Signal Transduction by the Platelet Fc Receptor

By George P. Anderson and Clark L. Anderson

We have evaluated the mechanism by which crosslinking human platelet Fc receptor (FcR) for IgG triggers platelet aggregation and the platelet release reaction. Platelet FcR was crosslinked by incubating purified human platelets with anti-FcR monoclonal antibody and F(ab')2 anti-mouse Ig. The resultant [Ca\(^{2+}\)] increase, monitored by Fura-2 and measured in the absence of extracellular Ca\(^{2+}\), reached a peak of 760 ± 50 nmol/L. The effects of cyclooxygenase inhibitors, aspirin and indomethacin, and a phospholipase A\(_2\) inhibitor, dibromocacetophenone, were examined. Regardless of the inhibitor, at least 25% of the [Ca\(^{2+}\)] increase remained. Thrombin (0.2 U/mL) stimulated an immediate [Ca\(^{2+}\)] increase that reached 1.95 ± 0.8 μmol/L. The [Ca\(^{2+}\)] increase generated by thrombin was only slightly reduced by these inhibitors. Crosslinking the FcRll of platelets resulted in a fivefold increase in the production of [\(^3\)H]inositol phosphates (IP) which, in the absence of extracellular Ca\(^{2+}\), was insensitive to aspirin. The activation of a [Ca\(^{2+}\)] increase along with the measured increases in IP indicate that FcRll crosslinking leads to the activation of phospholipase C (PLC). In contrast to thrombin, platelet activation via FcRll depends to a large extent on arachidonic acid metabolites. However, neither cyclooxygenase nor phospholipase A\(_2\) inhibitors completely blocked FcRll-stimulated [Ca\(^{2+}\)] increase. These observations led us to propose that crosslinking of platelet FcRll initially activates PLC.

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IMMUNE COMPLEXES comprised of IgG interact with the surface membrane of human platelets causing the release of several inflammatory mediators and initiating the platelet aggregation response. This interaction and its effects are likely involved in a number of normal physiologic and pathologic processes in humans. Thus, the molecular details of immune complex-mediated platelet activation are of considerable interest.

The site on platelets to which IgG immune complexes bind, the receptor for the Fc portion of IgG (FcR), has recently been identified. It is a 40-Kd integral membrane sialylglycoprotein nearly identical to an FcR (FcRII) found on monocytes, macrophages, granulocytes, and B cells. The outer surface of each platelet bears about 1,500 copies. The human FcRll protein family has been determined to be encoded by at least three different genes and to result in six distinct transcripts. The diversity of these transcripts lies principally in their cytoplasmic domain with their extracellular and transmembrane domains maintaining 85% identity. The function of these several different FcRll transcripts has not yet been determined, and the transcript or transcripts expressed in the platelet is still unknown.

A monoclonal antibody (MoAb) (IV.3) to this platelet FcRll blocks the binding of IgG immune complexes to the platelet, inhibits immune complex-mediated platelet responses, and when crosslinked with a second bridging antibody triggers platelet responses in the absence of ligand. In addition IV.3 was found to immunoprecipitate a 40-Kd molecule, identical in molecular weight to FcRll from other cells.

The mechanism by which FcR transduces a signal across the platelet plasma membrane is largely unknown. We regard the platelet FcR as an attractive model to study because of its relative simplicity. This cell bears a single FcR class and seems to manifest a single FcR-mediated response, whereas most other easily available cells bear several classes of FcR and generate two or more kinds of responses. Furthermore, signal generation in the platelet has been the focus of much recent investigation. Several platelet agonists such as thrombin and thromboxane A\(_2\) (TXA\(_2\)) have been shown to mediate signal transduction through activation of phospholipase C (PLC) and release of inositol 1,4,5-trisphosphate (IP\(_3\)) while other agonists such as adenosine 5'-diphosphate (ADP) and epinephrine have less direct mechanisms that appear to involve TXA\(_2\) stimulation. Thus, while significant differences exist in the exact manner by which several agonists activate the platelet, PLC activation appears common to all. Furthermore, for thrombin the activation of the Na\(^+\)/H\(^+\) antipporter and the influx of external Ca\(^{2+}\) are both secondary phenomena.

