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Subcellular localization and secretion of factor V from human platelets

(coagulation/collagen/prostaglandin I_2/platelet factor 4/aspirin)

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ABSTRACT **Factor V, a plasma protein cofactor necessary for optimal conversion of prothrombin to thrombin, is also present in considerable concentration in blood platelets (9.9 units per 10^9 platelets). Subcellular fractionation by two methods has localized factor V in the α granules of unstimulated platelets. ADP and epinephrine cause release of 4.6% and 6.4%, respectively, of the total factor V, a process completely inhibited by cyclooxygenase alkylation by aspirin. In contrast, collagen causes release of 25% of platelet factor V, a process only partially suppressed by aspirin. Secretion of factor V depends on the availability of metabolic energy, because antimycin A, an inhibitor of aerobic metabolism, and 2-deoxyglucose, an inhibitor of anaerobic glycolysis, together almost totally inhibited the secretion of factor V induced by collagen. The data establish that factor V is not normally available on unstimulated platelets but can be secreted from α granules upon stimulation with physiological agents such as ADP, epinephrine, and collagen. Because factor V is known to serve as a receptor for factor Xa, the exposure of factor V on platelets consequent to release would accelerate the process of blood coagulation.**

One year after the discovery of factor V in plasma, Ware *et al.* (1) recognized that platelets also contain an accelerator of prothrombin conversion. Though this coagulant activity was more stable to heat inactivation and storage than was plasma factor V, it was generally assumed to be the plasma factor adsorbed to the platelet surface (2, 3). In 1975, Breederveld *et al.* (4), using gel filtration to isolate platelets from plasma, found minimal activity of factor V associated with platelets but were able to increase the V activity by freeze–thawing. Østerud *et al.* (5) confirmed this observation and showed that platelets contain an activated form of factor V as well as a platelet activator of plasma factor V. Kane *et al.* (6) have presented convincing evidence that factor Xa binds to the platelet membrane at a binding site that is identical to or requires the presence of factor V.

Despite these important observations, several questions remain in evaluating the influence of platelet factor V on the hemostatic and possible thrombotic processes. What is the subcellular localization of factor V? Are prostaglandin synthesis and metabolic energy required for its release? Do collagen, ADP, and epinephrine release factor V as thrombin does? This study attempts to answer these questions.

MATERIALS AND METHODS

Bovine serum albumin (fraction V), ADP, epinephrine, and bovine fibrinogen (fraction 1, type IV, 93% clottable) were supplied by Sigma. Human fibrinogen (grade L, 95% clottable) was a product of Kabi (Stockholm, Sweden). Bovine thrombin was from Upjohn. Fibrillar equine tendon collagen (collagen reagent Horm) was obtained from Hormon-Chemie (Munich). Prostaglandin I_2 (PGI_2) was a gift of John Pike of Upjohn. Sepharose 2B was obtained from Pharmacia. Triton X-100 was purchased from New England Nuclear. Platelet factor 4 (PF4) radioimmunoassay kits were obtained from Abbott. All other chemicals were reagent-grade products of the best quality available.

Platelet Preparations. *Platelet-rich plasma (PRP).* Human blood from normal volunteers who had not ingested aspirin for 2 weeks was collected into an anticoagulant solution (1 part 3.8% sodium citrate to 9 parts whole blood) and centrifuged at 23°C for 20 min at 40 × *g*. Supernatant PRP was then removed by aspiration. Whole blood and plasma were exposed only to plastic surfaces.

Gel-filtered platelets (GFP). Platelets were filtered through Sepharose 2B by using a modification of the method of Tangen *et al.* (7). Three milliliters of PRP was applied to the top of a 1.7 × 17.5 cm column previously equilibrated with calcium-free Tyrode's buffer, pH 7.4, containing albumin (3.5 mg/ml). Fractions containing washed platelets appeared at the void volume of the column and were pooled. The platelet count was determined by phase-contrast microscopy. For aggregation and release studies human fibrinogen was added to give a final concentration of 1.67 mg/ml. In some studies the column was equilibrated and eluted with buffer containing PGI_2 (534 nM).

