# ADP stimulates $IP_3$ formation in human platelets

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Aspirinated human platelets labeled with $^{32}PO_4$ showed a 1.7-fold increase in [$^{32}$P]$IP_3$ when stimulated with ADP. ADP-stimulated mobilization of internal $Ca^{2+}$ and phosphorylation of myosin were enhanced in the presence of extracellular $Ca^{2+}$ but the increase in $IP_3$ was not significantly affected by external $Ca^{2+}$. The $Ca^{2+}$ ionophore, ionomycin, elevated internal $Ca^{2+}$ and induced myosin phosphorylation without a detectable change in $IP_3$. These results indicate that the mechanism of ADP stimulation of human platelets is similar to that of other platelet agonists and supports the theory that $IP_3$ functions to liberate internal $Ca^{2+}$.

*(Platelet) Polyphosphoinositide Inositol triphosphate Myosin phosphorylation $Ca^{2+}$ Phosphatidic acid*

## 1. INTRODUCTION

Activation of platelets with agonists is thought to occur through an elevation of intracellular calcium which can now be measured directly using intracellular $Ca^{2+}$ indicators such as quin2 [1]. Agonist-induced rises in cytoplasmic free calcium occur in the absence of external calcium indicating that calcium can be liberated from internal stores. The mechanism by which an agonist acting at the plasma membrane can release calcium from internal stores was at first obscure. Binding of an agonist to a receptor often results in the turnover of PI and its phosphorylated derivatives PIP and $PIP_2$ [2]. The earliest measurable change in phosphoinositides is a rapid breakdown and resynthesis of $PIP_2$ [2], a characteristic pattern shown to occur in platelets stimulated with thrombin and PAF [3–5]. The cleavage of $PIP_2$ involves a specific phospholipase C and results in the production of diacylglycerol and $IP_3$ [2]. Agonist-induced $IP_3$ release has been demonstrated for thrombin, collagen, PAF and vasopressin [6–10]. $IP_3$ has been shown to be a potential second messenger and link between the activation of plasma membrane receptors and the mobilization of calcium from intracellular reservoirs [11–13]. These data strongly indicate that agonist-dependent activation of a specific phospholipase C results in an increase in cytoplasmic concentration of $IP_3$ which in turn causes an elevation in cytoplasmic $Ca^{2+}$ necessary for subsequent activation of the platelets.

In recent experiments, Fisher et al. [14] failed to detect a hydrolysis of $^{32}$P-labeled $PIP_2$ when human platelets were stimulated with ADP. Furthermore, when platelets were labeled with [$^3$H]-inositol, these investigations also failed to detect an ADP-stimulated increase in [$^3$H]$IP_3$. In a different set of experiments, they showed that ADP stimulated an increase in cytoplasmic $Ca^{2+}$ in platelets loaded with quin2. Since these data pose a serious challenge to the idea that $IP_3$ is required to release $Ca^{2+}$ from intracellular stores, we decid-

*Abbreviations:* PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; $PIP_2$, phosphatidylinositol 4,5-phosphate; $IP_3$, inositol 1,4,5-triphosphate; PAF, platelet activating factor

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ed to reexamine the question of whether $IP_3$ is formed during platelet stimulation by ADP. We have used both an improved method for measuring $IP_3$ formation in $^{32}P$-labeled platelets [15] and the new intracellular fluorescent $Ca^{2+}$ indicator fura-2 [16] to monitor $Ca^{2+}$ mobilization and $IP_3$ formation in the same cells. We have found that $^{32}P$-labeled $IP_3$ levels increase almost 2-fold over basal levels supporting the idea that $IP_3$ formation is necessary for agonist-dependent $Ca^{2+}$ mobilization.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Hirudin, ADP, inositol triphosphate were obtained from Sigma (St. Louis, MO). Apyrase was a gift of Dr W. Figures of the Thrombosis Res. Ctr. Fura-2 was obtained from Molecular Probes (Junction City, OR), ionomycin from Calbiochem-Behring (La Jolla, CA) and bovine thrombin was from Armour Pharmaceutical (Kankakee, IL).