In this report, we test the hypothesis that FcR-mediated platelet triggering involves primarily the activation of PLC and phosphatidylinositol (PI) turnover.

MATERIALS AND METHODS

Platelet isolation. Platelets were prepared by a modification of the method of George et al. Blood was obtained from healthy donors who had abstained from nonsteroidal antiinflammatory drugs for 2 weeks and alcohol for 24 hours. Blood (48 mL) was drawn into a syringe containing 8 mL of 56 mmol/L Na citrate, 65 mmol/L citric acid, and 100 mmol/L glucose. The blood, in two tubes, was then centrifuged at 600g for 6 minutes. The platelet-rich plasma was removed and the red blood cell layer was diluted by the addition of 10 mL of wash out buffer to each tube. The wash out buffer consisted of 12 mmol/L Na citrate (pH 6.5), 120 mmol/L NaCl, 5 mmol/L EDTA, 30 mmol/L glucose, and 1 mg/mL bovine serum albumin (BSA). To obtain a higher yield of platelets the resuspension was centrifuged as before, and the supernatant was added to the platelet-rich plasma. For the initial experiments this step was repeated. The platelets were then pelleted by centrifugation (1,100g for 15 minutes), and were then resuspended in assay buffer. The
assay buffer for the initial aggregation and fluorescence experiments consisted of 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO<sub>4</sub>, 10 mmol/L Hepes (pH 7.4), and 1 mg/mL BSA. For the PI turnover experiment and the inhibitor [Ca<sup>2+</sup>], assays the buffer was 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl<sub>2</sub>, 3.3 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 20 mmol/L Hepes (pH 7.4), and 1 mg/mL BSA. A typical platelet yield was 55% ± 5%, 7.5 × 10<sup>9</sup> platelets. These platelets were contaminated with approximately 0.05% leukocytes as determined by a Coulter (Hialeah, FL) counter. In later experiments the blood was additionally anticoagulated by 50 ng/mL PGE<sub>2</sub>, and in experiments where the response to ADP was measured, 0.035 U/mL apyrase was included in all buffers prior to the final assay buffer, which was free of apyrase. These various isolation procedures did not appear to affect the manner in which the platelets responded to either thrombin or anti-FcRII MoAb.

**Platelet aggregometry.** Percent aggregation was determined by a Bio-Data (Hatboro, PA) aggregometer PAP-4. Platelets (0.45 mL at 3 × 10<sup>9</sup>/mL) were placed in aggregometer tubes. Ca<sup>2+</sup> (1 mmol/L) was added to each tube and for the appropriate tubes 10 μL of anti-FcRII MoAb IV.3 (1:4 dilution of the stock supernatants from cultures of the cloned cell line) was added to 5 or 10 minutes before stimulation. Three minutes before stimulation the tubes were warmed to 37°C. The platelets were then stimulated by the addition of thrombin (0.2 U/mL) or by the addition of a secondary crosslinking antibody, F(ab')<sub>2</sub>, anti-mouse IgG (12.5 μg/mL). The concentrations of the IV.3 MoAb and the secondary antibody were chosen empirically by determining concentrations that maximized the slope, the rate at which aggregation occurred, in addition to maximal percent aggregation. The slope and the percent aggregation were dependent on the concentrations of both antibodies. The slope was the determinant used for comparison because, as observed previously, the slope was the best quantitative correlate of the platelet aggregation response. The final percent aggregation was found to approach a maximum value at a lower antibody concentration and thus to be a less sensitive measurement than the slope.

