Platelet α-Granule Proteins: Studies on Release and Subcellular Localization

By Karen L. Kaplan, M. Johan Broekman, Arthur Chernoff, George R. Lesznik, and Michael Drillings

Four platelet proteins are secreted by platelets during the release reaction: platelet factor 4 (PF4), β-thromboglobulin (βTG), platelet-derived growth factor (PDGF), and fibrinogen. The characteristics of release of these proteins and 14C-serotonin (14C-5HT) were studied and compared with secretion of acid hydrolases as described by others. Distinct differences were found between the release of PF4, βTG, and fibrinogen and the release of acid hydrolases. Among these were the following: (1) ADP and epinephrine induced release of PF4 and βTG but not acid hydrolases. (2) Low concentrations of thrombin caused release of PF4, βTG, and fibrinogen, but high concentrations were required for secretion of acid hydrolases. (3) Aspirin and indomethacin inhibited PF4, βTG, and fibrinogen release, but in vitro addition of aspirin caused minimal inhibition of acid hydrolase secretion. Secretion of protein by platelets also differed from release of 14C-5HT in that PF4, βTG, and fibrinogen were secreted to a greater extent than was 14C-5HT by low concentrations of thrombin, collagen, and U46619 (a structural analogue of prostaglandin H2), and arachidonic acid caused more extensive release of PF4, βTG, and fibrinogen than of 14C-5HT at all concentrations that induced release. When platelets were subjected to subcellular fractionation, specific radioimmunoassay techniques indicated that PF4, βTG, and fibrinogen were localized in the platelet α-granule fraction. The PDGF was localized to the α-granule fraction by the use of a bioassay. This localization was distinct from that of platelet acid hydrolases and dense granule components. Thus, there were three types of platelet granule populations, each with unique constituents and with characteristic patterns of release. In vitro release of α-granule proteins by ADP, epinephrine, and arachidonic acid was prevented by inhibition of platelet cyclooxygenase, and this suggested that release was dependent on oxygenation of arachidonic acid by cyclooxygenase. In contrast, release by collagen and thrombin was only partially mediated by this pathway, since high concentrations of these agents bypassed the inactivated cyclooxygenase and induced α-granule protein release. Initial studies were performed to determine whether in vivo release could be inhibited by aspirin. Plasma levels of PF4 and βTG in normal volunteers were decreased following aspirin ingestion, thus suggesting a contribution of cyclooxygenase-related platelet release to circulating levels of PF4 and βTG in normal subjects.

Abbreviations used: PF4, platelet factor 4; βTG, β-thromboglobulin; PDGF, platelet-derived growth factor; PRP, platelet-rich plasma; FPA, fibrinopeptide A; 14C-5HT, 14C-serotonin; HNA, heparin-neutralizing activity. Release and secretion are used interchangeably.

From the Department of Medicine, Columbia University College of Physicians and Surgeons, the Department of Medicine, Cornell University Medical College, and the Department of Medicine, New York Veterans Administration Hospital, New York, N.Y.

Submitted September 18, 1978; accepted December 1, 1978.

Supported in part by grants from the National Institutes of Health (HL-15486, HL-18828, HL-07029, and HL-21006), by the New York Heart Association, by the S.M. Louis Memorial Fund, and by the Veterans Administration. Dr. Kaplan is a recipient of a New York Heart Association Senior Investigatorship.

Address for reprint requests: K. L. Kaplan, M.D., Columbia University College of Physicians and Surgeons, Department of Medicine 8-512, 630 West 168th Street, New York, N.Y. 10032.

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DURING the platelet release reaction platelet granular contents are secreted into the extracellular medium in an energy-requiring process. PF4 (a heparin-neutralizing protein) and βTG are platelet-specific proteins that have recently been characterized and for which specific radioimmunoassays have been developed. They are secreted during the platelet release reaction in vitro and plasma levels of these proteins may provide an index of platelet release in vivo. Fibrinogen, which is found primarily in the plasma, can also be detected in washed platelets, and it has been shown to be released from platelets by thrombin. PDGF has not yet been chemically characterized, but it has been shown to be released from platelets.

