked by the addition of 1 μM and thereafter by 4 μM PIP_2; Panel B: Additive effects of PIP_2 (1 μM) and IP_3 (1 μM) on the calcium release; Panel C: Independence of sequentially added PIP_2 (1 μM) and IP_3 (1 μM) in evoking calcium release. Calcium release was measured as described in the legend to Fig. 2.

induction of calcium release from the platelet membrane vesicles. As shown in Fig. 3, repeated calcium release could be obtained by the sequential additions of PIP_2 and/or IP_3, and no potentiation of the IP_3 effect could be obtained by a previous addition of PIP_2. In the case of the simultaneous addition of the two releasing agents an additive but not synergistic effect could be seen.

In order to examine the possible additivity of the maximum effective concentrations of PIP_2 and IP_3, we carried out the experiments demonstrated in Fig. 4. As shown, the applied saturating concentrations of PIP_2 (10–20 μM) or IP_3 (5–10 μM) each produced a maximum calcium release, while the simultaneous addition (Panel A) of the two compounds further increased the amount of calcium released. Sequential addition of the two agents within 30 s also increased the releasable calcium from 30–35 to 50–55% (Fig. 4, Panel B).

Effects of inorganic and organic modifiers and the temperature on the calcium release reaction

In these experiments first we examined the effects of EGTA-buffered free calcium concentrations in the media on the calcium release from platelet membrane vesicles. Loading of the vesicles was carried out at the free calcium concentrations indicated and the calcium releasing agent was added after reaching steady-state calcium incorporation. As shown in Table II, the effect of PIP_2 in inducing calcium release was practically independent of the medium calcium concentrations between 0.35–50 μM, while the effect of IP_3 was almost completely abolished when calcium concentration was increased above 5 μM. According to the kinetic analysis of this calcium effect on the IP_3-induced calcium release, the K_i of the free calcium concentration was 1.7 μM (we have tested six different free calcium concentrations between 0.12 to 50 μM, data not shown in detail). IP_3 and PIP_2 at low calcium concentrations (0.35 μM) both produced half-maximum calcium release in similar concentrations (about 0.7 μM).

In Table II we list several further compounds examined for their effects on calcium release and uptake in the platelet membrane vesicles. When the magnesium concentration was altered at a constant calcium concentration (0.35 μM) in the incubation medium, IP_3-induced calcium release was found to be independent of, while PIP_2-induced release was strongly reduced by magnesium. A similar strong inhibition of PIP_2-induced calcium release by magnesium was found at 5, or 50 μM calcium concentrations, too. Manganese, in a concentration of 1.0 mM also blocked PIP_2-induced calcium release from platelet membrane vesicles, while nickel ions were ineffective in this respect. Estimation of the effects of these divalent ions, which form extremely stable chelates with EGTA, could not be carried out at the low (EGTA-buffered) calcium concentrations which favour the IP_3-induced calcium release. In order to avoid this problem, the effect of lanthanum was studied at 3