Subcellular Fractionation of Platelets. *Method 1:* Two hundred milliliters of whole blood was collected from a normal volunteer after informed consent had been obtained, and PRP was prepared. The PRP was incubated with 2-deoxyglucose (16.67 mM) and antimycin A (8.5 μg/ml) for 30 min at 37°C. EDTA was added to give a final concentration of 1 mM and the PRP was centrifuged at 3000 × *g* for 10 min at 4°C. The platelet pellet was washed three times with a barbital/NaCl buffer, pH 6.5, containing EDTA (1 mM) and the following inhibitors were added (final concentrations are given): *N*-carbobenzoxy-L-glutamyl-L-tyrosine (1 mM), phenylmethylsulfonyl fluoride (2 mM), benzamidine·HCl (10 mM), 2-deoxyglucose (16.67 mM), antimycin A (8.5 μg/ml). A fourth wash contained all the inhibitors except EDTA. The platelet pellet was then suspended in 0.02 M barbital buffer containing 0.15 M NaCl, pH 6.5, and including the above inhibitors and 0.25 M sucrose. The platelets were homogenized twice by the nitrogen decompression technique (8), using a Mini-Bomb Cell Disruption Chamber (Kontes Glass, Vineland, NY). The homogenate then underwent differential centrifugation according to the method of Fukami *et al.* (9) to obtain the following fractions: F_1 (1000 × *g* pellet), F_2 (12,000 × *g* pellet), F_3 (100,000 × *g* pellet), F_4 (100,000 × *g*

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Abbreviations: PRP, platelet-rich plasma; GFP, gel-filtered platelets; PF4, platelet factor 4; LA-PF4, low-affinity PF4; PGI_2, prostaglandin I_2.

supernatant). F_2 was resuspended in buffer and subjected to ultracentrifugation by a modification of the method of Marcus *et al.* (10), using a discontinuous gradient ranging from 30% to 60% sucrose (11). Five fractions were identified by their light-scattering properties and collected by aspiration. They were then dialyzed against barbital/NaCl buffer at 4°C for 6 hr with three changes to remove sucrose and inhibitors prior to assay for factor V, enzyme markers, and proteins. With this method 75% of the factor V activity and more than 90% of the protein and enzyme markers applied to the gradient were recovered.

Method 2: Platelets from a fresh unit of whole blood in acid/citrate/dextrose were obtained from the Baptist Memorial Hospital Blood Bank (Memphis, TN). The normal volunteers had taken no medication for more than 2 weeks prior to the study and gave informed consent for the procedure. The PRP was centrifuged at 8000 × *g* for 30 min at 4°C. Platelet buttons were then washed with 0.02 M barbital buffer containing 0.15 M NaCl, pH 7.4, until the supernatant contained no detectable factor V activity (four washes). The platelets were disrupted by the nitrogen decompression method (8) and subjected to ultracentrifugation, as above. Fractions obtained were identified by their light-scattering properties, collected by aspiration, and dialyzed. The recovery of factor V activity was 58% and that of the protein and enzyme markers was greater than 90% of that applied to the gradient.

The results obtained by both these methods are expressed as relative specific activity, calculated by dividing the activity recovered (%) by the protein recovered (%). β-Glucuronidase was assayed according to the method of Fishman *et al.* (12); β-*N*-acetylglucosaminidase, according to the method of Holmsen *et al.* (13); acid phosphatase, by the method of Linhardt (14); and protein, by the method of Lowry *et al.* (15).

Platelet Release Studies. Studies with human platelets were performed by stirring 0.5 ml of GFP in 12 × 75 mm plastic tubes, at 1200 rpm for 15 min at 37°C in the presence of the aggregating agent and fibrinogen.

PF4. Low-affinity PF4 (LA-PF4) was determined by radial immunodiffusion (16). PF4 was determined by radioimmunoassay.

Assay of factor V. This assay was performed by the one-stage method described by Lewis and Ware (17) as modified by Colman (18). One milliliter of normal human plasma (pool of 10 separate plasmas) was defined as containing 1 unit of factor V. The addition of Triton X-100 up to 0.1% or the presence of collagen did not alter the activity.

Inhibitor Studies. *Metabolic inhibition study.* GFP (0.3 ml) were preincubated with 1 μl of antimycin A (2.5 mg/ml in ethanol) and 10 μl of 2-deoxyglucose (500 mM) for 30 min at 37°C. This procedure, which depletes the platelet metabolic pool of ATP as previously reported by Holmsen and Day (19), was performed as described by Colman *et al.* (20).