Interpretation of the significance of plasma levels of PF4 and βTG would be facilitated if the mechanism of release and the effects of inhibitors on the release of these proteins were known. The platelet release reaction has previously been divided into release I (release of serotonin and nonmetabolize adenine nucleotides) and release II (release of acid hydrolases), which are distinguished on the basis of their differences in inducers, inhibitors, and rates of release, their metabolic requirements for release, and the subcellular localization of the released materials. Inhibitors that primarily affect release I are those such as aspirin and indomethacin that inhibit oxygenation of arachidonic acid by cyclooxygenase to form prostaglandin cyclic endoperoxides. These endoperoxides are converted to thromboxane A2, an important mediator of release.

The present studies have attempted to define the characteristics of in vitro release produced by different stimuli and the effects of inhibitors of platelet cyclooxygenase on in vitro release of the platelet proteins. They have also demonstrated the subcellular localization of PF4, βTG, PDGF, and fibrinogen in relation to the localization of serotonin and acid hydrolases. A major advantage of the new radioimmunoassay techniques is their applicability to measurement of in vivo release. Thus, along with the in vitro studies, the effects of cyclooxygenase inhibitors on in vivo release of PF4 and βTG were examined by comparing plasma levels of the proteins in normal individuals before and after ingestion of aspirin.

MATERIALS AND METHODS

Assay Procedures

PF4 and βTG were measured by radioimmunoassay as previously described. It is possible that subsequent technical improvements in the βTG and PF4 assays and blood collection techniques will alter the absolute levels of these materials determined in plasma, but the relative levels should remain similar to those reported. Standards for both the PF4 and βTG radioimmunoassays were compared with purified materials generously provided by Dr. D. S. Pepper. For determination of PF4 levels in normal plasma, the assay was modified by preincubation of all the reagents (except 125I-PF4) overnight, followed by addition of the radiolabeled tracer. After an additional 2-hr incubation at 4°C the bound tracer was separated from the free form. Under these conditions binding of the radiolabeled tracer did not reach equilibrium, and the sensitivity of the assay was thereby increased. With this modification the lower limit of sensitivity of the PF4 radioimmunoassay in plasma was 0.8 ng/ml. Fibrinogen was quantified using the radioimmunoassay for fibrinopeptide A (FPA) after treatment of samples with thrombin (1 U/ml) for 30 min at 37°C. Purified human thrombin (specific activity 2500 U/mg) kindly provided by Dr. D. Aronson was used. PDGF was measured by a bioassay using 3T3 cells as previously described. β-N-acetylglucosaminidase was measured as described by Holmsen et al. following an incubation period of 60 min. Total protein in subcellular fractions was estimated by absorbance at 280
nm. "C-5HT was determined in a liquid scintillation counter (Packard model 3320) using Instabray scintillation fluid (Yorktown Research, Hackensack, N.J.) as previously described.16

**Subcellular Fractionation Studies**

For each study two units of fresh ACD blood were obtained from the New York Blood Center. Each donor denied having taken any medication during the preceding 2 wk. Blood was drawn into a plastic pack system (Fenwal, 4R1709, 1-465, Deerfield, Ill.). Processing at 4°C was initiated within 50 min of blood collection.

Initial centrifugation was carried out at 1000 g for 10 min in a Sorvall RC-3 centrifuge. Rotor speed was brought up over 60 sec, thereby obviating the problem of formation of creases in the bags, with consequent trapping of red cells. Platelet-rich plasma (PRP) was expressed into the first satellite bag, and the bag containing residual erythrocytes and leukocytes was discarded. Prior to all subsequent centrifugation steps the satellite bags were blown taut with air, thus facilitating separation of the liquid phase from the pellet. The PRP was spun at 225 g (10 min) to eliminate remaining erythrocytes and leukocytes. PRP was then expressed into the second satellite bag, and the first bag was discarded. For every 10 g of PRP contained in the bag, 1 ml of citrate solution (38-mM citric acid, 75-mM sodium citrate) was added. The air-filled PRP-citrate bags were centrifuged at 2500 rpm (1600 g) for 10 min, and the platelet-poor plasma was discarded. The sedimented platelets were suspended in 3 ml of Tris-citrate (75-mM Tris, 100-mM KCl, 12-mM citrate, pH 6.4) by gentle massage of the bag. An additional 50 ml of Tris-citrate were added when suspension was complete.31 Following centrifugation at 2500 rpm (1600 g) for 10 min, the washing procedure was repeated. The final sediment was resuspended in a total of 18 ml of Tris-citrate containing the following protease inhibitors: 0.4-mM α-toluenesulfonic acid (Eastman Kodak, Rochester, N.Y.), Trasylol (1000 U/ml) (Sigma Chemical, St. Louis, Mo.), pepstatin (10 μg/ml) (Sigma), and 2-mM benzamidine HCl (Sigma). In one fractionation experiment the PRP was incubated with "C-serotonin. The platelets were homogenized three times using the nitrogen decompression technique (cell distribution bomb, Parr Instrument, Moline, Ill.) with 1200 psi at 1°C.31 The homogenate was distributed over six linear sucrose density gradients (30%-60% gradients with 5-mM EDTA, pH 7.4) and centrifuged for 2 hr at 200,000 g in a Beckman SW41 rotor. The fractions were collected, pooled,31 and stored at −50°C until assayed. The quantity of each of the substances under study in a given fraction was calculated as a percentage of the total amount of that substance recovered after ultracentrifugation. Relative specific activity was calculated as percentage substance in the fraction divided by percentage total protein in the fraction.