**Measurement of [Ca<sup>2+</sup>]<sub>i</sub>, by Fura-2.** Platelets at approximately 3 × 10<sup>9</sup>/mL were incubated with 1 μmol/L Fura-2/AM for 30 minutes at 37°C. EDTA (1 mmol/L) was added and the platelets were pelleted by centrifugation (10 minutes at 1,100g). The pellet was rinsed twice with assay buffer to remove excess Fura-2/AM. The platelets were then resuspended with assay buffer to a concentration of 1 to 1.2 × 10<sup>9</sup>/mL. EDTA was added at 1,100g for 10 minutes, and then rinsed twice with assay buffer plus 1 mmol/L EDTA to remove excess label before being resuspended in assay buffer to a concentration of 1 to 1.2 × 10<sup>9</sup>/mL. The platelets were incubated for 10 minutes at 37°C before stimulation. The anti-FcRII MoAb (20 μL of a 1:4 supernatant dilution/mliliter) and 10 mmol/L lithium chloride to inhibit the inositol monophosphatase were added to the platelets before the final incubation. At time zero the platelets were stimulated by the addition of 0.2 μmol/L of thrombin or 25 μg/mL of the secondary crosslinking antibody. At appropriate intervals 0.5-mL aliquots were removed and added to 1.5 mL of 1:2 chloroform/methanol to stop the reaction. Phase separation was achieved by the addition of 0.5 mL of chloroform. The aqueous phase was removed and diluted to 4 mL with H<sub>2</sub>O. The insoluble material was removed by centrifugation at 1,000g for 10 minutes. The aqueous phase was then mixed with 0.5 mL of a Dowex-1 slurry (Sigma, St Louis, MO) for 10 minutes to allow the IP to bind. The free inositol and glycerophosphoinositol were washed from the Dowex-1 by 4 mL of H<sub>2</sub>O and twice with 4 mL of 60 mmol/mL Na formate and 5 mmol/mL Na tetraborate. Then the IP as a group were released from the Dowex-1 by the addition of 1 mL of 1.0 mol/L Na formate and 0.1 mol/L formic acid. However, in initial experiments the IP were obtained in a stepwise manner (not shown), separating the IP, inositol bisphosphate (IP<sub>2</sub>), and IP<sub>3</sub>, with 0.2, 0.4, and 1.0 mol/L of ammonium formate and 0.1 mol/L of formic acid, as described by Downes and Michell. The counts per minute of IP<sub>1</sub> released were determined by liquid scintillation counting. Neither procedure yields information on the isomer structure of the IP obtained.

**Materials.** Preparation and characterization of the anti-FcRII MoAb (IV.3) have been described previously. The goat anti-mouse IgGs were obtained from Cappel Worthington Biochemicals (Malvern, PA). The anti-FcRII MoAb Ka79 was the generous gift of Dr Mohanakumar, Washington University, St Louis, MO. The H<sub>2</sub>O was obtained from New England Nuclear (Boston, MA). The Fura-2/AM was obtained from Molecular Probes (Eugene, OR). The 2,4-dihydroxyacetophenone (DBA) was obtained from Aldrich (Milwaukee, WI). The d-phenylalanyl-l-phenylalanyl-L-arginine chloromethyl ketone (PPACK) was obtained from Calbiochem (La Jolla, CA). Human thrombin, ADP, and EGTA were obtained from Sigma Chemical Co (St Louis, MO). All other chemicals were of reagent grade.