Aspirin inhibition study. Two normal volunteers ingested 600 mg of aspirin (acetylsalicylic acid) 4 hr before venepuncture. PRP was gel filtered as described, with PGI_2.

Thrombin Activation of Factor V Released by Collagen. GFP (0.5 ml) were stirred with collagen (4 μg/ml) for 15 min and the supernatant containing released substances was separated by centrifugation. The supernatant (0.2 ml) was then incubated at 37°C with thrombin (0.2 ml) to give final concentration of 0.05 unit/ml. Aliquots were tested at 0, 1, 5, and 10 min in the one-stage assay for factor V. Fresh human plasma diluted 1:100 was tested in parallel.

RESULTS

Density Gradient Localization of Factor V. When platelets were subjected to subcellular fractionation by method 1, which includes a number of inhibitors of proteolytic enzymes, the membrane marker acid phosphatase showed the highest specific activity in the 100,000 × *g* pellet (fraction F_3, Fig. 1). In contrast, the highest relative specific activity of β-*N*-acetylglucosaminidase, β-glucuronidase, LA-PF4, and factor V emerged in the 12,000 × *g* pellet (fraction F_2). In the case of factor V, 68% of the activity appeared in fraction F_2. In an attempt to further separate the components of the granular fraction, F_2 was subjected to sucrose density ultracentrifugation (Fig. 2). The lysosomal markers β-glucuronidase and β-*N*-acetylglucosaminidase peaked in fractions B and C, respectively, while factor V and LA-PF4 had maximal activity in fractions D and E, respectively.

Though not shown, electron microscopy has been performed in our laboratory on the fractions obtained in Figs. 1 and 2. Results are identical to those reported by Fukami *et al.* (9). Subfraction D (Fig. 2) consisted primarily of α granules.

In order to confirm the separation of lysosomal enzymes from factor V, subcellular localization was also performed by method 2. No inhibitors were present. The lysosomal marker β-*N*-acetylglucosaminidase had its highest relative specific activity in fraction 6, whereas factor V and LA-PF4 peaked in fraction 8 (Fig. 3). By this method [^{14}C]serotonin, not shown, has peak concentration in fraction 9 (21).

With method 2 the recovery of factor V activity was only 58%. A lower pH and the presence of protease inhibitors in method 1 increased the recovery to 75%. Despite the difference in recovery by these two methods, factor V activity and LA-PF4 were

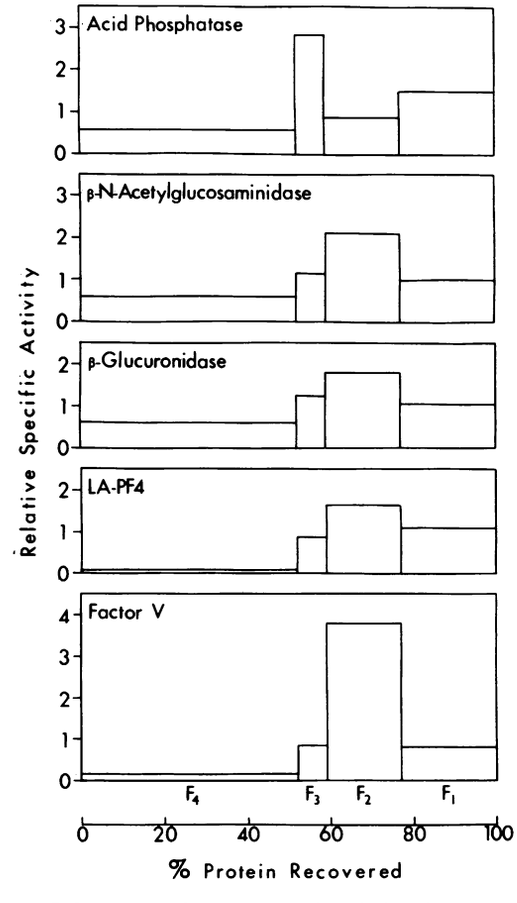

FIG. 1. Relative specific activities of acid phosphatase, β-*N*-acetylglucosaminidase, β-glucuronidase, LA-PF4, and factor V in disrupted platelets subjected to differential centrifugation to obtain the following fractions: F_1 (1000 × *g* pellet), F_2 (12,000 × *g* pellet), F_3 (100,000 × *g* pellet), F_4 (100,000 × *g* supernatant).