**Preparation of PRP and Gel-Filtered Platelets for In Vitro Release Studies**

Blood collected from normal volunteers through 19-gauge butterfly needles into plastic syringes was mixed with 4% (w/v) citrate in plastic tubes in a ratio of 9 parts to 1 part. The blood was centrifuged for 15 min at 150 g to prepare PRP. The PRP was applied to a column of Sepharose 2B, and gel-filtered platelets were eluted with Ca²⁺-free Tyrode’s buffer.16 "C-5HT [5-(2-'C)-hydroxytryptamine binoxalate (New England Nuclear, Boston, Mass.) labeling of gel-filtered platelets was performed as described by Valdorf-Hansen and Zucker.40 All in vitro release studies were performed in a Payton dual-channel aggregometer at 37°C with stirring at 900 rpm.

**Preparation of Plasma for Assay for PF4 and βTG**

Samples collected into plastic syringes through 19-gauge butterfly needles were mixed with 0.1 volume 4% citrate containing 0.01 M adenosine 0.02 M theophylline and Adenosine was obtained from Nutritional Biochemical, Cleveland, Ohio, and theophylline from Sigma and placed at 0°C. This anticoagulant mixture is a modification of that employed by Ludlam et al.31 The blood samples were centrifuged within 60 min of collection at 3000 g for 20 min at 4°C, the supernatant plasma was removed and centrifuged for 10 min at 46000 g at 4°C, and the supernatant was removed and frozen for radioimmunoassay for PF4 and βTG.

**Indomethacin Inhibition Studies**

Gel-filtered platelets suspended in Ca²⁺-free Tyrode’s buffer with 0.35% bovine serum albumin (reagent grade, Miles Chemical, Kankakee, Ill.) were used. For studies of indomethacin inhibition of thrombin-induced release, purified human thrombin was reconstituted and diluted with 0.9% saline.
Indomethacin (Sigma) was dissolved in absolute ethanol. Either indomethacin solution or an ethanol control was added to the platelet suspension in a volume of 0.5% of the platelet suspension and stirred for 1 min prior to the addition of thrombin. Somewhat higher concentrations of thrombin were required to induce release in the presence of 0.5% ethanol than in its absence. Samples were stirred with thrombin for 3 min. Thrombin was inactivated by addition of hirudin (Koch-Light, Colnbrook Bucks, England), 0.5 U/ml final concentration, and the aggregometer cuvettes were immersed in an ice bath. Cuvettes were centrifuged for 20 min at 3000 g at 4°C, and the supernatant was removed. An aliquot of the supernatant was used for determination of ³H-5HT, and the remainder was stored for radioimmunoassay for PF4, βTG, and FPA. Control experiments demonstrated that indomethacin in the concentrations used did not affect thrombin-induced FPA cleavage from purified fibrinogen in solution. Since the fibrinogen concentration in gel-filtered platelets (plasma fibrinogen remaining after gel filtration plus granule-bound platelet fibrinogen) is well below the Kₘ for fibrinogen, this substrate should be rate-limiting for FPA cleavage, and the amount of FPA cleaved should reflect the amount of fibrinogen available. Thus a lower concentration of FPA in the presence of indomethacin than in its absence implies inhibition of release of fibrinogen by indomethacin. Indomethacin inhibition of collagen-induced release was performed in the same manner as in the thrombin experiments, except that indomethacin in ethanol was diluted with Ca²⁺-free Tyrode’s solution prior to addition to the platelet suspension, and the release reaction was stopped by immersion of cuvettes in an ice bath. The collagen was an acid-soluble preparation obtained from Hormonchemie, Munich, West Germany. Dilutions were made in the pH-2.8 buffer supplied with the collagen.