**RESULTS**

**Preliminary tests of platelet aggregation.** The initial experiments were performed to determine the proper concentration of primary (anti-FcRII MoAb) and secondary [F(ab')<sub>2</sub>] goat anti-mouse IgG antibody to be used in subsequent experiments. Figure 1 shows the typical aggregation curve induced by thrombin or induced by the sequential addition of primary and secondary antibodies. Thrombin, as has been shown by others, elicits a prompt aggregation response, while crosslinking of FcRII results in aggregation after a time delay. The two curves, however, are similar in slope and in final percent aggregation attained. The specificity of IV.3 was shown to be the same as before, by the failure of MOPC-141, an IgG2b protein like IV.3, to stimulate platelet activation on addition of a secondary antibody. The general ability of anti-FcRII MoAbs to stimulate platelet aggregation was determined by Grynkiewicz et al to be 224 nmol/L. In later experiments, the procedure described by Berridge et al was performed with 50 μCi/mL 3H-myo-inositol and 25 μmol/L adenosine for 2 hours at 37°C to allow the incorporation of the label into the phosphatidylinositides. The platelets were then pelleted at 1,100g for 10 minutes, and then rinsed twice with assay buffer plus 1 mmol/L EDTA to remove excess label before being resuspended in assay buffer to a concentration of 1 to 1.2 × 10<sup>9</sup>/mL. The platelets were incubated for 10 minutes at 37°C before stimulation. The anti-FcRII MoAb (20 μL of a 1:4 supernatant dilution/mliliter) and 10 mmol/L lithium chloride to inhibit the inositol monophosphatase were added to the platelets before the final incubation. At time zero the platelets were stimulated by the addition of 0.2 μmol/L of thrombin or 25 μg/mL of the secondary crosslinking antibody. At appropriate intervals 0.5-mL aliquots were removed and added to 1.5 mL of 1:2 chloroform/methanol to stop the reaction. Phase separation was achieved by the addition of 0.5 mL of chloroform. The aqueous phase was removed and diluted to 4 mL with H<sub>2</sub>O. The insoluble material was removed by centrifugation at 1,000g for 10 minutes. The aqueous phase was then mixed with 0.5 mL of a Dowex-1 slurry (Sigma, St Louis, MO) for 10 minutes to allow the IP to bind. The free inositol and glycerophosphoinositol were washed from the Dowex-1 by 4 mL of H<sub>2</sub>O and twice with 4 mL of 60 mmol/mL Na formate and 5 mmol/mL Na tetraborate. Then the IP as a group were released from the Dowex-1 by the addition of 1 mL of 1.0 mol/L Na formate and 0.1 mol/L formic acid. However, in initial experiments the IP were obtained in a stepwise manner (not shown), separating the IP, inositol bisphosphate (IP<sub>2</sub>), and IP<sub>3</sub>, with 0.2, 0.4, and 1.0 mol/L of ammonium formate and 0.1 mol/L of formic acid, as described by Downes and Michell. The counts per minute of IP<sub>1</sub> released were determined by liquid scintillation counting. Neither procedure yields information on the isomer structure of the IP obtained.
confirmed by the use of Ku79. Ku79 was able to stimulate platelet aggregation when crosslinked by a secondary antibody (not shown). In addition, the platelets were found to be insensitive either to IV.3 by itself or to both primary and secondary antibodies together if the secondary antibody was added before the primary antibody.

Due to the lag time between the addition of the F(ab')₂ anti-mouse Ig and the subsequent aggregation it was necessary to confirm that stimulation of FcRII resulted in the primary signal and not simply the generation of a more effective agonist. Of prime concern was that FcRII might be stimulating the release of ADP from a small number of platelets, which would then be the actual agonist for the remainder of the platelets. A secondary concern was that perhaps a residual amount of prothrombin was being activated. The first was rejected partially because the washed platelets isolated in the absence of apyrase were unresponsive to exogenous ADP. When apyrase was included in the isolation buffers to act as an ADP sink the isolated platelets retained their responsiveness to ADP. The stimulation of these platelets with 10 μmol/L of ADP could be totally inhibited by 50 μmol/L of adenosine triphosphate (ATP); however, 50 μmol/L of ATP inhibited the rate of anti-FcRII MoAb stimulated aggregation by only 50% (data not shown), while thrombin-stimulated aggregation was unaffected. This indicates that the FcRII-stimulated aggregation is augmented by ADP secretion, but not dependent on it. The second concern was allayed by testing the ability of the thrombin inhibitor α-phenylalanine-β-phenylalanine-L-arginine chloromethyl ketone (PPACK) to prevent stimulation through FcRII. Platelet aggregation stimulated by crosslinking FcRII was found to be totally insensitive to 45 μmol/L PPACK, a concentration theoretically sufficient to inhibit approximately 100 U/mL of thrombin, and experimentally found to totally inhibit platelet response to 0.2 U/mL of thrombin (data not shown).

