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-FcRII 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, dibromoacetophenone, 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 1.96 ± 0.8 μmol/L. The [Ca\(^{2+}\)] increase generated by thrombin was only slightly inhibited by these inhibitors. Crosslinking the FcRII of platelets resulted in a fivefold increase in the production of \([\text{H}]\)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 FcRII crosslinking leads to the activation of phospholipase C (PLC). In contrast to thrombin, platelet activation via FcRII depends to a large extent on arachidonic acid metabolites. However, neither cyclooxygenase nor phospholipase A\(_2\) inhibitors completely blocked FcRII-stimulated [Ca\(^{2+}\)] increase. These observations led us to propose that crosslinking of platelet FcRII initially activates PLC.

© 1990 by The American Society of Hematology.

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 sialoglycoprotein 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 FcRRII 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 FcRRII 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 FcRII 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 FcRII 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

From the Departments of Internal Medicine, Physiological Chemistry, and Molecular Genetics, The Ohio State University, Columbus, OH.

Submitted October 10, 1989; accepted May 22, 1990.

Supported in part by US Public Health Service grants no. ROI CA24067, T32 CA09498, and F32 AI07880, and by the Bremer Foundation.

Address reprint requests to Clark L. Anderson, MD, Davis Medical Research Center, Room 2054, 480 W 9th Ave, Columbus, OH 43210.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

0006-4971/90/7606-0015$3.00/0

Blood, Vol 76, No 6 (September 15), 1990: pp 1165-1172

1165
assay buffer for the initial aggregation and fluorescence experiments consisted of 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO₄, 10 mmol/L NaH₂PO₄, 2.7 mmol/L NaHCO₃, 1 mmol/L MgCl₂, 3.3 mmol/L NaCl, and 0.1 mmol/L BSA. For the PI turnover experiments, the measuring instrument was a SLM 8000C spectrofluorimeter thermostated to 37°C. The platelets were pelleted by centrifugation (10 minutes at 1,100g) and then resuspended with assay buffer to a concentration of 1 × 10⁸/mL. The cytoplasmic Ca²⁺ concentration was determined by the addition of 2 mmol/L Ca²⁺ and 50 μmol/L digitonin. The Fmax was determined by the addition of 2 mmol/L EGTA and 50 μmol/L digitonin. A small percentage of the Fura-2 (5% to 10%) was found to have leaked out of the platelets. The fluorescence due to this extracellular Fura-2 was corrected by measuring the quenching yield difference between the intact platelets in the presence of 1 mmol/L Ca²⁺ and 1 mmol/L EGTA. The dissociation constant used was determined by Grynkiewicz et al.²⁶ to be 224 μmol/L. In later experiments, which monitored the effect of the inhibitors, the [Ca²⁺] of the platelets was monitored by a Perkin-Elmer (Norwalk, CT) LS-B luminescence spectrophotometer. With this instrument the platelets were studied with an excitation at 335 nm with a 5-nm band pass and the emission was measured by the Perkin-Elmer at 380 nm with a 5-nm band pass with adjustments of the label. The peak [Ca²⁺] was measured by the Perkin-Elmer was smaller.

Measurement of PI turnover. The inositol phosphates (IP) were quantified by the procedure described by Berridge et al. A² Platelets (2 × 10⁹/mL) were incubated 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 phosphatidylinositols. 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 × 10⁹/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/milliliter) 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 U/mL 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 H₂O. The insoluble material was removed by centrifugation at 1,100g 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 glycero-phosphoinositols were washed from the Dowex-1 by 4 mL of H₂O and twice with 4 mL of 60 mmol/L Na formate and 5 mmol/L 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 (IP₂), and IP₃, 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 3H-IP 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 Ku79 was the generous gift of Dr Mohanakumar, Washington University, St. Louis, MO. The goat anti-mouse IgG2b protein like IV.3, to stimulate platelet activation on agonist stimulation, was purchased from SouthernBiotech. The Fura-Z/AM was obtained from Molecular Probes (Eugene, OR). The 2,4-dibromoacetophenone (DBA) was obtained from Aldrich (Milwaukee, WI). The 3,4-dihydroxybenzaldehyde (DHBA) 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'), anti-mouse IgG (12.5 pg/mL). The concentration of primary antibody was determined by the addition of 2 mmol/L 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 H₂O. The insoluble material was removed by centrifugation at 1,100g 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 glycero-phosphoinositols were washed from the Dowex-1 by 4 mL of H₂O and twice with 4 mL of 60 mmol/L Na formate and 5 mmol/L 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 (IP₂), and IP₃, 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 3H-IP 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 Ku79 was the generous gift of Dr Mohanakumar, Washington University, St. Louis, MO. The 3H-myo-inositol was obtained from New England Nuclear (Boston, MA). The Fura-2/AM was obtained from Molecular Probes (Eugene, OR). The 2,4-dibromoacetophenone (DBA) was obtained from Aldrich (Milwaukee, WI). The 3,4-dihydroxybenzaldehyde (DHBA) 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'), anti-mouse IgG (12.5 pg/mL). The goat anti-mouse IgG (12.5 pg/mL). The secondary antibodies were then incubated with 1 pmol/L Fura-2/AM and 10 mmol/L EGTA and 50 μmol/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 U/mL of thrombin or 25 μg/mL of the secondary crosslinking antibody. The slope and the percent aggregation were determined by the addition of 10 mmol/L Ca²⁺ and 50 μmol/L digitonin. The Fmax was determined by the addition of 2 mmol/L EGTA and 50 μmol/L digitonin. Diclofenac acid was used as a control to inhibit the 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.³ The measurement of [Ca²⁺] by Fura-2. Platelets at approximately 3 × 10⁹/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 3 × 10⁹/mL and kept in the dark at room temperature until tested. Initial fluorescence measurements were performed using an SLM Aminco (Urbana, IL) SPF 500C spectrofluorimeter thermostated to 37°C. The Fura-2-loaded platelets were excited at 335 nm with a 2-nm band pass, and the emission monitored at 510 nm with a 5-nm band pass. The samples were handled in the same manner as in the aggregation experiments.