Studies on Release Induced by Arachidonic Acid and U46619

These studies were performed using gel-filtered platelets suspended in Ca²⁺-free Tyrode’s buffer with 0.2% human serum albumin (Sigma). Potassium arachidate (Nu Check Prep, Elysian, Minn.) was diluted in 0.15-M saline. U46619, a structural analogue of PGH₂ [(15S)-hydroxy-11α, 9α-(epoxymethano)-prosta-5Z, 13E-dienoic acid], kindly provided by Dr. G. L. Bundy of the Upjohn Laboratories, Kalamazoo, Mich., was dissolved in absolute ethanol to a concentration of 10 mg/ml and stored at 4°C. Further dilutions were made with 0.15-M saline. In some experiments imipramine (Sigma) was added to the gel-filtered platelets in the aggregometer cuvette at a final concentration of 1.7 μM.

Studies with Aspirin in Normal Volunteers

Twelve normal laboratory personnel participated in the study. Ten subjects took 600 mg of aspirin (Norwich Pharmaceuticals, Norwich, N.Y., lot C-06027-Z) twice a day for 3.5 days. Blood samples were collected (as described previously for PF4 and βTG plasma levels and for platelet release studies) before aspirin, at ½ hr after aspirin, and after the final dose. Two additional subjects received a single dose of 600 mg of aspirin, and plasma levels of PF4 and βTG were measured before and after the aspirin. For in vitro release studies the platelet count of the PRP was adjusted to 250,000/μl with autologous platelet-poor plasma. The final concentrations of substances used to induce release were 2.5-μM ADP (Nutritional Biochemical) dissolved in 0.9% NaCl, 455-μM epinephrine (Parke-Davis adrenaline chloride 1/1000 solution for injection, used undiluted), collagen (4.2 μg/ml), U46619 (10 μg/ml), 0.66-mM arachidonic acid (used as ethanol solution of the free acid), and thrombin (0.25 U/ml). Two aliquots of PRP from each subject were frozen and thawed (dry ice–ethanol) three times for total platelet content of PF4 and βTG. A control incubation with saline was performed with each PRP sample, and release with the other agents was expressed as the difference between the PF4 and βTG content of the experimental samples and the saline control. Levels in the saline control did not change with aspirin administration.

Consent

All normal volunteers who participated in these studies gave informed consent. The protocol for this study was approved by the Human Investigation Committee of Columbia University.

Statistical Analysis

All comparisons of pre- and post-aspirin data were made using Student’s paired t test.
RESULTS

Subcellular Localization

Distributions of PF4, βTG, fibrinogen, PDGF, β-N-acetylg glucosaminidase, and 14C-5HT in the sucrose density-gradient fractions are shown in Fig. 1. The fractions obtained were comparable to those previously described by Broekman et al. Activity of the acid hydrolase β-N-acetylg glucosaminidase in relation to protein content was highest in fraction 4 (mostly mitochondria), whereas PF4, βTG, PDGF, and fibrinogen were most abundant in fraction 7 (α granules). 14C-5HT was concentrated in fraction 9 (dense granules). Thus the four secreted platelet proteins PF4, βTG, PDGF, and fibrinogen were located in subcellular fractions rich in α granules and not in fractions that contained lysosomal enzymes or in the dense granules containing serotonin.

Inhibition of α-Granule and Dense-Granule Release by Indomethacin

Since in vitro inhibition of cyclooxygenase has been shown to prevent release of dense granules but not release of lysosomal enzymes the effect of such inhibition on α-granule release was examined.

Fig. 1. Relative specific activities of PF4, βTG, fibrinogen, PDGF, β-N-acetylg glucosaminidase, and 14C-serotonin in each fraction from sucrose density-gradient fractionations of disrupted platelets are plotted versus the percentage recovered protein in each fraction. Values plotted are means of three platelet fractionations, except for 14C-serotonin, which was a single study. Mean total recoveries of PF4, βTG, and fibrinogen were 106.3 nmoles, 35.9 nmoles, and 9.5 nmoles, respectively, per fractionation.
Dose–response curves for thrombin-induced release of the α-granule proteins PF4, βTG, and fibrinogen and the dense-granule amine ^14C-5HT from gel-filtered platelets in the presence and absence of 50-μM indomethacin are shown in Fig. 2. Release of the three proteins and of ^14C-5HT was inhibited over the range of thrombin concentrations tested, with inhibition most prominent at intermediate concentrations and decreasing at 0.1 U/ml. A clear separation of release of the proteins and serotonin was not observed in these studies, apparently because of the presence of 0.5% ethanol.