### 2.2. *Preparation of $^{32}PO_4$, fura-2-labeled platelets*

Human blood was taken from informed healthy volunteers into acid/citrate/dextrose. Platelet-rich plasma obtained by centrifugation at 180 × *g* for 15 min at ambient temperature was recentrifuged (800 × *g*, 15 min, ambient temperature). The platelet pellet was resuspended in 0.5 vol. of autologous platelet-poor plasma and incubated with $^{32}PO_4$ (0.25 mCi/ml; ICN, Irvine, CA) at 37°C. After 30 min, fura-2 and aspirin were added to concentrations of 5 μM and 1 mM, respectively, and the incubation continued for another 30 min. The platelets were isolated from the incubation medium by centrifugation (800 × *g*, 15 min) and resuspended in a buffer composed of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM $NaH_2PO_4$, 5 mM glucose, 10 mM Hepes (pH 7.4), 0.01 U/ml hiridin and 0.5 ng/ml apyrase. The cell suspension was adjusted to a final density of $2 \times 10^8$ cells/ml.

### 2.3. *Stimulation of platelets and analytical procedures*

Prior to all experiments, platelets were checked for shape change by addition of 20 μM ADP to the platelets in a Payton aggregometer. For all experimental determinations, agonists were added to a prewarmed (37°C) and stirred platelet suspension (2.5 ml) in a Perkin Elmer LS-5 spectrofluorometer. Changes in fura-2 fluorescence were followed using an excitation of 340 nm and emission of 510 nm. At various times during the $Ca^{2+}$ transient, the excitation wavelength was momentarily switched to 380 nm and the cytosolic free $[Ca^{2+}]$ was calculated from the ratio of 340 nm/380 nm fluorescence as described [16,17]. Reactions were stopped either by addition of 1 ml of ice-cold 2.1 N $HClO_4$ for myosin phosphorylation and $IP_3$ determinations or, for phospholipid determinations, by addition of an aliquot of the platelet suspension to 3.75 vols of ice-cold chloroform/methanol (1:2, v/v) containing 25 mM sodium EDTA.

Samples treated with $HClO_4$ were separated into supernatant and pellet by centrifugation at 10000 × *g* for 10 min. The pellet was used for determination of myosin phosphorylation as described [18]. The supernatant was neutralized by adding an appropriate volume of 6 N KOH and the amount of $^{32}P$-labeled $IP_3$ formed was determined as described [15]. Samples for phospholipid analysis were resolved oxalate-treated thin layer plates (Merck, Darmstadt, silica gel 60) using the solvent system, chloroform/acetone/methanol/glacial acetic acid/$H_2O$ (40:15:13:12:7 by vol.) [19]. The resolved phospholipids were detected by radioautography and the radioactivity of the pertinent compounds measured by liquid scintillation counting.

## 3. RESULTS

### 3.1. *Measurement of ADP-induced inositol-triphosphate production*

In our experience, the response of platelets to ADP can be variable and dependent on the method of platelet isolation. Thus, after labeling the platelets with fura-2 and $^{32}PO_4$, each platelet suspension was tested for its shape change response to ADP in an aggregometer. The preparations that were responsive were studied in the fluorometer and, in all cases, stimulation with ADP caused an increase in cytoplasmic free $Ca^{2+}$. The same samples in which $Ca^{2+}$ mobilization was determined were used to measure levels of $^{32}P$-labeled $IP_3$ and myosin phosphorylation. The results of 5 experiments are summarized in fig.1.

