Activation of phospholipase C in thrombin-stimulated platelets does not depend on cytoplasmic free calcium concentration

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Human platelets loaded with the fluorescent Ca^{2+} indicator quin2 and with different radioactive compounds including [^{3}H]serotonin, [^{14}C]arachidonic acid (AA) and [^{32}P]orthophosphate were stimulated by thrombin under conditions producing secretion. In the absence of external Ca^{2+} (Ca_e^{2+}), cytoplasmic free $[Ca^{2+}]$, $[Ca^{2+}]_i$, increased to 340 nM, against 1685 nM at 1 mM $[Ca^{2+}]_e$. In both cases, diglyceride and phosphatidic acid production proceeded at the same rate, whereas AA release was inhibited at low $[Ca^{2+}]_i$. It is concluded that, at variance with phospholipase A_2, phospholipase C activation does not depend on $[Ca^{2+}]_i$. These results give further support to the hypothesis of a Ca^{2+}-independent pathway of cell activation involving phospholipase C and protein kinase C.

Platelet activation *Thrombin* *Phospholipase C* *Inositol phospholipid* *Calcium* *Fluorescent indicator*

1. INTRODUCTION

An increased turnover of inositol-phospholipids triggered by specific agonists able to mobilize calcium has been recognized as a widespread phenomenon accompanying the activation of a number of cells, including platelets [1–6]. Three fundamental advances were recently realized in this field:

(i) Phosphodiesteratic cleavage of PI-P_2 occurs as one of the earliest events of cell activation and promotes the mobilization of intracellular calcium through the liberation of inositol-1,4,5-triphosphate [7–9];

(ii) DG, the other product of PLC action, are specific activators of protein kinase C [10]. This leads, in platelets, to the phosphorylation of a 40-kDa protein directly involved in secretion [11]. The most recent evidence indicates that such a process occurs at $[Ca^{2+}]$ as low as 10^{-7} M [11]. In contrast, μM $[Ca^{2+}]$ are required to activate the calmodulin-dependent myosin light chain kinase [11].

(iii) The use of the fluorescent calcium indicator, quin2, has enabled us to measure the cytoplasmic free calcium concentration $[Ca^{2+}]_i$ in platelets under various conditions of stimulation [12]. It was found that thrombin can elevate $[Ca^{2+}]_i$ to the micromolar range only if external calcium, $[Ca^{2+}]_e$, is present. At zero $[Ca^{2+}]_e$, $[Ca^{2+}]_i$ increases only to 200–300 nM and this is still accompanied by a full secretory response. However, when such a small $[Ca^{2+}]_i$ elevation is induced by ionophores, platelet activation no longer occurs. This has raised the concept of a 'Ca^{2+}-independent pathway' of platelet activation involving DG and protein kinase C [12,13].

If this concept is correct, one has to admit that the activation of PLC, a Ca^{2+}-dependent enzyme

Abbreviations: PI, phosphatidylinositol; PI-P, phosphatidylinositol 4-phosphate; PI-P_2, phosphatidylinositol 4,5-bisphosphate; DG, diglyceride; PA, phosphatidic acid; PLC, phospholipase C; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; $[Ca^{2+}]_e$, external free calcium concentration; PRP, platelet-rich plasma; AA, arachidonic acid

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[14–19], should also occur at basal $[Ca^{2+}]_i$, just as described for protein kinase C. This study was thus undertaken with quin2 loaded platelets in order to check for phospholipase C activation upon thrombin challenge under conditions where $[Ca^{2+}]_i$ was monitored.

2. MATERIALS AND METHODS

Quin2 was obtained from the Radiochemical Centre (Amersham). [^{32}P]orthophosphate was from C.E.A. (Saclay). [U-^{14}C]AA (390 mCi/mmol) and 5-[^{3}H]hydroxytryptamine (serotonin, 33.8 Ci/mmol) were purchased from NEN (Dreieich). Human thrombin (3000 NIH units/mg protein) and imipramine were from Sigma (St. Louis, MO).

2.1. *Preparation of platelets*

Fresh human blood from human volunteer donors not having taken any medication for at least a week was drawn onto acid citrate dextrose (ACD) as an anticoagulant [20]. PRP was obtained by centrifugation at 120 × *g* for 20 min (20°C). PRP was incubated at 37°C in the presence of 15 μM quin2 (30 min), 100 μCi/ml [^{32}P]orthophosphate (60 min), 1 μCi/ml [^{3}H]serotonin (30 min) or 0.15 μCi/ml [^{14}C]AA (90 min). Quin2 was added in all incubation procedures, whereas radioactive precursors were included alternatively. In all cases, the whole labelling time did not exceed 90 min. Platelets were sedimented at 1000 × *g* for 20 min and suspended in 0.5 vol. (compared to PRP) of a Tyrode–Hepes buffer (pH 7.35) lacking calcium [20]. Platelet concentration under these conditions was around 5×10^8 cells/ml.