Dose–response curves for collagen-induced release of PF4, βTG, and fibrinogen, as well as ^14C-5HT, from gel-filtered platelets in the presence and absence of 20-μM indomethacin are shown in Fig. 3. Protein release was greater than ^14C-5HT release at low concentrations of collagen, but about 80% release of all four

Fig. 2. Thrombin dose–response curves in absence (solid line) and presence (dash line) of 50-μM indomethacin. Ordinate indicates release as percentage of material in frozen and thawed samples after subtracting the content of the saline control; abscissa indicates thrombin concentration. All incubations of thrombin with platelets were for 3 min. Panel A shows βTG release, panel B shows PF4 release, panel C shows FPA levels, and panel D shows ^14C-serotonin release. Error bars: ± 1 SEM. Panels A and B are means of three experiments; panel C, two experiments; panel D, five experiments.
Fig. 3. Collagen dose–response curves in absence (solid line) and presence (dash line) of 20-μM indomethacin. Ordinate indicates release as percentage of material in frozen and thawed samples after subtracting the content of the saline control; abscissa indicates collagen concentration. All incubations of collagen with platelets were for 3 min. Panel A shows βTG release, panel B shows PF4 release, panel C shows fibrinogen release, and panel D shows 14C-serotonin release. Error bars: ± 1 SEM. All panels show means of three experiments.

substances was achieved with the highest collagen concentration tested (50 μg/ml). Indomethacin caused nearly complete inhibition of release of all four substances at low collagen concentrations and partial inhibition at higher concentrations. In the presence of indomethacin the difference in extent of release of the proteins and serotonin at low concentrations of collagen was no longer seen.

Release of α-Granule Proteins by Arachidonic Acid

Inhibition of α-granule protein release by indomethacin suggested that release was dependent on products of the cyclooxygenase pathway. This dependence was examined more directly by studying the release of PF4, βTG, and fibrinogen from gel-filtered platelets by arachidonic acid. Dose–response curves for arachidonic-acid-induced release of PF4, βTG, fibrinogen, and 14C-5HT are shown in Fig. 4. Release of the three proteins and 14C-5HT becomes maximal within a very narrow concentration range. The threshold concentrations of arachidonic acid for release of the α-granule proteins and the dense-granule amine were identical, but the extent of protein release was greater than the extent of serotonin release. This was also true when release was induced in the presence of 1.7-μM imipramine (data not shown). Detailed studies of the release of α-granule proteins by arachidonic acid will be described in a separate communication.43
Release of α-Granule Proteins by an Endoperoxide Analogue

Since release of α-granule proteins could be correlated with arachidonic acid transformation, the effect of the endoperoxide analogue U46619 on α-granule release was examined. Dose–response curves for release of PF4, βTG, fibrinogen, and $^{14}\text{C}$-5HT by U46619 are shown in Fig. 5. In contrast to the results with arachidonic acid, increasing amounts of U46619 resulted in increased release over a 10-fold range. Protein release exceeded serotonin release, and in the case of the endoperoxide analogue, release of protein was detected at a lower concentration of the stimulus (0.08 μg/ml) than was release of $^{14}\text{C}$-5HT (0.1 μg/ml). These differences were also seen when 1.7-μM imipramine was included in the incubation mixture. The time course of release of α-granule proteins by the analogue (at concentrations of 0.1 and 0.4 μg/ml) did not differ from the time course of release of serotonin (data not shown).