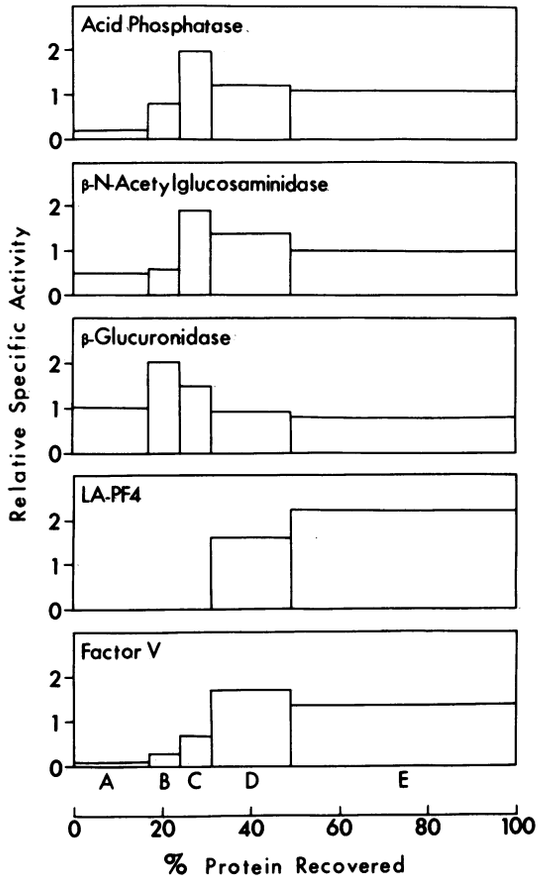

FIG. 2. Relative specific activities of acid phosphatase, β-*N*-acetylglucosaminidase, β-glucuronidase, LA-PF4, and factor V in fractions obtained when F_2 was resuspended and subfractionated by ultracentrifugation on a sucrose gradient (method 1). Fraction A represents the top and fraction E the bottom of the gradient.

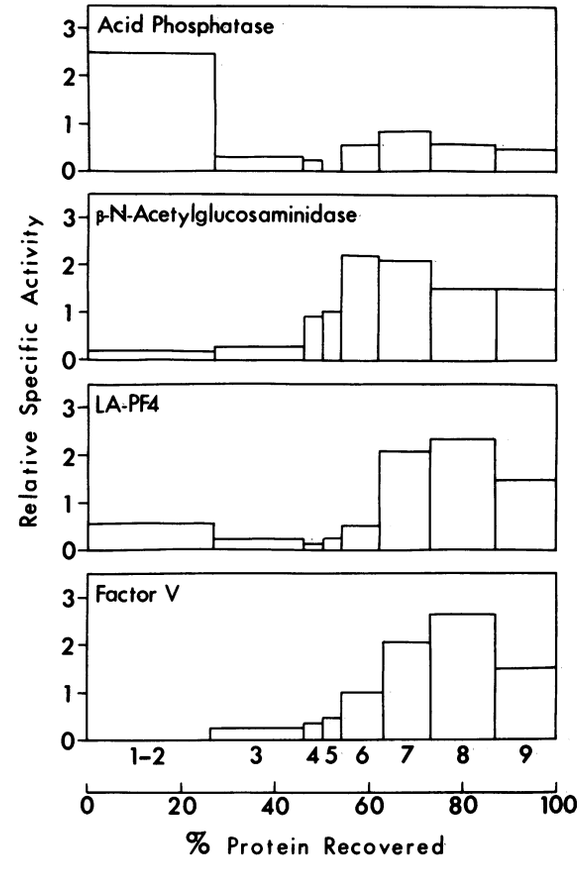

FIG. 3. Relative specific activities of acid phosphatase, β-*N*-acetylglucosaminidase, LA-PF4, and factor V in each fraction obtained when disrupted platelets were subjected to sucrose gradient ultracentrifugation (method 2). Fractions 1 and 2 at the top of the gradient were combined. The gradient was prepared by this method four times, with similar results each time.

in fractions of relatively high density, higher than the lysosomal enzymes or membrane marker.

Release of Factor V from GFP. Base line factor V activity in unstimulated platelets obtained by gel filtration was low (Table 1). Exposure of the platelet suspension to Triton X-100 at a final concentration of 0.1% increased the activity 14-fold and allowed solubilization of about 80% of the factor activity. ADP and epinephrine failed to release any factor V activity in excess of control, despite the usual release of serotonin. However, collagen released about 32% of the total factor V activity.