Effect of the concentration of F(ab')₂ anti-mouse IgG. As the concentration of F(ab')₂ anti-mouse IgG was increased from 3 to 25 μg/mL the lag time between the addition of secondary antibody and subsequent aggregation decreased from 2 to less than 0.5 minutes (Fig 2). This observation, together with the fact that crosslinking of FcRII does not appear to stimulate platelet aggregation solely by initiating ADP release or prothrombin activation, indicates that lag time is likely due to the accumulation of a sufficient degree of FcR crosslinking.

Release of internally stored Ca²⁺ initiated by thrombin or by anti-FcRII MoAb crosslinked with a secondary antibody. Experiments were performed to examine whether stimulation of FcRII resulted in a [Ca²⁺] increase and to compare the FcRII-mediated increase with that stimulated by thrombin. Figure 3A and B shows this comparison in the presence of 1 mmol/L EGTA. Under these conditions the extracellular concentration of Ca²⁺ is essentially zero and the platelet's basal [Ca²⁺] averaged 104 ± 12 nmol/L. Therefore, the measured [Ca²⁺] increase can be attributed only to release from internal stores. The [Ca²⁺] increase stimulated by thrombin was found to be instantaneous and reached 1.95 ± 0.8 μmol/L, consistent with the findings of Detwiler.
Fig 3. Measurement of platelet \([\text{Ca}^{2+}]\) flux using Fura-2. Platelets loaded with Fura-2, in the presence of 1 mmol/L of EGTA (A, B) or 1 mmol/L \([\text{Ca}^{2+}]\) (C, D), were stimulated with thrombin (0.2 U/mL; A, C) added at the gap in the tracing, or were incubated with anti-FcRII MoAb IV.3 (B, D) before the addition of the crosslinking secondary antibody (F(ab')2 anti-mouse IgG (12.5 \(\mu\)g/mL) at the gap in the tracing. Each curve is representative of three separate experiments that yielded comparable results. As in Fig 1, thrombin stimulated an immediate response while a significant lag preceded the antibody-mediated response.

Figure 3C and D shows the comparison of the \([\text{Ca}^{2+}]\) increase generated by thrombin and anti-FcRII MoAb crosslinked by a secondary antibody in the presence of 1 mmol/L \([\text{Ca}^{2+}]\). Although the peak \([\text{Ca}^{2+}]\) did not vary significantly from Figure 3A and B the final \([\text{Ca}^{2+}]\) remained elevated to approximately 300 nmol/L for both thrombin and crosslinked anti-FcRII MoAb. This finding, indicative of the influx of external \([\text{Ca}^{2+}]\), has been described for thrombin by Pollock and Rink.

Inhibition of the \([\text{Ca}^{2+}]\) increase by aspirin. The cyclooxygenase inhibitor, aspirin, had a dramatic effect on the \([\text{Ca}^{2+}]\) increase stimulated by FcRII. Figure 4 shows the effect of increasing the aspirin concentration from 0 to 2 mmol/L on the subsequent \([\text{Ca}^{2+}]\) increase. Aspirin in the absence of agonist did not induce a \([\text{Ca}^{2+}]\) increase. While aspirin is able to inhibit up to 75% of the \([\text{Ca}^{2+}]\), increase, it did not totally block the increase indicating that TXA2 formation occurs subsequent to PLC activation.
Fig 5. Comparison of the PI turnover induced by thrombin versus anti-FcRII MoAb in the presence of 1 mM EGTA. [3H]-myo-inositol–treated platelets (1.0 to 1.2 × 10⁷/mL) were stimulated as described in Fig 3. Aliquots were removed at indicated times and the 3H-IP were isolated by batch elution from Dowex-1. The experiment presented is representative of three separate experiments. The release of IP occurred in harmony with the 

\[ [\text{Ca}^{2+}] \] flux and platelet aggregation.

![Graph](image)

Fig 6. Effect of aspirin (1 mmol/L) on the PI turnover stimulated by anti-FcRII MoAb IV.3 IgG and secondary crosslinking antibody in the presence of 1 mmol/L of EGTA. Aspirin (1 mmol/L) was added to the platelets 15 minutes before stimulation. The experiment was performed as described in Figs 3 and 5. The data represent the mean and SD of three separate experiments, standardized to the average unstimulated value. Aspirin does not produce a measurable inhibition in the absence of extracellular Ca²⁺.