ADP induced a rapid increase in $IP_3$ to about 1.7-fold above the basal level in 5 s. A paired Student's $t$-test of this data showed that at all times the increase was statistically significant at least at the 95% level and that the initial rise ($t$ = 5 s) was significant at the 99.5% confidence level. For comparison, thrombin (1 U/ml) in a single experiment was found to cause a 3.2-fold increase in $IP_3$ in 5 s and a maximum 9.2-fold increase at 60 s. The ADP-induced increase in $IP_3$ was paralleled by rapid changes in both cytoplasmic $Ca^{2+}$ levels and myosin phosphorylation. The increase in internal $Ca^{2+}$ and the extent of myosin phosphorylation were dependent on extracellular $Ca^{2+}$, differences for myosin phosphorylation that were statistically significant at 10, 30 and 60 s. In contrast, there was no statistical significance between the ADP-induced increase in $IP_3$ levels in the presence or absence of external $Ca^{2+}$. In addition, 200 nM ionomycin elicited a greater increase in cytoplasmic $Ca^{2+}$ than ADP and comparable levels of myosin phosphorylation but caused no significant increase in $IP_3$ formation (103 ± 15% at 5 s and 101 ± 20% at 10 s; $n$ = 6).

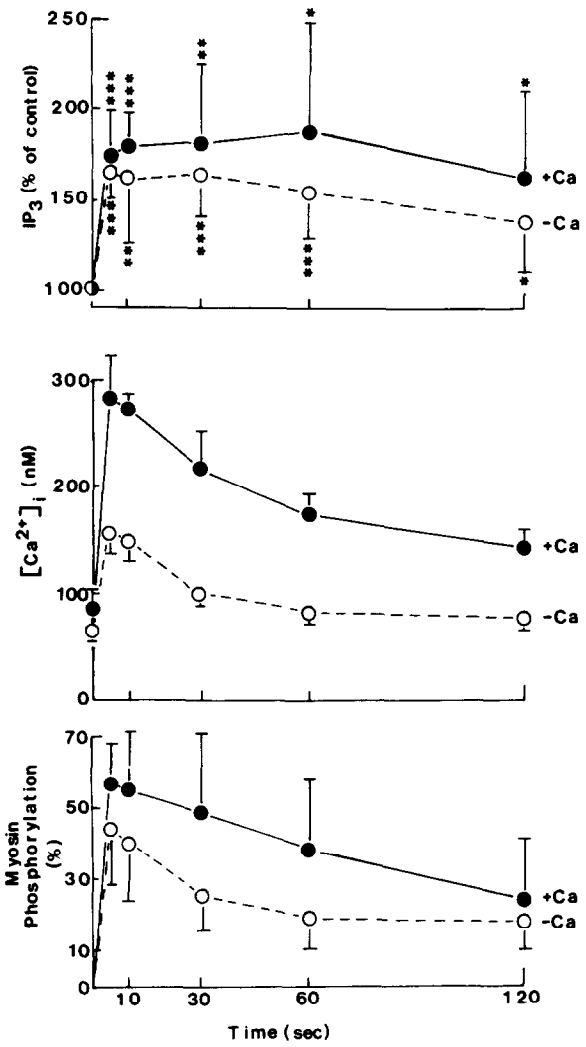


Fig.1. Time course of ADP-induced $IP_3$ formation, $Ca^{2+}$ mobilization and myosin phosphorylation. All experiments were done using 20 $\mu$M ADP on the same set of platelet samples. The bars indicate SD of 5 experiments. For $IP_3$ determinations, significance at any time relative to basal is indicated: * $p < 0.5$; ** $p < 0.01$; *** $p < 0.005$.

### 3.2. *ADP-induced changes in phosphoinositide metabolism*

ADP caused neither an increase nor decrease in $^{32}P$-labeled PIP or $PIP_2$ (table 1) confirming the results of Fisher et al. [14]. In contrast, a slight increase in $^{32}P$-labeled PI (about 25%) was detected and more strikingly (table 1), ADP caused a 2.2-fold increase in $^{32}P$-labeled PA. By comparison, thrombin (1 U/ml) was found to increase the $^{32}P$-labeled PA level about 17-fold in 2 min in platelets from the same group of donors.

