RESEARCH COMMUNICATION

Sub-second oscillations of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate during platelet activation by ADP and thrombin: lack of correlation with calcium kinetics

Sanghamitra RAHA, Glen D. JONES* and Adrian R. L. GEAR†

Department of Biochemistry, Box 440, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A.

The hypothesis that ADP and thrombin liberate $\text{Ins}(1,4,5)\text{P}_3$ in blood platelets, with kinetics consistent for releasing Ca^{2+} within 2 s, was tested by quenched-flow techniques. Both agonists stimulated transient and equal synthesis of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ near 200 ms and later short-lived peaks, which

were not correlated with the slower steady increase in intracellular $[\text{Ca}^{2+}]$ between 0.5 to 2 s detected by Indo-1. Shear forces alone caused transient liberation of these inositol phosphates within 0.5 s and up to 4 s, yet failed to increase intracellular $[\text{Ca}^{2+}]$.

INTRODUCTION

Platelet aggregation and 'shape change' induced by ADP and thrombin begin within 1 s of activation and may be nearly complete by 5 s, as revealed by quenched-flow approaches and by single-particle sizing and counting [1–3]. Cytoplasmic Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) and protein phosphorylation (20 and 47 kDa) increase by 0.5 s, whereas much less change occurs after 2–3 s [4–6]. The initial rapid rise in $[\text{Ca}^{2+}]_i$ originates from internal stores, since chelation of external Ca^{2+} has no effect on the kinetics with either ADP or thrombin as agonists. These results were obtained by a continuous-flow technique [4]. However, use of stopped-flow methods suggests that ADP, but not thrombin, opens a plasma-membrane channel at about 50 ms, causing a small influx of Ca^{2+} [7,8].

The initiation and regulation of these very rapid biochemical and functional events of platelets are poorly understood. Whether both ADP and thrombin stimulate phosphatidylinositol turnover has been a controversial question [9–16]. The study by Daniel et al. [16] is noteworthy in revealing the appearance of $\text{Ins}(1,4,5)\text{P}_3$ as early as 5 s, which did not require extracellular Ca^{2+} . Murphy et al. [17] discovered little temporal correlation between $\text{Ins}(1,4,5)\text{P}_3$ formation and the increase in $[\text{Ca}^{2+}]_i$ when rabbit platelets were stimulated by platelet-activating factor.

We have examined the initial kinetics of inositol phosphate liberation in platelets activated by ADP or thrombin under physiological blood-flow conditions, taking advantage of the quenched-flow approach for studying rapid platelet reactions [1]. We also used electroporation to improve platelet labelling efficiency with $[\text{H}^3]\text{inositol}$ [18]. Surprisingly, both ADP and thrombin elicited significant transient increases of similar magnitude in $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ near 200 ms, as well as later (< 5 s) peaks in these inositol phosphates. Shear forces acting alone also caused significant transient increases.

MATERIALS AND METHODS

Preparation of platelet-rich plasma, electro-permeabilization for $[\text{H}^3]\text{inositol}$ incorporation and platelet washing

Human venous blood was used to prepare platelet-rich plasma

and washed platelets [4]. Electroporation (eight discharges at 20 kV/cm) was in 140 mM potassium glutamate, 20 mM Pipes, 5 mM ATP and 0.2 μM CaCl_2 (free Ca^{2+} [19]), pH 7.4, with resealing for 90 min at 37 °C in the presence of 40 $\mu\text{Ci}/\text{ml}$ D-myo-[1,2- ^3H]inositol (American Radiochemicals Inc.) to improve $[\text{H}^3]\text{inositol}$ incorporation [18], together with 40 μM inositol (Sigma). Stainless-steel plates in the discharge cell (1.3 ml) minimized potential artifacts via inositol phosphate kinases and phosphatases [20]. The labelled and resealed platelets were washed with an Eagle's-Hepes buffer [4] containing 5 units/ml apyrase (Sigma grade VII) to maintain ADP reactivity, and resuspended in this buffer with 0.5 mg/ml fibrinogen and 1.5 mM CaCl_2 . Labelling efficiency with $[\text{H}^3]\text{inositol}$ (typically 3×10^5 d.p.m./ 10^8 platelets) was about 25 times that for intact platelets, and similar to values reported by Authi et al. [18].