Effect of In Vivo Inhibition of Cyclooxygenase on α-Granule Proteins

Since α-granule proteins were released following stimulation by arachidonic acid and U46619, and the release of these proteins by thrombin and collagen could be partially blocked following in vitro addition of indomethacin, we decided to study the effects of inhibiting cyclooxygenase in vivo. Following the ingestion of aspirin by normal volunteers, the following measurements were made: (1) plasma level of PF4 and βTG; (2) platelet content of PF4 and βTG; (3) the ability of platelets from “aspirinized” donors to release PF4 and βTG in vitro in response to agents that induce the platelet release reaction. Table 1 demonstrates the mean plasma levels and mean platelet contents of PF4 and βTG in normal subjects prior to and following aspirin ingestion. The mean plasma level of βTG in normal subjects prior to and following aspirin ingestion. The mean plasma level of βTG showed a small but significant ($p < 0.02$) decrease 90 min after aspirin ingestion; 3½ days later the βTG levels were not different from normal. The mean plasma level of PF4 fell at 90 min and remained low at 3½ days, but the $p$ value for the decrease was 0.1 > $p > 0.05$. Ten of the 12 subjects showed decreases in plasma levels of PF4 90 min after aspirin ingestion. The mean platelet content of βTG increased significantly.
Fig. 5. U46619 dose–response curve. Ordinate indicates release as percentage of material in frozen and thawed samples after subtracting the content of the saline control; abscissa indicates concentration of U46619. All incubations of U46619 with platelets were for 3 min. Points are mean values of duplicate samples from a representative experiment. Black circles, ^3H-serotonin; white circles, $TG; black triangles, PF4; white triangles, fibrinogen.

after 3½ days of aspirin ingestion. Mean PF4 content increased by approximately the same amount as the increase in $TG, but because of the greater variation among subjects the change in mean content with aspirin was not statistically significant. As a control for a possible in vitro effect of aspirin, blood samples were collected from 10 additional normal volunteers and divided between the standard anticoagulant and the same anticoagulant with aspirin added. Levels of PF4 and $TG were not different between the two groups.

Table 2 shows the amount of PF4 and $TG release in citrated PRP prepared from the volunteers before and after aspirin ingestion. Release induced by ADP, epinephrine, and arachidonic acid was 90%–99% inhibited by aspirin ingestion. Collagen-induced release was significantly, although less completely, inhibited at both 90 min and 3½ days after aspirin ingestion. Thrombin-induced release of PF4 was significantly inhibited at both intervals, but the amount of $TG released by thrombin after 3.5 days was not significantly less than that released before aspirin because of wide scatter in the data. However, the total platelet content of $TG rose significantly during the period of aspirin administration, and if the thrombin release data were expressed as percentage of $TG released, the values would be as follows: before aspirin 69.1%, 1½ hr after aspirin 38.1%; 3½ days after aspirin 29.5%. Since the analogue U46619 does not require conversion by cyclooxygenase for its effect on platelets, it was anticipated that aspirin would not inhibit release by this agent, and no inhibition was found.
Table 1. Plasma Levels and Platelet Contents of PF4 and βTG Before and After Aspirin Ingestion in Normal Volunteers

<table>
<thead>
<tr>
<th></th>
<th>Before Aspirin (mean ± SEM)</th>
<th>1 ½ hr After 600 mg Aspirin (mean ± SEM)</th>
<th>After 600 mg Aspirin b.i.d. for 3 ½ Days (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma level (pmoles/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF4</td>
<td>0.16 ± 0.03</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>βTG</td>
<td>0.50 ± 0.05</td>
<td>0.39 ± 0.05</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td><strong>Platelet content (pmoles/10⁶ platelets)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF4</td>
<td>236 ± 21</td>
<td>255 ± 10</td>
<td>315 ± 10</td>
</tr>
<tr>
<td>βTG</td>
<td>230 ± 12</td>
<td>248 ± 12</td>
<td>317 ± 24</td>
</tr>
</tbody>
</table>

*Data analyzed by Student's paired t test. Aspirin was given to 12 normal volunteers, and blood for determination of plasma levels was collected at the times indicated. Only 10 of the subjects were tested at 3 ½ days. Duplicate samples for determination of platelet content were prepared from 10 subjects at each time.

**DISCUSSION**

Previous subcellular fractionation studies of platelets have indicated that serotonin and metabolically inert adenine nucleotides are contained in the dense granules and that platelet acid hydrolases are stored in organelles distinct from both the dense granules and the granules that have been called α granules.

Fibrinogen (identified by the staphylococcal clumping technique) has been reported to be in the α granules, as has platelet heparin-neutralizing activity (HNA). However, recent studies have indicated that proteins other than PF4 contribute to HNA.

Studies of release of PF4, βTG, and PDGF by thrombin and analysis of these materials in patients with storage pool disease have provided indirect evidence of their localization in α granules.