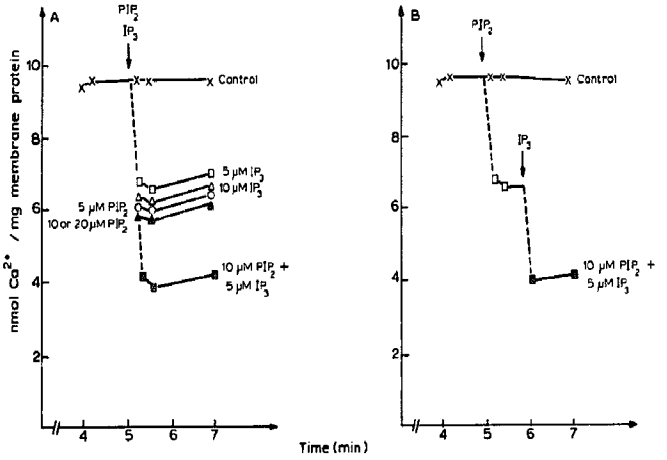

Fig. 4. Additivity of the calcium release evoked by maximally effective concentrations of PIP_2 and IP_3. Panel A: X——X, no addition; □——□, 5 μM IP_3; △——△, 10 μM IP_3; ○——○, 5 μM PIP_2; ▲——▲, 10 or 20 μM PIP_2; □——□, 5 μM IP_3 + 10 μM PIP_2. Panel B: X——X, no addition; addition of 10 μM PIP_2 (□——□), followed by the addition of 5 μM IP_3 (■——■). Calcium release was measured as described in the legend to Fig. 2, at 0.35 μM free calcium concentration in the media at 37 °C.

μM unbuffered, nominal initial calcium concentration where 15 μM lanthanum significantly inhibited both the IP_3-, and the PIP_2-induced calcium release. The inhibition of the ATP-dependent calcium uptake at these low lanthanum concentrations was not significant. There was no noticable effect of exchanging the medium potassium for sodium ions, or varying the pH between 7.0 to 7.6 on the calcium release.

The effects of various organic modifiers of the phosphoinositide-dependent transport systems were also tested. Quinacrine, an inhibitor of phospholipase A [32] did not affect the calcium release reaction induced either by PIP_2 or by IP_3 (Fig. 5, panel A, B). Neomycin, an aminoglycoside antibiotic, strongly binds polyphosphoinositides [33] and is known to inhibit the aggregation in intact or permeabilized platelets [34,35]. In our hands,

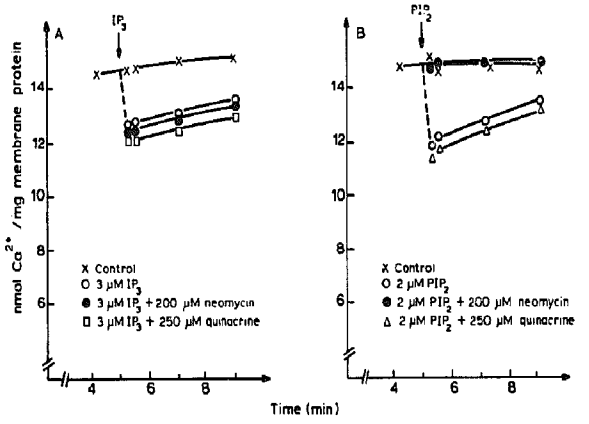

Fig. 5. Effects of quinacrine and neomycin on the calcium release from platelet membrane vesicles. Panel A: X——X, no addition; ○——○, 3 μM IP_3; ●——●, 3 μM IP_3 + 200 μM neomycin; □——□, 3 μM IP_3 + 250 μM quinacrine. Panel B: X——X, no addition; ○——○, 2.0 μM PIP_2; ●——●, 2.0 μM PIP_2 + 200 μM neomycin; □——□, 2.0 μM PIP_2 + 250 μM quinacrine. Calcium release was measured from calcium-loaded platelet membrane vesicles as described for Fig. 2.

neomycin in a concentration of 200 μM inhibited the calcium release reaction evoked by PIP or PIP_2, but not by IP_3 (Fig. 5, panels A and B). Orthovanadate (50 μM) or quercetin (50 μM), inhibitors of the calcium pump [36,37], did not alter the release reaction in calcium-preloaded platelet membrane vesicles (Table II). We have also examined the effect of the protein kinase C activator phorbol ester PMA (10–200 nM), calmodulin (20 μg/ml), cyclic AMP (10 nM–10 μM) and forskolin (20 μM) on the calcium release but found no significant changes in the calcium release induced either by PIP_2 and IP_3 (data not shown).

When examining the effects of various organic calcium channel blockers on the calcium release (Table II), we found no significant effects of verapamil (10–100 μM) or sensit (fendilinum, 5–50 μM) on these reactions (50 μM sensit produced a measurable inhibition of the ATP-dependent calcium uptake). In contrast, cinnarizine, which has been reported to inhibit IP_3-induced calcium release in platelet membrane vesicles [38], blocked the IP_3-induced but not the PIP_2-induced calcium release in our preparation.