Quenched-flow aggregometry and analysis of $[\text{H}^3]\text{inositol}$ phosphates

Platelet aggregation is measured as the percentage loss of single platelets detected by resistive-particle counting [1]. The platelet suspensions (about 4×10^8 particles/ml) were pre-warmed for 5 min at 37 °C and made to react for 130 ms to 4 s with ADP (10 μM), thrombin (1 unit/ml) or NaCl (0.154 M) in the quenched-flow system under physiological flow conditions [1]. Samples were quenched by 0.3 M HClO_4 , neutralized with K_2CO_3 , and analysed by h.p.l.c. coupled to a flow-through radioactivity detector (Berthold, LB 504), using a gradient of 0.015–1.49 M ammonium phosphate [21]. Standards for $[\text{H}^3]\text{Ins}(1,4,5)\text{P}_3$, $[\text{H}^3]\text{Ins}(1,3,4)\text{P}_3$ and $[\text{H}^3]\text{Ins}(1,3,4,5)\text{P}_4$ (American Radiochemicals Inc. or NEN) were run before and after each day's h.p.l.c. samples. Transient peaks of inositol phosphates were found in all experiments. However, some preparations revealed slightly different times for the first peak near 200 ms, and, to align this peak in all experiments, peak times were averaged and assigned to the nearest time point. Student's unpaired *t* test was used to assess statistical significance.

Abbreviation used: $[\text{Ca}^{2+}]_i$, cytoplasmic Ca^{2+} concentration.

* Present address: Centre for Biologics and Research, Hematology Division, Bldg. 29, 8800 Rockville Pike, Bethesda, MD 20892, U.S.A.

† To whom correspondence should be addressed.

RESULTS AND DISCUSSION

Electroporation and ADP-responsive platelets

Although radioisotopic measurements of [^3H]inositol phosphates may not correlate directly with their chemical amount, such data are important for understanding the functional role of these compounds and control mechanisms during cell activation [22]. Binding assays to detect $\text{Ins}(1,4,5)\text{P}_3$ were not used, since these

Figure 1 Aggregation of electroporated platelets after resealing and [^3H]inositol incorporation: efficiency of ADP and thrombin as agonists

Aggregation in the quenched-flow apparatus is measured as the loss of single platelets [1] and data are expressed as means $\pm$ S.E.M. ($n = 3$ platelet preparations). Shear forces were 15–50 dyn/cm^2 (saline control, $\triangle$), ADP was 10 μM ($\square$), and thrombin 1 unit/ml ($\circ$). The free Ca^{2+} was set at 0.2 μM [19] for resealing after electroporation, and the final suspension medium contained 1.5 mM Ca^{2+} and fibrinogen (0.75 mg/ml) to ensure aggregation.

are rather insensitive, requiring at least 4×10^9 platelets/ml (results not shown; [17,23]). Our quenched-flow system used platelets at about $0.4 \times 10^9/\text{ml}$, which is close to normal plasma concentrations. Another benefit of using ^3H -labelled platelets is the ability to detect other inositol phosphates in only 10^8 platelets.

We therefore developed new conditions for permeabilization and resealing at 37 $^\circ\text{C}$ in the presence of 0.2 μM Ca^{2+} . Platelet preparations exhibited normal aggregation kinetics in response to ADP or to thrombin during the first 5 s, without evidence for shear-induced aggregation or preactivation [0.15–0.5 mN (15–50 dyn/cm^2 ; Figure 1]. Omitting Ca^{2+} from the resealing medium yielded poorly and variably reactive platelets, whereas the presence of 0.2 or 1 μM Ca^{2+} (results not shown) gave good aggregation efficiency in response to ADP. We employed 0.2 μM Ca^{2+} rather than 1 μM Ca^{2+} to minimize activation of Ca^{2+} -dependent proteases.

Early increases (< 2 s) in $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and Ca^{2+}

Since the rapid rise in intra-platelet Ca^{2+} elicited by ADP or thrombin during the first 2 s of platelet activation is well documented [4,7,8], we evaluated $\text{Ins}(1,4,5)\text{P}_3$ generation at short intervals during the first 4 s. Shear forces acting alone (Figure 2a, control) produced moderate amounts of $\text{Ins}(1,4,5)\text{P}_3$ at 200 ms, 350 ms and 1 s, which were significantly higher than static values ($P < 0.01$). Within the first 1 s, ADP induced only one transient peak in $\text{Ins}(1,4,5)\text{P}_3$, around 200 ms, significantly higher than the flowing control ($P < 0.002$, Figure 2a). Thrombin also evoked an early transient increase in $\text{Ins}(1,4,5)\text{P}_3$ near 150 ms. However, unlike ADP, thrombin produced significant amounts of $\text{Ins}(1,4,5)\text{P}_3$ at 500 and 750 ms ($P < 0.003$) compared with the

Figure 2 Initial effects of shear forces, ADP or thrombin on platelet production of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (0–2 s)