The cytoplasmic Ca²⁺ concentration was determined by the method of Grynkiewicz et al.²² The Fmax was determined by the addition of 2 mmol/L Ca²⁺ and 50 μmol/L digitonin. The Fmin was determined by the addition of 2 mmol/L EGTA and 50 μmol/L digitonin. A small percentage of the Fura-2 (5% to 10%) was found to have leaked out of the platelets. The fluorescence due to this extracellular Fura-2 was corrected by measuring the quenching yield difference between the intact platelets in the presence of 1 mmol/L Ca²⁺ and 1 mmol/L EGTA. The dissociation constant used was determined by Grynkiewicz et al.²² to be 224 μmol/L. In later experiments, which monitored the effect of the inhibitors, the [Ca²⁺] of the platelets was monitored by a Perkin-Elmer (Norwalk, CT) LS-B luminescence spectrophotometer. With this instrument the platelets were excited at 335 nm with a 5-nm band pass and the emission was monitored at 500 nm with a 10-nm band pass. While qualitatively the two instruments yielded the same results, the peak [Ca²⁺], measured by the Perkin-Elmer was smaller.
Fig 1. Platelet aggregation stimulated by thrombin and anti-FcRII MoAb. With an aggregometer, the aggregation of stirred platelets was measured after the addition of (A) thrombin (0.2 U/mL) and (B) anti-FcRII MoAb IV.3 plus a crosslinking secondary anti-murine antibody (12.5 μg/mL). Thrombin stimulated rapid aggregation while FcRII crosslinking required a long lag phase before the appearance of platelet aggregation.

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-phenylalaninyl-L-phenylalaninyl-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 FcRII 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 [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 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 μg/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. The [Ca\(^{2+}\)] increase stimulated by the FcRII reached an average peak concentration of 750 ± 50 nmol/L. The [Ca\(^{2+}\)] for both agonists returned to near basal levels as Ca\(^{2+}\) exited the cells or was resequestered. As seen in the aggregation studies, a significant lag occurred between the addition of secondary antibody and the [Ca\(^{2+}\)] increase. This lag phase was also dependent on the concentration of secondary antibody, similar to Fig 2 (not shown). The same controls used for platelet aggregation showed the specificity of IV.3 in the generation of a [Ca\(^{2+}\)] increase.

Figure 3C and D shows the comparison of the [Ca\(^{2+}\)] increase generated by thrombin and anti-FcRII MoAb crosslinked by a secondary antibody in the presence of 1 mmol/L Ca\(^{2+}\). Although the peak [Ca\(^{2+}\)] did not vary significantly from Figure 3A and B the final [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 Ca\(^{2+}\) on stimulation, has been described for thrombin by Pollock and Rink.32

Inhibition of the [Ca\(^{2+}\)] increase by aspirin. The cyclooxygenase inhibitor, aspirin, had a dramatic effect on the [Ca\(^{2+}\)] increase stimulated by crosslinked anti-FcRII MoAb. Figure 4 shows the effect of increasing the aspirin concentration from 0 to 2 mmol/L on the subsequent [Ca\(^{2+}\)] increase. Aspirin in the absence of agonist did not induce a [Ca\(^{2+}\)] increase. While aspirin is able to inhibit up to 75% of the [Ca\(^{2+}\)] increase, it did not totally block the increase indicating that TXA\(_2\) formation occurs subsequent to PLC activation.