In the calcium uptake experiments decreasing temperatures strongly inhibited the rate of ATP-dependent calcium influx and at 8°C active calcium accumulation was only 20% of that measured at 37°C (data not shown). Thus we examined the temperature-dependence of the calcium release reaction as well. As shown in Fig. 6, the apparent time-course and the relative magnitude of the calcium release was practically independent of the change in the temperature from 37°C to 8°C, both in the presence of PIP_2 and IP_3 (for controls see Fig. 4).

Metabolism of added PIP and PIP_2 during the calcium release reaction

PIP and PIP_2, labelled by ^{32}P on the inositol rings were prepared in human red cell membranes as described in Materials and Methods. Their metabolism was followed in the platelet membrane vesicles by simulating the calcium release conditions with unlabelled calcium. The concentrations of PIP and PIP_2 were adjusted to 12 μM and 2 μM, respectively, which produced a near maximum calcium release in parallel studies

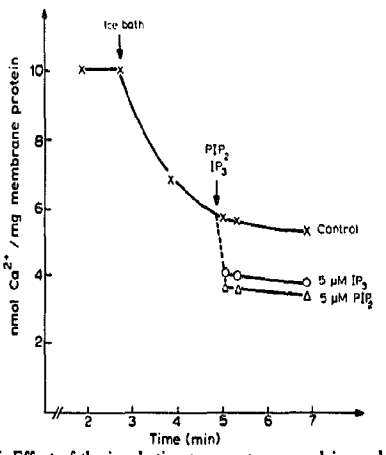

Fig. 6. Effect of the incubation temperature on calcium release from platelet membrane vesicles evoked by PIP_2 and IP_3. The medium temperature after calcium loading at 37°C is rapidly decreased to 8°C. Control (× ——— ×). At the times indicated by the arrows 5 μM PIP_2 (Δ ——— Δ) or 5 μM IP_3 (○ ——— ○) was added to the medium. The free calcium concentration in the medium was 0.35 μM, calcium release was measured as described in the legend to Fig. 2.

with tracer calcium. The reaction was terminated by the addition of cold, acidic chloroform/methanol solution to the media and the lipids were extracted as described in Materials and Methods. The radioactivity was determined both in the aqueous and in the organic phases. In these studies the degradation of polyphosphoinositides by phosphatases or phospholipase C gave the same increase in the aqueous phase radioactivity. In the control experiments membranes inactivated by heat-treatment of 100°C for 3 min were used.

As shown in Table III/A, during the period of a maximum calcium release reaction (30 s) the maximum degradation of PIP_2 was about 16–17% at 1.7 μM calcium concentration in the medium. This value was even smaller (below 10%) at 0.35 μM calcium, but significantly increased (up to 40%) if the calcium concentration in the medium was increased to 50–100 μM. If a 10 min exposure period was used, most of the added polyphosphoinositides were degraded by the platelet membrane vesicles. Similar findings were obtained by using ^{32}P-labelled PIP in these experiments (data not shown). Fig. 7 shows the autoradiogram of the ^{32}P-labelled lipids separated by TLC, after

TABLE III

DEGRADATION OF PIP_2 DURING CALCIUM MOBILIZATION

The calcium release experiments were carried out at 20 °C at 1.7 μM free Ca^{2+} concentration. The vesicles were loaded with unlabelled Ca^{2+} as described in Materials and Methods. 2 μM PIP_2 labelled with ^{32}P or with ^{3}H in the inositol ring was added to the vesicles, and after 0.5 or 10 min the reaction was stopped by acidic chloroform-methanol solution. After lipid extraction the radioactivity in the different phases was determined. Each value represents the data of three independent experiments.