In another set of experiments PGI_2 (534 nM) was included in the elution buffer in order to prevent any stimulation of the platelets by the process of gel filtration. The concentration of PGI_2 was high in order to maintain inhibition throughout the experiment. The half-life of PGI_2 is about 20 min in plasma or buffer containing albumin, pH 7.5 (22). Platelets obtained in this manner showed normal aggregation with collagen in 1–2 hr and with epinephrine at 3–4 hr after elution. Under these conditions minimal factor V activity was present in the supernatant of unstimulated platelets (Table 2). When platelet suspensions were stirred with ADP or epinephrine, a small but definite amount of factor V activity was released. Greater amounts of factor V were released with collagen. PF4 was released in parallel.

Table 1. Release of factor V from GFP*

Sample	Factor V activity, units/10^9 platelets: Suspension	Factor V activity, units/10^9 platelets: Supernatant	Factor V release, % of total factor V†	Serotonin, % release
Control	0.415	0.268	7.3	7.0
ADP, 4 μM	0.438	0.254	6.7	42.8
Epinephrine, 25 μM	0.395	0.290	7.8	51.9
Collagen, 4 μg/ml	1.500	1.165	31.6	58.6
Triton, 0.1%	3.700	2.975	80.2	

* Average of four studies.

† Factor V release = [(factor V in supernatant)/(factor V in Triton suspension)] × 100.

It is of interest that Triton solubilization of these platelets resulted in factor V activity of 9.9 units per 10^9 platelets, whereas that of the platelets collected in the absence of PGI_2 was 3.7 units per 10^9 platelets. These data suggest that release of factor V occurred during gel filtration if PGI_2 was not included.

Inhibition of the Release of Factor V. When gel-filtered platelets were preincubated with antimycin A and 2-deoxyglucose prior to stimulation by collagen, the release of both factor V and LA-PF4 was diminished to less than 10% of the control value.

In order to evaluate the role of prostaglandin synthesis in the release of platelet factor V, platelets were exposed to aspirin *in vivo* (Table 3). Platelets were obtained from two donors prior to and 4 hr after ingestion of 600 mg of aspirin. Release of both factor V activity and PF4 by ADP and epinephrine was com-

Table 2. Release of factor V from GFP*

Sample	Factor V		PF4	
	Units/10^9 platelets	% release	μg/10^9 platelets	% release†
Control	0.07	0.7	0.145	1.0
ADP, 4 μM	0.46	4.6	2.07	13.8
Epinephrine, 25 μM	0.64	6.4	2.35	15.6
Collagen, 4 μg/ml	2.56	25.7	6.17	41.1
Triton, 0.1%	9.94		15.00	

Sepharose 2B was equilibrated with calcium-free Tyrode's buffer containing PGI_2 (534 nM) and eluted with the same buffer. For each sample, 0.5 ml of platelet suspension was stirred with the aggregating reagent in the presence of fibrinogen until aggregation was complete. The sample was then centrifuged at 12,000 × *g* for 2 min at 23°C and supernatant was assayed.

* Average of four studies.

† PF4 release = [(PF4 in supernatant)/(PF4 in Triton sample)] × 100.

pletely inhibited with aspirin. However, release by collagen was only partially inhibited.

Activation of Factor V Released from Platelets. The activity of factor V released by collagen may reflect an increase in factor V protein secreted. Alternatively, the factor V activity might reflect the activation of factor V by proteolytic enzymes during or after the release reaction.

Human plasma factor V was activated 9.3-fold by thrombin at 0.05 unit/ml. Under the same conditions factor V released by collagen from GFP was activated 11.2-fold (1.10 to 12.34 units per 10^9 platelets). Maximal activation was reached in 5 min for factor V from plasma and in 10 min for factor V from platelets. These results suggest that virtually all of factor V in platelets is released in a procoagulant form.