![Graph](image)

at a high enough concentration to strongly stimulate phospholipase A₂.

Effect of DBA on platelet aggregation and \([\text{Ca}^{2+}]\) increase. DBA, a potent phospholipase A₂ inhibitor, was used to examine the role that the arachidonic acid metabolites play in the activation cascade. Platelet aggregation stimulated by FcRII crosslinking could be totally inhibited by as little as 2 μmol/L of DBA, while thrombin was not completely blocked by 5 μmol/L of DBA (Fig 7). In addition to FcRII crosslinking, ADP and arachidonic acid failed to aggregate platelets inhibited by 5 μmol/L of DBA for 3 minutes (not shown). The maximum concentration of DBA used was 5 μmol/L due to the observation that at higher concentrations (10 μmol/L) platelet Ca²⁺ stores were disrupted. The [Ca²⁺], increase stimulated by FcRII crosslinking was maximally inhibited (74%) by approximately 2.5 μmol/L of DBA (Fig 8). The inability of DBA to totally inhibit the [Ca²⁺], increase implies that stimulation of phospholipase A₂ is not FcRII’s primary mechanism of platelet activation.

![Graph](image)

Effect of cyclooxygenase and phospholipase A₂ inhibitors on the [Ca²⁺], increase generated by platelet agonists. The [Ca²⁺], increase generated by FcRII crosslinking and by other agonists was compared in their response with three different inhibitors (Table 1). Arachidonic acid was used as a positive control to insure the effectiveness of aspirin and indomethacin; both inhibitors completely blocked platelet stimulation. None of the other agonists were completely blocked by any of the inhibitors, which further implies that the activation of phospholipase A₂ and the subsequent formation of arachidonic acid is not the primary activation pathway.
Platelet activation is thought to occur initially via PLC in response to thrombin. We have simulated immune complex-mediated platelet activation by crosslinking the FcRII on human platelets with an anti-FcRII MoAb and a second bridging antibody. Such crosslinking results in platelet aggregation that resembles the effect seen with the well-studied platelet agonist thrombin except that the response to FcRII crosslinking is preceded by a lag (Fig 1). The duration of this lag period bears an inverse relationship to the concentration of secondary bridging antibody (Fig 2) and thus is most likely due to the time-consuming formation of a threshold amount of crosslinked receptors necessary for triggering. The other possible explanation for a lag phase would be to allow for the activation of the actual platelet agonist. However, thrombin activation does not appear to be involved because the thrombin inhibitor PPACK has no effect on FcRII-mediated aggregation. ADP secretion likewise is not responsible since competitively inhibiting ADP with ATP decreases the aggregation rate by only 50% and has no effect on the lag phase. This does mean, however, that the subsequent secretion of ADP has a significant role in recruitment of additional platelets in the aggregation process.

Platelet activation is thought to occur initially via PLC stimulation or through the release of arachidonic acid by other phospholipases. Other potential activating events such as 