	A. ^{32}P-PIP_2 [a]				B. [*inositol*-^{3}H]PIP_2 [b]			
	organic phase % of total activity		aqueous phase % of total activity		organic phase % of total activity		aqueous phase % of total activity	
	0.5 min	10 min	0.5 min	10 min	0.5 min	10 min	0.5 min	10 min
Inactivated membrane vesicles	89 ±3.2	84.5±3.3	7.8±0.8	10.4±1.1	90.2±2.5	87.4±2.5	5.4±0.5	7.1±0.6
Active membrane vesicles	79.5±3.0	40.2±1.7	16.6±1.4	51.3±2.2	88.7±2.4	86.8±2.5	6.4±0.6	8.9±0.7

[a] Original activity in the samples 20000–25000 cpm.
[b] Original activity in the samples 40000–45000 cpm.

a 30 s incubation with the platelet membrane vesicles. As shown, the degradation of these lipids is small and there is no detectable conversion of PIP to PIP_2 during this period.

Table III/B demonstrates similar experiments carried out by [^{3}H]PIP_2, tritiated on the inositol ring. As shown, there is no measurable liberation of water-soluble radioactive products, that is phospholipase C activity is negligible in these membranes. Red blood cell ghosts, studied with the same tritiated PIP_2, exhibited a phospholipase C activity of 1.6 nmol/mg protein per min in the presence of 25 μM calcium at 37 °C.

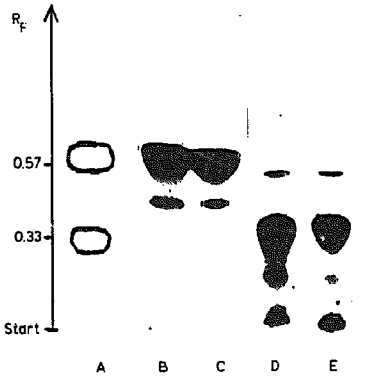

Fig. 7. Recovery of labelled polyphosphoinositides after inducing a calcium release in platelet membrane vesicles. ^{32}P-labelled polyphosphoinositides were produced in red cell membranes, incubated with control or heat-inactivated (100 °C for 3 min) platelet membranes (0.3 mg membrane protein in 1 ml) for 30 s, extracted and separated on TLC plates as described in Materials and Methods. Track A: standard non-labelled PIP_2 (R_f = 0.33) and PIP (R_f = 0.57); Track B: 12 μM labelled PIP added to heat-inactivated membranes: Track C: 12 μM labelled PIP added to membranes; Track D: 2 μM labelled PIP_2 added to heat-inactivated membranes; Track E: 2 μM labelled PIP_2 added to membranes.

Discussion

The experiments presented in this work demonstrate that polyphosphoinositides reduce the net active calcium uptake into human platelet membrane vesicles by inducing a calcium release reaction. This is in agreement with the recent findings of Oho and Takisawa [16] in ox platelet membrane vesicles, but in contrast with the effect of these lipids in the red cell membranes, where they significantly accelerate calcium uptake by producing a calmodulin-like activation of the calcium pump (see Ref. 39). The human platelet membrane preparation used in these studies is a mixture of surface- and endoplasmic reticulum type membranes, as indicated by the enzyme assays in this and a previous study [19]. Calmodulin has no measurable effect on the rate of calcium uptake into the platelet membrane vesicles and previous studies have also indicated an altered composition of the particular pump protein which may be related to that isolated from red cell or from another plasma membranes [19,40].

It has been shown by numerous studies on various endoplasmic reticulum membrane vesicles, including those isolated from platelets, that the phosphodiesteric cleavage product of PIP_2, inositol trisphosphate (IP_3), induces a rapid calcium mobilization [6,41]. Thus the most conspicuous explanation for the effect of polyphosphoinositides would be the liberation of IP_3 – either directly from PIP_2 or after its formation from PIP. However, the experimental data presented above indicate a direct calcium releasing effect of the inositol lipids, clearly different from that caused by IP_3. The evidence suggesting these direct and independent effects can be summarized as follows:

(a) The effect of IP_3 is antagonized by increasing calcium concentrations in the medium, while the calcium release evoked by PIP or PIP_2 is not. In contrast, this latter effect is inhibited by magnesium and manganese ions. Moreover, the action of IP_3 is selectively blocked by cinnarizine, while the action of PIP_2 is selectively blocked by neomycin.