[^3H]Inositol-labelled platelets were treated at 37 $^\circ\text{C}$ in the quenched-flow system for up to 2 s and exposed to: control, or shear forces only (15–50 dyn/cm^2 ; $\triangle$, $\blacktriangle$); 10 μM ADP ($\square$, $\blacksquare$); 1 unit/ml thrombin ($\circ$, $\bullet$). $\text{Ins}(1,4,5)\text{P}_3$ ($\triangle$, $\square$, $\circ$) and $\text{Ins}(1,3,4,5)\text{P}_4$ ($\blacktriangle$, $\blacksquare$, $\bullet$) were separated by h.p.l.c. as described in the Materials and methods section. [^3H]inositol phosphate data are presented as means $\pm$ S.E.M. of 4–9 experiments and are given as $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (d.p.m./ 10^6 platelets) values above the static level. Significance of a peak ($P < 0.02$, compared with preceding or following data) is indicated by an asterisk (*). The [^3H] $\text{Ins}(1,4,5)\text{P}_3$ and [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ levels in resting static controls were 0.103 ± 0.055 and 0.211 ± 0.079 d.p.m./ 10^6 platelets respectively.

Figure 3 Longer-term effects of ADP and thrombin on the production of $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ (0–4 s)

Conditions and symbols are as in Figure 2, except production of inositol phosphates was monitored for up to 4 s reaction in the quenched-flow apparatus and $\text{Ins}(1,3,4)\text{P}_3$ was assessed as a measure of inositol phosphate breakdown (∇). ADP and thrombin are represented by the (—) and (---) lines respectively. Static control levels of $\text{Ins}(1,3,4)\text{P}_3$ were 0.337 d.p.m./ 10^6 platelets. Significance of a peak is shown for $\text{Ins}(1,3,4)\text{P}_3$ only, with an asterisk (*) representing a P value of 0.02 or less, compared with preceding or following data.

corresponding controls (Figure 2a). ADP also caused a transient later peak at 1.5 s ($P < 0.002$, versus 1 s).

The initial $\text{Ins}(1,4,5)\text{P}_3$ peak around 200 ms produced by ADP and thrombin coincided with one of equal magnitude in $\text{Ins}(1,3,4,5)\text{P}_4$ (Figure 2b). Shear forces acting alone also generated a significant early peak at 0.5 s in $\text{Ins}(1,3,4,5)\text{P}_4$ ($P < 0.001$) versus static controls or ADP at 0.5 s. Both inducing agents caused transient peaks between 1.0 and 2.0 s in $\text{Ins}(1,3,4,5)\text{P}_4$ (Figure 2b).

Overall transients in $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ (< 4 s); Ca^{2+} relationships

Later changes (up to 4 s) for not just $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, but also $\text{Ins}(1,3,4)\text{P}_3$ as a possible index of inositol phosphate breakdown, are shown in Figure 3. Production of all three inositol phosphates appeared to oscillate. The observed peaks were significantly higher than immediately preceding or following values (*, $P < 0.02$ or less). The first increases in $\text{Ins}(1,3,4)\text{P}_3$ at 0.35 and 0.5 s caused by ADP or thrombin occurred after the initial peaks noted for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, consistent with this inositol phosphate being derived from the other two compounds [22,24]. Not shown are data on the ability of shear forces acting alone to induce rapid formation of $\text{Ins}(1,3,4)\text{P}_3$. Highly significant transients ($P < 0.02$) were found at 0.5, 1.5, 2.5 and 4 s exposure to shear stress in the reaction loop [1].

We have thus established that ADP and thrombin can rapidly produce $\text{Ins}(1,4,5)\text{P}_3$ in the presence of fibrinogen and mM external Ca^{2+} levels under physiological flow conditions (15–50 dyn/cm²) and that levels oscillate. This synthesis and its kinetics were not influenced by the presence of indomethacin (1 $\mu\text{g}/\text{ml}$), ruling out involvement of prostaglandin endoperoxides (results not shown) and suggesting that secretion of dense-granule ADP is also unlikely to be responsible for the rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ observed under shear forces acting alone (Figures 2a and 2b).