## 4. DISCUSSION

We have shown that ADP stimulation of aspirinated human platelets is able to induce an increase in both $^{32}P$-labeled $IP_3$ and $^{32}P$-labeled PA. The magnitudes of these increases are both approx. 6-fold lower than produced by 1 U/ml thrombin and are consistent with the generally accepted view that ADP is a weak agonist compared to thrombin. However, since thrombin liberates sufficient $IP_3$ to achieve an intracellular concentration of greater than 10 $\mu$M [7,15], stimulation with ADP would produce enough $IP_3$ to nearly saturate the $IP_3$ sensitive internal store of platelets which has a $K_m$ of 1 $\mu$M in saponized platelets [13]. The lower level of phosphoinositide metabolism induced by ADP can explain the lack of an initial decrease in $PIP_2$. At a lower level of phospholipase C activation, the hydrolysis of $PIP_2$ may be compensated by resynthesis from PI.

Both the ADP-stimulated increase in in-

Table 1

Changes in platelet phosphoinositides induced by ADP compared to thrombin and ionomycin

| Agonist | Phospholipid | 5 s | 10 s | 30 s | 60 s | 120 s |
|---|---|---|---|---|---|---|
| ADP | | | | | | |
| | $PIP_2$ | 99 ± 11 | 107 ± 7 | 108 ± 12 | 105 ± 7 | 102 ± 8 |
| | PIP | 94 ± 9 | 102 ± 7 | 102 ± 7 | 106 ± 9 | 109 ± 7 |
| | PI | 99 ± 12 | 106 ± 11 | 105 ± 11 | 109 ± 11 | 123 ± 16 |
| | PA | 116 ± 12 | 140 ± 16 | 190 ± 20 | 219 ± 21 | 216 ± 25 |
| Thrombin | | | | | | |
| | PA | 310 ± 64 | 510 ± 138 | 993 ± 180 | 1369 ± 239 | 1731 ± 271 |
| Ionomycin | | | | | | |
| | PA | 101 ± 12 | 109 ± 9 | 100 ± 8 | 124 ± 10 | 115 ± 14 |

Results are expressed as mean percentages of resting levels ± SD of 4 determinations for ADP (20 $\mu$M), 2 for thrombin (1 U/ml) and 3 for ionomycin (200 nM)

tracellular $Ca^{2+}$ and myosin phosphorylation were enhanced in the presence of extracellular $Ca^{2+}$. In contrast, the level of $IP_3$ produced in these cells was not greatly affected by the extracellular $Ca^{2+}$ concentration. These results suggest that $IP_3$ was not produced as a consequence of $Ca^{2+}$ mobilization but rather is consistent with a causal role for $IP_3$ in $Ca^{2+}$ mobilization. Furthermore, ionomycin which increases intracellular $Ca^{2+}$ by bypassing receptor-dependent mechanisms does not increase either PA [20] or $IP_3$.

The failure of Fisher et al. [14] to detect an ADP-stimulated increase in $IP_3$ cannot be attributed solely to our use of a more sensitive method to measure $IP_3$ since we were able to detect an increase in $^{32}P$-labeled PA by a similar technique to that used by Fisher et al. [14]. In our experiments, we measured both $Ca^{2+}$ mobilization, myosin phosphorylation and $IP_3$ in the same samples thereby assuring internal consistency and identical cellular responsiveness. In the studies of Fisher et al. [14], $Ca^{2+}$-mobilization and $IP_3$ release were measured in different preparations of platelets.

Vickers et al. [21] studying rabbit platelets found that ADP did cause a significant decrease in $PIP_2$. Other platelet agonists, which appear to be only quantitatively different from ADP, have been shown to cause increases in $IP_3$. Rather than invoking different mechanisms for different agonists, it is more satisfactory to propose that only one basic mechanism of stimulus-response coupling is operative for all agonists and while there may be quantitative differences between platelets from different mammalian species, the cells are qualitatively similar. The data presented here demonstrate that ADP conforms to the pattern common to other platelet agonists.

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