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\text{Ca}^{2+} \text{ channel formation or activation of the Na}^{+}/\text{H}^{+} \text{ antiporter have been determined to be insufficient primary stimuli and are considered secondary effects.}^{23,24} \text{ Thrombin has been shown to stimulate initially PLC. We hypothesized that the FcRII might also function by activating PLC. To examine this hypothesis, the response of platelets to thrombin and to FcRII crosslinking were studied in parallel. Both agonists stimulate platelets in a Ca^{2+}-depleted medium to increase the [Ca^{2+}]_i. In the absence of extracellular Ca^{2+} (+1 mmol/L of EGTA), FcRII crosslinking on Fura-2-loaded platelets exhibited an increase in [Ca^{2+}]_i to 750 ± 50 nmol/L compared with 1.9 ± 0.8 μmol/L by thrombin (Fig 3). This increase could only have originated from internal Ca^{2+} stores.}^{27} \text{ Whether this flux was due directly to stimulation of PLC by FcRII or whether FcRII activation of PLC was mediated by arachidonic acid metabolites was determined by measuring FcRII-mediated [Ca^{2+}]_i, increase in the presence of aspirin, indomethacin, and DBA. Aspirin and indomethacin, which block the cyclooxygenase pathway of the arachidonic acid cascade,}\text{ inhibit 70% (Fig 4 and Table 1) of [Ca^{2+}]_i, increase indicating that a majority of the flux was due to TXA, formation. However, a residual 25% was refractory to aspirin or indomethacin inhibition and was likely due to the direct activation of PLC by FcRII crosslinking. A similar result was observed for inhibition of phospholipase A_2 by DBA. We surmise that the [Ca^{2+}]_i, increase due to PLC activation resulted in the subsequent activation of Ca^{2+}-sensitive phospholipase A_2, which in turn amplified the [Ca^{2+}]_i, increase by stimulating further PLC activation.}^{13} \text{ Thrombin, as observed by others, has little requirement for secondary mediators. This may be a function of thrombin's ability to stimulate an intense [Ca^{2+}]_i, increase. In contrast, when [Ca^{2+}]_i, was measured in the presence of external Ca^{2+}, no difference was seen in the peak [Ca^{2+}]_i, reached; rather, the signal failed to return to baseline as it did in the absence of Ca^{2+}. This feature would indicate that nearly all the initial flux can be accounted for by the release of internal Ca^{2+} stores and that the increased postflux [Ca^{2+}]_i, is maintained by the presence of external Ca^{2+}. The stimulation of PI turnover was the other factor compared between thrombin and anti-FcRII MoAb. We measured by anion exchange chromatography the accumulation of total IP versus time in the presence of Li+, which inhibits the inositol monophosphatase.}\text{ Under these conditions a sustained increase in PI was seen following FcRII crosslinking (Fig 5) similar to that observed upon thrombin activation.}

<table>
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<th>Agonists</th>
<th>Aspirin (1 mmol/L)</th>
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<th>DBA (5 μmol/L)</th>
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<td>ADP</td>
<td>47 ± 18 (n = 2)</td>
<td>47 ± 23 (n = 2)</td>
<td>78 ± 6 (n = 2)</td>
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The inhibitors were added to Fura-2-loaded platelets (3 × 10⁸/mL) 3 minutes before addition of agonist. The peak [Ca^{2+}]_i was measured. Aspirin- and DBA-treated samples contained 1 mmol/L of EGTA, while the indomethacin-treated samples contained 1 mmol/L of Ca^{2+}. The mean and range of the percent inhibition is listed followed by the replicate number. The response to thrombin was only weakly inhibited, whereas the response to anti-FcRII MoAb was inhibited up to 75%.
stimulation. Others have shown that such an accumulation in total IP is invariably initiated by an early increase in the concentration of IP,
which acts on the dense tubular system to release Ca2+ stores.3,9-10

In the absence of external Ca2+ (1 mmol/L of EGTA) the effect aspirin had on the formation of IP, was not reflected in the total accumulation of IP after FcRII crosslinking (Fig 6). This finding implies that the [Ca2+] increase is a much more sensitive indicator of IP production than the measurement of total release of IP. This is likely since it has been shown that an IP, concentration of only 20 nmol/L gives maximal accumulation an average of 31% (not shown) suggesting that a maintained [Ca2+] increase was necessary for sustained phospholipase A2 activation.

It is clear that the inhibition of arachidonic acid metabolic pathway does not prevent stimulation of PLC by FcRII. The stimulation of phospholipase A2 appears to have an amplifying effect on the initial stimulation of PLC, and while it is possible that FcRII could be costimulating PLC and phospholipase A2, it seems certain that PLC activation occurs independent of phospholipase A2. More work will be necessary to determine whether intermediary molecules are involved in the FcRII activation of PLC. It is presumed that like other PLC activators FcRII interacts directly with a G protein. Future efforts are being focused on defining the nature of this interaction.

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