When platelets react with ADP or thrombin, intracellular Ca^{2+} levels rise rapidly within the first 1–2 s of activation from 0.1 μM to near 0.3 μM at 1 s and to about 1.0 μM at 2 s [4]. This early increase does not require external Ca^{2+} , and therefore internal stores are involved. The electroporated and resealed platelets also revealed rapid increases of $[\text{Ca}^{2+}]_i$ similar to those seen in control washed platelets, using a stopped-flow apparatus (results not shown). Onset times of about 0.2 and 0.4 s for ADP and thrombin, respectively, are comparable with those reported by Sage and Rink [7]. Although shear forces acting alone generated transient but significant amounts of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (Figures 2a and 2b), they failed to increase $[\text{Ca}^{2+}]_i$ [4] or to initiate platelet aggregation (Figure 1). Indeed, the total integrated productions of $\text{Ins}(1,4,5)\text{P}_3$ and even $\text{Ins}(1,3,4,5)\text{P}_4$ elicited by either shear forces or ADP were similar up to 1 s (Figures 2a and 2b). This suggests that a transient production of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ is not sufficient by itself to release $[\text{Ca}^{2+}]_i$ and cause cell activation. Thrombin evokes comparable increases in $[\text{Ca}^{2+}]_i$ up to 2 s [4] and similar aggregation as ADP does (Figure 1), whereas the total amount of $\text{Ins}(1,4,5)\text{P}_3$ produced up to 1 s was more than twice that seen with ADP. Both agonists did, however, cause rapid and nearly equal formation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ near 200 ms. It is possible that this early rise in the inositol polyphosphates may serve as a trigger [24,25] for the later increase in $[\text{Ca}^{2+}]_i$ between 0.5 and 2 s [4,7,8].

Evidence for multiphasic production of inositol phosphates or diacylglycerols in platelets and other cells

A major finding from our use of the quenched-flow approach is therefore that soluble inositol phosphates are transiently formed within 200 ms of platelet activation. Transient synthesis of $\text{Ins}(1,4,5)\text{P}_3$ in this time frame has not been previously demonstrated in human platelets or in other human cells. Earlier studies [9–16] in human platelets have focused on times greater than 5 s, which are not early enough to be causally linked to the rapid rise in internal Ca^{2+} [4,7,8]. Our results relate directly to a study by Breer et al. [26], who showed sub-second and transient generation of $\text{Ins}(1,4,5)\text{P}_3$ in rodent cilia and insect antennal preparations which were rapid enough for olfactory transduction. $\text{Ins}(1,4,5)\text{P}_3$ fluctuations have also been demonstrated in REF52 fibroblasts between 30 and 120 s of activation [27]. It is clear from our study that ADP and thrombin did not cause a prolonged and steady increase in $\text{Ins}(1,4,5)\text{P}_3$, but rather rapid synthesis and breakdown. Whether activation of protein kinase C, which may inhibit phospholipase C [28] and stimulate $\text{Ins}(1,4,5)\text{P}_3$ removal [29], was responsible for the observed fluctuations remains to be elucidated.

Several recent reports suggest that diacylglycerol levels may increase in phases after thrombin stimulation. Both Murphy et al. [17] and Nakashima et al. [23] showed an early peak near 10 s, similar to the original observation by Rittenhouse-Simmons [30]. However, Nakashima et al. [23] noted a later peak near 2–3 min, seen only in the presence of extracellular Ca^{2+} . Subsequently, Werner et al. [31,32] observed oscillating production of diacylglycerol, with peaks at 0.3–0.6 min intervals.

Concluding remarks

Our study highlights the complexity of mechanisms involved in agonist-induced intracellular Ca^{2+} fluxes and suggests that inositol phosphate-independent mechanisms of internal Ca^{2+} release may operate during rapid platelet activation. Some intriguing additional questions arise: what is the source and function of the $\text{Ins}(1,3,4,5)\text{P}_4$ generated near 200 ms in equal amounts to $\text{Ins}(1,4,5)\text{P}_3$? How is the synthesis of $\text{Ins}(1,3,4,5)\text{P}_4$

regulated, and is there a role for PtdIns 3-kinases which are active during platelet stimulation? Irvine [33] discusses existing evidence that Ins(1,3,4,5) P_4 can mobilize intracellular Ca^{2+} , acting in conjunction with Ins(1,4,5) P_3 . Also, Sorisky et al. [34] provide interesting data that platelet stimulation by thrombin for 20 s activates PtdIns 3-kinase and leads to formation of PtdIns(3,4,5) P_3 , but not PtdIns(3,4) P_2 , and that this occurs in the absence of extracellular Ca^{2+} . The kinetics and extent of PtdIns(3,4,5) P_3 synthesis are controversial [33], since other groups have little evidence for its formation in thrombin-activated platelets [35,36], and a role as a substrate for phospholipase-C-generated Ins(1,3,4,5) P_4 must remain an open question. Finally, the mechanisms are unknown by which shear forces acting alone stimulate liberation of both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 in amounts equal to those seen with ADP during the first 1 s of exposure, yet fail to release intra-platelet Ca^{2+} .