(b) Breakdown products of phospholipids, such as DAG, PA, or arachidonic acid do not act as calcium releasing agents in the relevant concentrations and the inhibition of the phospholipase A activity by quinacrine does not affect PIP or PIP_2-induced calcium release.

(c) Direct measurements by adding pre-labelled inositol lipids show no conversion or degradation which could produce enough IP_3 to induce commeasurable calcium release under the same conditions.

(d) The simultaneous addition of maximum effective concentrations of PIP_2 and IP_3 produce much greater (nearly additive) calcium release than that could be evoked by the compounds alone.

A recent report by Oho and Takisawa [16] demonstrated a similarly calcium-insensitive but magnesium- and neomycin-sensitive calcium release induced by PIP_2 in a bovine platelet membrane preparation. We confirm their basic findings in human platelet membrane vesicles and extend these observations by looking for selective inhibitors of the two transport pathways and by demonstrating the lack of degradation or interconversion of the polyphosphoinositides when inducing calcium release. This latter finding is supported by the data of Rock et al. [42] who also found the PIP_2 phosphodiesterase activity in their platelet membrane preparations negligible. In contrast to the findings in Ref. 16, in our preparation PIP_2 induced a calcium release of similar magnitude than that evoked by IP_3 and the two effects were found to be additive. Such an additivity could be easily overlooked under the experimental conditions of Oho and Takisawa [16] or may be due to species and/or membrane composition differences.

The major questions raised by these experiments are: what may be the physiological role of a calcium mobilization induced by polyphosphoinositides (PPI) and where is the site of their action.

The membrane preparation used in this study is a mixture of plasma-, and endoplasmic reticulum (ER) membranes, moreover, the fusion of these membranes during the formation of individual vesicles is also likely to occur [19]. As judged by the enzyme assays, at least some of the plasma membrane forms inside-out vesicles. Thus ATP-dependent calcium uptake includes both sequestration into the endoplasmic reticulum and extrusion through the plasma membrane, while the observed calcium release represents both liberation from internal stores and influx from the extracellular medium.

One possible site of calcium mobilization by PPI under our steady-state calcium accumulation and efflux conditions could be the inhibition of ATP-dependent calcium transport pathways. However, the different drug sensitivities of the uptake and release pathways, as well as the findings that calcium accumulation is highly temperature-sensitive, while the release reaction is immediate and maximal even at lower temperatures, point into the direction of the opening of specific, calcium-selective channels. An additional argument against the role of ATP-dependent pump in the release reaction is that both PPI and IP_3 could induce substantial calcium release from platelet membrane vesicles loaded with calcium passively, that is in the absence of ATP (data not shown).

As to the possible physiological role of PPI-induced calcium permeation pathway, we suggest its involvement in the sustained increase of intracellular calcium levels after stimuli, particularly by

increasing the influx of external calcium through the plasma membrane. While the initial trigger of platelet activation is most probably the IP_3-dependent calcium liberation from internal stores (controlled by a receptor–G protein–phospholipase C-dependent regulatory mechanism), this calcium release pathway is strongly inhibited by an increase in cytoplasmic calcium (see Table II, and Ref. 43). It has been demonstrated that DAG, liberated together with IP_3, activates protein kinase C thus increasing the formation of PPI [14,15]. This may block any further phospholipase C action [44] and modulate the calcium signal [45]. Thus PPI, this partially water-soluble lipid fraction formed or redistributed after the initial trigger, may provide a sustained, intracellular calcium-independent calcium influx through the plasma membrane, required to the suicidal function of platelets in hemostasis.

Acknowledgements

The authors wish to thank to Drs. Ilma Szász and A. Egyed for the helpful discussions and Mrs. M. Berényi, M., Sarkadi and M. Marjai for the technical assistance in the experiments. This work was supported by a grant (6/968/OTKA-EUM) from the Hungarian Academy of Sciences.

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