2.2. *Incubation of platelets*

Platelet suspensions were equilibrated for 1 min at 30°C in 2 ml fluorimeter cuvettes. $[Ca^{2+}]_e$ was kept near zero or brought to 1 mM upon addition of 0.1 M $CaCl_2$. Thrombin (1 units/ml, final concentration) or buffer (in controls) was added under a minimal volume (0.01 ml/ml suspension), followed by brief mixing using a plastic bar. Various experiments under identical conditions included determination of $[Ca^{2+}]_i$, of serotonin secretion and lipid studies.

2.3. *Measurement of $[Ca^{2+}]_i$*

This was performed as in [12] using a Perkin Elmer spectrofluorimeter (model MPF-3 L).

2.4. *Determination of serotonin secretion*

Release of [^{3}H]serotonin was assessed essentially as in [21] in the presence of 2 μM imipramine in order to inhibit reuptake.

2.5. *Lipid studies*

Lipids were extracted as in [22] after acidification with 10 N HCl in the case of ^{32}P-labelled phospholipids [23], or with formic acid when [^{14}C]AA was used [24]. ^{32}P-labelled lipids were isolated by monodimensional thin-layer chromatography [25], which allowed a good separation of PI, PI-P, PI-P_2 and PA. In the case of [^{14}C]AA experiments, development was performed as in [26]. This system separated from the bulk of phospholipids, PA, various cyclo-oxygenase and lipoxygenase derivatives of AA, non-converted AA and DG.

3. RESULTS

As shown in fig.1, human platelets loaded with quin2 remained fully responsive to thrombin with respect to serotonin secretion. However, release proceeded faster with 1 mM $[Ca^{2+}]_e$. This difference could be related to the variations of $[Ca^{2+}]_i$ (fig.2). Starting from a basal concentration of 140 nM in the resting state, $[Ca^{2+}]_i$ increased to 340 nM within 15 s, when $[Ca^{2+}]_e$ was kept near zero. With 1 mM $[Ca^{2+}]_e$, basal and maximal $[Ca^{2+}]_i$ were 217 nM and 1685 nM, respectively.

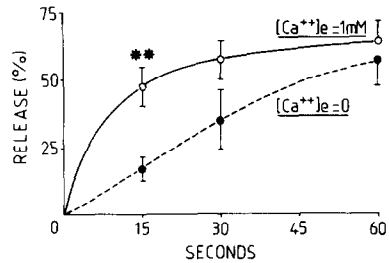

Fig.1. Time course of serotonin secretion from platelets stimulated by thrombin. Release is expressed as percentage of total platelet serotonin and corresponds to the mean ± SE (4 experiments); ** $p < 0.02$ according to Student's *t*-test.

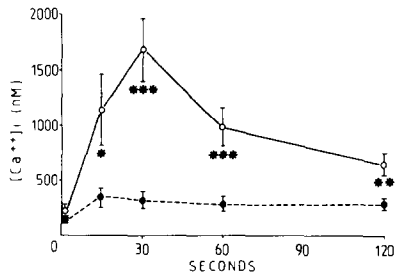

Fig.2. Variation of cytoplasmic free calcium concentration $[Ca^{2+}]_i$ during thrombin stimulation of human platelets. Values correspond to means ± SE (5 experiments). Probability of significance according to Student's *t*-test: * $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$.