Comparison of PI turnover induced by thrombin and anti-FcRII MoAb crosslinked by a secondary antibody. It is well known that the release of stored Ca\(^{2+}\) is due to the formation of IP\(_1\).35 To confirm that measurable PI turnover was being stimulated by FcRII we compared the increase in the IP generated by thrombin with the increase caused by anti-FcRII MoAb crosslinked by a secondary antibody. Initial experiments in which the IP were separated into IP\(_1\), IP\(_2\), and IP\(_3\) fractions indicated the majority of the label appeared in the IP\(_1\) fraction (not shown). This is presumably due to the addition of Li\(^+\), as has been described previously.35,36 Since our purpose was to examine if FcRII was stimulating measurable PI turnover as has been observed for thrombin, only the net labeling of the entire IP pool was examined. Figure 5 shows the results of this comparison. As was observed before with the aggregation response and with [Ca\(^{2+}\)] flux, thrombin initiates an immediate response while a lag follows the addition of F(ab')\(_2\), anti-mouse IgG. It is clear that thrombin, as is well known, and anti-FcRII crosslinked by a secondary antibody both stimulate PI turnover.

Effect of aspirin on PI turnover. Since the cyclooxygenase inhibitor, aspirin, is such a potent inhibitor of the [Ca\(^{2+}\)] increase generated by FcRII, we examined the effect of aspirin on PI turnover. As Fig 6 shows there was no measurable inhibition by aspirin on PI turnover in the presence of 1 mmol/L of EGTA. On the other hand, aspirin inhibited PI turnover an average of 31% in the presence of 1 mmol/L of Ca\(^{2+}\) (data not shown). It appears that inhibition could be observed only when Ca\(^{2+}\) was present, presumably...
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 x 10⁸/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 [Ca²⁺]⁴ flux and platelet aggregation.

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⁺.

Fig 7. Effect of DBA concentration on platelet aggregation stimulated by anti-FcRII MoAb or thrombin. In an assay similar to that described in Fig 1, DBA (0 to 5 μmol/L) was added to the platelets 5 minutes before agonist addition. The slope as calculated by the Bio-Data aggregometer is in terms of % full scale/30 seconds. The 0 and 5 μmol/L of DBA points represent the mean and SD of four separate experiments. The other points represent the mean and SD of two separate experiments. DBA (5 μmol/L) is 2.5 x the concentration required to achieve maximal inhibition of anti-FcRII–stimulated aggregation.

Effect of DBA on platelet aggregation and [Ca²⁺] 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.

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.
Other potential activating events such as Ca\(^{2+}\) channel formation or activation of the Na\(^+\)/H\(^+\) antiporter have been determined to be insufficient primary stimuli and are considered secondary effects.\(^{23,24}\) 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.\(^{37}\)

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,\(^{35}\) inhibit 70% (Fig 4 and Table 1) of [Ca\(^{2+}\)]\(_i\), increase indicating that a majority of the flux was due to TXA\(_2\) 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.\(^{13}\) 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.\(^{39}\) Under these conditions a sustained increase in IP was seen following FcRII crosslinking (Fig 5) similar to that observed upon thrombin

**DISCUSSION**

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-requiring 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. The inhibitors were added to Fura-2-loaded platelets (3 × 10\(^5\)/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 IP₃, which acts on the dense tubular system to release Ca²⁺ stores.⁵,¹⁰,¹¹

In the absence of external Ca²⁺ (1 mmol/L of EGTA) the effect aspirin had on the formation of IP₃ was not reflected in the total accumulation of IP after FcRII crosslinking (Fig 6). This finding implies that the [Ca²⁺]ᵢ increase is a much more sensitive indicator of IP₃ production than the measurement of total release of IP. This is likely since it has been shown that an IP₃ concentration of only 20 nmol/L gives maximal release of stored Ca²⁺.¹² Thus a small and transient decrease in the rate of IP₃ release caused by aspirin inhibition of the cyclooxygenase could have a dramatic effect on the [Ca²⁺]ᵢ increase, yet produce no measurable difference in the total release of IP as measured over a 10-minute period. In addition, TXA₂ would function to activate the platelets in a more simultaneous manner, which could greatly accentuate the peak [Ca²⁺]ᵢ observed. In the presence of external Ca²⁺ (1 mmol/L) the addition of 1 mmol/L aspirin inhibited IP accumulation an average of 31% (not shown) suggesting that a maintained [Ca²⁺]ᵢ 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.

ACKNOWLEDGMENT

We would like to thank Dr John Brandt and the Department of Biochemistry for their generosity.

REFERENCES

5. Stengelin S, Stamenkovic I, Seed B: Isolation of cDNAs for two distinct human Fc receptors by ligand affinity cloning. Embo J 7:1053, 1988
10. Stengelin S, Stamenkovic I, Seed B: Isolation of cDNAs for two distinct human Fc receptors by ligand affinity cloning. Embo J 7:1053, 1988
32. Pollock WK, Rink TJ: Thrombin and ionomycin can raise platelet cytosolic Ca\(^{2+}\) to micromolar levels by discharge of internal Ca\(^{2+}\) stores: Studies using fura-2. Biochem Biophys Res Comm 139:308, 1986
Signal transduction by the platelet Fc receptor

GP Anderson and CL Anderson