The results reported in this article using specific radioimmunoassays demonstrate that platelet factor 4, β-thromboglobulin, and fibrinogen are concentrated in

Table 2. PF4 and βTG Released in PRP Before and After Aspirin Ingestion

<table>
<thead>
<tr>
<th>Agent</th>
<th>Number of Subjects</th>
<th>Before Aspirin (mean ± SEM)</th>
<th>1 ½ hr After 600 mg Aspirin (mean ± SEM)</th>
<th>p (in comparison with before aspirin)</th>
<th>After 600 mg Aspirin b.i.d. for 3 ½ Days (mean ± SEM)</th>
<th>p (in comparison with before aspirin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (2.5 μM)</td>
<td>6</td>
<td>60.3 ± 5.3</td>
<td>1.6 ± 0.7</td>
<td>&lt;0.02</td>
<td>0.4 ± 0.2</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>41.1 ± 8.1</td>
<td>1.9 ± 0.6</td>
<td>&lt;0.01</td>
<td>2.2 ± 0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Epi (45 μM)</td>
<td>6</td>
<td>50.6 ± 2.5</td>
<td>5.0 ± 1.9</td>
<td>&lt;0.001</td>
<td>7.5 ± 1.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acid (0.66 mM)</td>
<td>7</td>
<td>33.3 ± 4.4</td>
<td>0.3 ± 0.1</td>
<td>&lt;0.001</td>
<td>1.1 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Collagen (4.2 μg/ml)</td>
<td>6</td>
<td>73.4 ± 7.3</td>
<td>16.1 ± 3.1</td>
<td>&lt;0.0001</td>
<td>16.1 ± 15.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Thrombin (0.25 U/ml)</td>
<td>9</td>
<td>44.0 ± 8.2</td>
<td>16.8 ± 5.3</td>
<td>&lt;0.05</td>
<td>15.9 ± 4.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U46619 (10 μM)</td>
<td>10</td>
<td>44.7 ± 5.5</td>
<td>48.6 ± 5.8</td>
<td>NS</td>
<td>56.4 ± 10.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Data analyzed by Student's paired t test. Subjects were excluded from analysis for any agent if less than 25% release occurred with that agent before aspirin. All PRP samples were adjusted to a platelet count of 250,000/μl with autologous platelet-poor plasma before release studies were performed.

**Picomoles per milliliter.**
platelet subfractions rich in α granules and not in those fractions rich in dense granules or the acid-hydrolase-containing organelles. PDGF, as measured by its activity in a bioassay, is concentrated in the same fractions as PF4, βTG, and fibrinogen. Whether each α granule contains all four proteins or there are subpopulations of α granules, each with its own protein or proteins, remains to be determined. It is tempting to speculate that other platelet proteins that may be secreted, such as the von Willebrand protein, factor V, and low-affinity platelet factor 4, are also located in the α granules. Concurrently with the fractionation studies reported here, Dr. D. Kaplan and associates have reported localization of PDGF, βTG, and fibrinogen to the α-granule fractions.

Previous studies of the release of the α-granule proteins have also been limited. ADP and collagen have been reported to induce release of PF4 antigen and βTG, whereas thrombin has been reported to induce release of PF4, βTG, and fibrinogen. Systematic parallel studies of release of the three proteins by these and other agents in vitro and of the effects of cyclooxygenase inhibition on in vitro and in vivo release have not been reported.

An interesting finding of the in vitro release studies described here is that there appear to be three types of platelet release rather than two. What has previously been called release II or α-granule release should perhaps be referred to as lysosomal enzyme release, since the lysosomal enzymes have been shown previously and in the present study to be localized in subcellular fractions different from the α granules. Lysosomal enzyme release is not induced by ADP or epinephrine, and it requires higher concentration of thrombin for induction than does dense-granule release. Also, lysosomal enzyme release by thrombin is minimally inhibited by in vitro cyclooxygenase inhibitors. In contrast, release of the three α-granule proteins PF4, βTG, and fibrinogen is induced by ADP and epinephrine and low concentrations of thrombin and collagen. Thrombin-induced release of α-granule proteins is blocked by cyclooxygenase inhibitors. PF4, βTG, and fibrinogen release also differs from dense-granule release as quantitated by 14C-serotonin release in that (1) there is greater percentage release of the proteins by low concentrations of thrombin and collagen, and the analogue U46619 and (2) there is less complete release of 14C-serotonin than of the proteins by arachidonic acid and U46619. Although it might be anticipated that these differences between α-granule release and dense-granule release would also be reflected by different time courses of release, these have not been found. In previous studies the time course of release of dense-granule components (14C-serotonin, ADP, and ATP) by thrombin (0.05 U/ml) was not different from the time course of release of PF4, βTG, and PDGF. Studies of the time course of release of PF4, βTG, and 14C-serotonin by collagen in citrated PRP also showed similar rates of release of these three components. The present study shows similar time courses of release for 14C-serotonin and the α-granule proteins by U46619. Further studies will be required to establish the basis for the observed differences between α-granule release and dense-granule release and to determine if different mechanisms are involved.