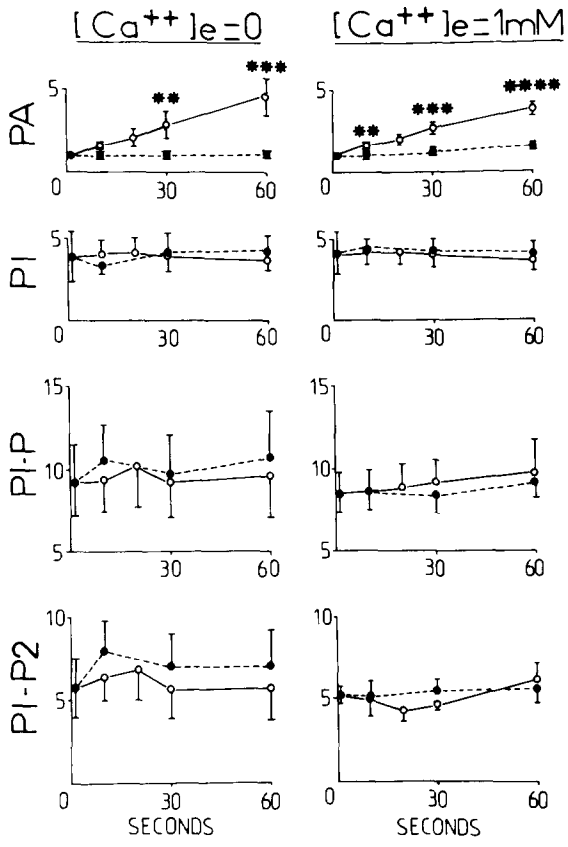

Fig.3. Variation of phospholipid radioactivity in ^{32}P-labelled platelets upon thrombin stimulation. Values (means ± SE, 3 experiments) for controls (●---●) and thrombin-stimulated cells (○—○) are expressed in arbitrary units taking the radioactivity of PA at zero time as 1 unit [23]. Probability of significance according to Student's *t*-test: ** $p < 0.02$; *** $p < 0.01$; **** $p < 0.001$.

The latter concentration was reached within 30 s, but $[Ca^{2+}]_i$ was already 1144 nM at 15 s. $[Ca^{2+}]_i$ remained in the range of 250 nM at zero $[Ca^{2+}]_e$, whereas it decreased progressively to 650 nM after 2 min at 1 mM $[Ca^{2+}]_e$.

Phospholipid changes occurring in ^{32}P-labelled platelets are reported in fig.3. No major differences were observed in the three phosphoinositides, whatever $[Ca^{2+}]_e$ was. The only significant variation concerned PA, which appeared immediately after thrombin addition. After 1 min, it displayed a 320 and a 250% increase over controls, in the presence and absence of Ca^{2+}_e. However, dif-

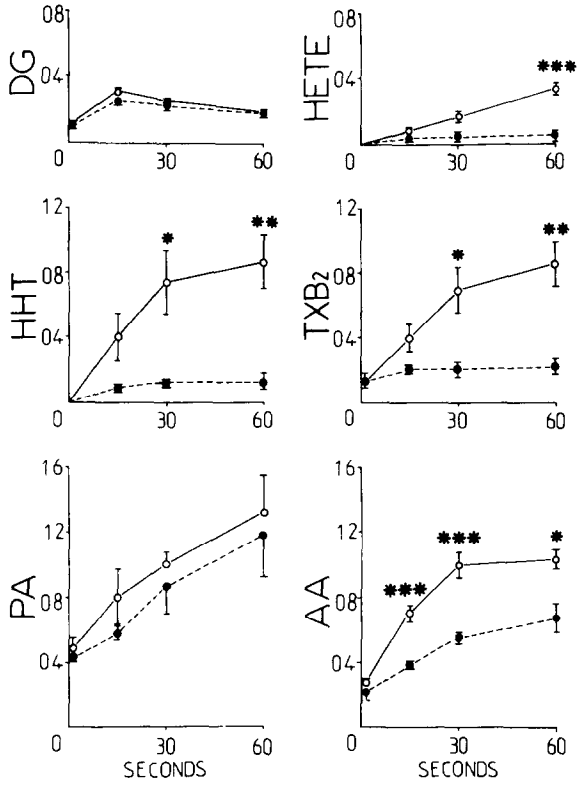

Fig.4. Formation of DG, PA, AA and its oxygenated derivatives in [^{14}C]AA-labelled platelets upon thrombin stimulation. Values (means ± SE, 3 experiments) correspond to percentages of total radioactivity present in platelets; (●---●) $[Ca^{2+}]_e$ = 0; (○—○) $[Ca^{2+}]_e$ = 1 mM. Controls are not represented because they were essentially identical at zero and 60 s. Probability of significance according to Student's *t*-test: * $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$. TXB_2, thromboxane B_2; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid.

Table 1

Modifications of polyphosphoinositide radioactivity in thrombin-stimulated platelets

Conditions	Phospholipids	No thrombin	Plus thrombin	Thrombin-induced decrease	p
$[Ca^{2+}]_e = 0$	PI-P_2	6.9 ± 1.0	5.5 ± 0.8	1.4 ± 0.4	<0.02
	PI-P	9.3 ± 1.2	8.4 ± 1.1	1.0 ± 0.3	<0.05
$[Ca^{2+}]_e$ = 1 mM	PI-P_2	4.6 ± 0.6	4.4 ± 0.6	0.2 ± 0.1	NS
	PI-P	7.6 ± 0.9	7.5 ± 1.0	0.1 ± 0.3	NS

Platelets loaded with quin2 and labelled with [^{32}P]orthophosphate were incubated for 10 s as described in the text. Radioactivity is expressed in arbitrary units as in [23] and corresponds to the mean ± SE (6 and 5 experiments in the absence and presence of Ca_e^{2+}, respectively). p, probability of significance according to paired Student's t-test (differences were never significant when using the non-paired test)

ferences between the two cases were not significant.