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REFERENCES

- Gear, A. R. L. (1982) *J. Lab. Clin. Med.* **100**, 866–886
- Gear, A. R. L. (1984) *Br. J. Haematol.* **56**, 387–398
- Gear, A. R. L. (1986) in *Platelet Function and Metabolism* (Holmsen, H., ed.), vol. 1, pp. 97–114, C.R.C. Press, Boca Raton, FL
- Jones, D. G. and Gear, A. R. L. (1988) *Blood* **71**, 1539–1543
- Carty, D. J., Spielberg, F. and Gear, A. R. L. (1986) *Blood* **67**, 1738–1743
- Carty, D. J., Freas, D. L. and Gear, A. R. L. (1987) *Blood* **70**, 511–515
- Sage, S. O. and Rink, T. J. (1987) *J. Biol. Chem.* **262**, 16364–16369
- Sage, S. O., Reast, R. and Rink, T. J. (1990) *Biochem. J.* **265**, 675–680
- Fisher, G. J., Bakshian, S. and Baldassare, J. J. (1985) *Biochem. Biophys. Res. Commun.* **129**, 958–964
- Sweatt, J. D., Blair, I. A., Cragoe, E. J. and Limbird, L. E. (1986) *J. Biol. Chem.* **261**, 8660–8666
- Vickers, J. D., Kinlough-Rathbone, R. L., Packham, M. A. and Mustard, J. F. (1990) *Eur. J. Biochem.* **193**, 521–528
- Lloyd, J. V., Nishizawa, E. E., Joist, J. H. and Mustard, J. F. (1973) *Br. J. Haematol.* **24**, 589–604
- Vickers, J. D., Kinlough-Rathbone, R. L. and Mustard, J. F. (1982) *Blood* **60**, 1247–1250
- Vickers, J. D., Kinlough-Rathbone, R. L. and Mustard, J. F. (1986) *Biochem. J.* **237**, 327–332
- Feliste, R., Simon, M. F., Chap, H., Douste-Blazy, L., Defreyn, G. and Maffrand, J. P. (1988) *Biochem. Pharmacol.* **37**, 2559–2564
- Daniel, J. L., Dangelmaier, C. A., Selak, M. and Smith, J. B. (1986) *FEBS Lett.* **206**, 299–303
- Murphy, C. T., Elmore, M., Kellie, S. and Westwick, J. (1991) *Biochem. J.* **278**, 255–261
- Authi, K. S., Hughes, K. and Crawford, N. (1989) *FEBS Lett.* **254**, 52–58
- Fabiato, A. and Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Loomis-Husselbee, J. W., Cullen, P. J., Irvine, R. F. and Dawson, A. P. (1991) *Biochem. J.* **277**, 883–885
- Johnson, R. M., Wasilenko, W. J., Mattingly, R. R., Weber, M. J. and Garrison, J. C. (1989) *Science* **246**, 121–124
- Shears, S. B. (1989) *Biochem. J.* **260**, 313–324
- Nakashima, S., Suganuma, A., Matsui, A. and Nozawa, Y. (1991) *Biochem. J.* **275**, 355–361
- Berridge, M. J. (1991) *Cell Calcium* **12**, 63–85
- Missiaen, L., Taylor, C. W. and Berridge, M. J. (1991) *Nature (London)* **352**, 241–244
- Breer, H., Boekhoff, I. and Tareilus, E. (1990) *Nature (London)* **345**, 65–68
- Harootunian, A. T., Kao, J. P. Y., Paranjape, S. L. and Tsien, R. Y. (1991) *Science* **251**, 75–78
- Zavoico, G. B., Halenda, S. P., Shaafi, R. I. and Feinstein, M. B. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3859–3862
- King, W. G. and Rittenhouse, S. E. (1989) *J. Biol. Chem.* **264**, 6070–6074
- Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63**, 580–587
- Werner, M. H., Bielawska, A. E. and Hannun, Y. A. (1992) *Biochem. J.* **282**, 815–820
- Werner, M. H., Bielawska, A. E. and Hannun, Y. A. (1992) *Mol. Pharmacol.* **41**, 382–386
- Irvine, R. F. (1992) *Curr. Opin. Cell Biol.* **4**, 212–219
- Sorisky, A., King, W. G. and Rittenhouse, S. E. (1992) *Biochem. J.* **286**, 581–584
- Cunningham, T. N., Lips, D. L., Bansal, V. S., Caldwell, K. K., Mitchell, C. A. and Majerus, P. W. (1990) *J. Biol. Chem.* **265**, 21676–21683
- Yatomi, Y., Ozaki, Y. and Kume, S. (1992) *Biochem. Biophys. Res. Commun.* **186**, 1480–1486