Inhibition of cyclooxygenase blocked α-granule protein release in vitro in the same manner as has been described for dense-granule components. In vitro release of PF4, βTG, and fibrinogen by ADP, epinephrine, and arachidonic acid
was completely inhibited by aspirin ingestion by the platelet donor, thus indicating that functioning cyclooxygenase is essential for release of the proteins by these agents. In contrast, protein release by thrombin and collagen was only partially inhibited in PRP from aspirin-treated volunteers. In studies with gel-filtered platelets, protein release by thrombin was partially inhibited, but the inhibition could be overcome at high thrombin concentrations, suggesting that at low thrombin concentrations the cyclooxygenase pathway contributes to release of $\alpha$-granule proteins, but at high concentrations other pathways can completely circumvent the blocked cyclooxygenase. It has been proposed that thrombin induces dense-granule release by three mechanisms—(ADP release, endoperoxide and thromboxane formation, and another pathway not yet defined$^{559}$), and the same may be true for $\alpha$-granule release by thrombin. Other authors have stated that ADP release is not a mechanism for thrombin induction of dense-granule release.$^{60}$ The question of ADP involvement in $\alpha$-granule release by thrombin requires study. With low concentrations of collagen, release of $\alpha$-granule proteins was completely inhibited by indomethacin, thus indicating that the cyclooxygenase pathway was required as for release induced by ADP, epinephrine, and arachidonic acid. At higher concentrations inhibition was partial, as with thrombin, and again at sufficiently high collagen concentrations inhibition could be almost completely overcome, again implying the involvement of other pathways.

The final question in these studies concerned the origin of the low levels of PF4 and $\beta$TG in plasma from normal individuals and the possible contribution of cyclooxygenase-dependent release to these levels. A major problem in interpreting any studies of plasma levels of PF4 and $\beta$TG is distinguishing between in vivo phenomena and in vitro release during blood collection and processing for radioimmunoassay. Measuring plasma levels of both proteins allows this distinction to be made. Previous studies from this laboratory,$^{16}$ as well as data on in vitro release and platelet content of the proteins from the normal volunteers in the present study, have shown that mean in vitro release of PF4 and $\beta$TG occurs in equimolar amounts and that their mean concentrations in platelets are equivalent. However, mean plasma levels of the two proteins in normal individuals are different, with $\beta$TG levels $3\frac{1}{2}$ times higher than PF4 levels ($p < 0.001$). Such a difference in plasma levels is consistent with equimolar release of PF4 and $\beta$TG but slower clearance of $\beta$TG from the blood.$^{61}$ If release occurred in vitro, equal levels of the two proteins would be expected. In normal individuals the mean plasma levels of both PF4 and $\beta$TG were lower 90 min after aspirin ingestion and after $3\frac{1}{2}$ days of aspirin than they were before aspirin, but only the initial decrease in $\beta$TG levels was statistically significant. These results suggest that most of the material measured in normal plasma appears by mechanisms not involving arachidonic acid metabolism, although a small fraction of normal circulating PF4 and $\beta$TG may be released by a cyclooxygenase-dependent pathway. In vitro inhibition of a small amount of release occurring during sample collection and preparation is unlikely, since aspirin added to the anticoagulant did not affect the levels. Also, the increases in total platelet content of the proteins with aspirin support an in vivo effect, since the higher total platelet contents could have resulted from decreased in vivo release because of the cyclooxygenase inhibition. The increase in total platelet content could also have resulted from an effect of aspirin on the synthesis or packaging of the proteins in
megakaryocytes. If aspirin does inhibit in vivo release, this could represent partial inhibition of release induced by thrombin or collagen or complete inhibition of release due to ADP, epinephrine, or arachidonic acid.

ACKNOWLEDGMENT

The authors would like to acknowledge many helpful discussions with Drs. H. L. Nossel and D. S. Goodman and Miss B. Linder.

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KL Kaplan, MJ Broekman, A Chernoff, GR Lesznik and M Drillings