As platelet PLC has been shown to selectively degrade PI-P_2, we brought special attention to polyphosphoinositides in a series of experiments involving a 10 s incubation time. As reported in table 1, thrombin induced a 20 and 10% decrease of PI-P_2 and PI-P, respectively, only when Ca_e^{2+} was omitted. In no case was any variation of PI observed (not shown).

Phosphatidic acid production upon thrombin stimulation was also evidenced in [^{14}C]AA-labelled platelets and displayed the same characteristics (fig.4). But a transient DG increase could also be detected under these conditions. As shown in table 2, DG displayed the same variation in the presence or absence of Ca_e^{2+}.

Table 2

Modifications of diglyceride radioactivity in thrombin-stimulated platelets

Conditions	Controls	Assays	p
$[Ca^{2+}]_e = 0$	0.30 ± 0.07	0.67 ± 0.06	<0.01
$[Ca^{2+}]_e = 1$ mM	0.28 ± 0.05	0.62 ± 0.04	<0.01

Platelets loaded with quin2 and labelled with [^{14}C]AA were incubated for 15 s in the absence or presence of thrombin. Values correspond to percentages of total radioactivity present in platelets and are means ± SE of 4 and 3 experiments in the absence and presence of Ca_e^{2+}, respectively. p, probability of significance according to Student's t-test performed between controls and assays

In contrast, the release of AA and of its oxygenated derivatives displayed profound differences between the two cases, since the increase of radioactivity triggered by thrombin was 7 times higher at 60 s for TXB_2, HHT and HETE and 1.5 times higher for AA when Ca_e^{2+} was present.

4. DISCUSSION

The main goal of this study was to check whether PLC activation in thrombin-stimulated platelets can occur at basal $[Ca^{2+}]_i$ under conditions similar to those allowing activation of protein kinase C. Taken together, our data indicate that this is indeed the case, since PA as well as DG are produced to the same level, whatever the extent of Ca^{2+} mobilization. Moreover, we also obtained evidence for a small PI-P_2 and PI-P decrease upon thrombin stimulation in the absence of Ca_e^{2+}. However, this decrease was significant only when using the paired Student's t-test. Such variations are difficult to evidence, since polyphosphoinositides are simultaneously degraded by phosphodiesteratic cleavage [7] and regenerated by PI phosphorylation [27,28]. Thus a PI-P_2 decrease does not reflect quantitatively the extent of PLC activation and in this respect a great variability was found between different studies [7,27–30]. This probably explains why such a decrease was no longer observed at 1 mM Ca_e^{2+}, whereas DG and PA production were essentially the same in both cases.

In contrast, major differences were observed for the release of AA and eicosanoids. Two pathways

have been described for AA liberation from platelet phospholipids. One includes a Ca^{2+}-dependent phospholipase A_2 [31–33], whereas the other one involves the sequential action of PLC and DG-lipase [23,24], which is still controversial [35,36]. PLC remains active at very low $[Ca^{2+}]$, (here), whereas DG-lipase is equally activated by Ca^{2+} and Mg^{2+} (Mauco, G. et al., in preparation), so that it should be fully active in the absence of Ca^{2+} mobilization. It would thus be tempting to speculate that phospholipase A_2 was only active under conditions where $[Ca^{2+}]_i$ was increased to micromolar concentrations, whereas the small release occurring at near basal $[Ca^{2+}]_i$ could be the fact of PLC-DG-lipase. But further investigations are still required to determine the respective part of each pathway.

In summary, our study brings evidence that platelet PLC activation occurs in the absence of a large Ca^{2+} mobilization. This is to our knowledge the first time that PLC activity was followed in parallel to $[Ca^{2+}]_i$ determination. In a previous work dealing with in vitro measurement of PLC [15], we found an optimal $[Ca^{2+}]$ of 1 mM for the enzyme, which still displayed a significant and almost constant activity between 10^{-7} and 10^{-6} M $[Ca^{2+}]$. So our present study confirms and extends these data by showing that PLC activation occurs in vivo at basal $[Ca^{2+}]_i$, which appears to be sufficient for the enzyme to operate. In addition to our recent work on the properties of lysophosphatidic acids [37], this would give further support to the hypothesis that PLC together with protein kinase C are involved in cell activation through a Ca^{2+}-independent pathway [11–13]. It also fits with the proposed role of inositol-tri-phosphate, the liberation of which by PLC would trigger Ca^{2+} mobilization from an intracellular store [9].

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