DISCUSSION

When platelets were disrupted and subjected to differential centrifugation, the maximal relative specific activity of factor V, LA-PF4, and the acid hydrolases β-glucuronidase and β-*N*-acetylglucosaminidase was in the granule fraction, whereas that of acid phosphatase appeared in the membrane fraction. Further separation of the granule fraction by isopycnic centrifugation on a sucrose gradient revealed factor V and LA-PF4 to be maximal in a denser fraction of the gradient than the acid hydrolases. The acid hydrolases are not located in the α granules (23, 24), whereas the heparin-neutralizing proteins (25, 26), fibrinogen (27), fibronectin (28), von Willebrand factor (29), and platelet-deprived growth factor (26) are. Our data suggest that factor V is also an α granule constituent.

In the present study factor V activity was found to be associated with intact GFP, thus confirming the results of others (4, 5). This factor V activity most likely reflects a small degree of activation of the platelet due to gel filtration, which can be suppressed by including PGI_2 in the gel filtration buffer. Salzman *et al.* (30) have shown stimulation of prostaglandin synthesis with exposure of platelets to Sepharose.

Previously, we (31) and others (5) reported no detectable release of factor V activity from GFP by ADP or epinephrine. Vicic *et al.* (32), using platelets separated from plasma by albumin density gradient centrifugation, noted a small but significant release of factor V by these agents. Our present data show a small but definite release of factor V by ADP and epinephrine when platelet aggregation returns to normal after filtration in the presence of PGI_2. When PGI_2 is omitted from the buffer, the factor V releasable by ADP and epinephrine is apparently released during the process of gel filtration. In contrast to ADP and epinephrine, which released 5% and 6% of the total factor V activity, respectively, collagen released 25%. PF4, an α granule marker, was released in parallel with factor V activity in response to all three agents tested, further supporting the subcellular localization of factor V.

Total factor V protein is difficult to assess by activity measurements. Triton itself does not activate factor V. However, it may solubilize activators of factor V as well as factor V protein. Ittyerah *et al.* (33) showed proteolytic alteration of bovine factor V during Triton solubilization of supernatant obtained after collagen stimulation. Although only a small decrease of molecular size was noted, a major change in migration was detected by immunoelectrophoresis. Similar changes occur in LA-PF4 after release, where a loss of amino acids results in a new peptide, β-thromboglobulin, with a change in pI of about 1.0 pH unit (34).

The factor V released by collagen is capable of further activation by thrombin. Because the activation quotient is very similar to that of plasma V, which is not in activated form, our study demonstrates that factor V released by collagen is mainly in the procoagulant form. Similar results have been reported by Kane *et al.* (35), using thrombin and sodium arachidonate to release factor V.

The total concentration of factor V in human platelets (9.9 units per 10^9 platelets) is higher than that detected immunochemically in bovine platelets (0.125 units per 10^9 platelets) (36). This finding could be due to species differences. The activity measurements in human platelets obtained by Kane *et al.* after freeze–thawing (3.5 units per 10^9 platelets) are also similar to the levels we observe in platelets prepared in the absence of PGI_2 (3.7 units per 10^9 platelets). Miletich and colleagues (6, 37, 38) have presented evidence that platelet factor V is required for the binding of factor Xa to platelets that have been stimulated by thrombin. Because factor V is detectable only in minimal concentration in the unstimulated platelet, these findings suggest that platelet stimulation may be required to release factor V from an intracellular location.

Data obtained from the release experiments in the presence of metabolic inhibitors are also consistent with a granular localization and a true release reaction. Preincubation of the GFP with antimycin A, which inhibits oxidative phosphorylation, and 2-deoxyglucose, which blocks anaerobic glycolysis, decreased the collagen-induced release of factor V and LA-PF4 to 10% of the control value.

The dependence of the release of factor V from platelets on prostaglandin synthesis was investigated by observing the effects of aspirin *in vivo* on two subjects. Inhibition of cyclooxygenase totally inhibited release of factor V from platelets by ADP and epinephrine. Similar inhibition of the α granule constituent PF4 was observed. Collagen-induced release of factor V and PF4 from platelets was only partially inhibited, indicating a lack of absolute requirement for intermediates of prostaglan-

Table 3. Inhibition of factor V release by aspirin

Sample	Factor V activity, units/10^9 platelets		PF4, μg/10^9 platelets	
	Before aspirin	After aspirin	Before aspirin	After aspirin
Control	0.06	0.12	0.14	0.14
ADP, 4 μM	0.48	0.16	2.00	0.19
Epinephrine, 25 μM	0.79	0.13	4.78	0.21
Collagen, 4 μg/ml	2.64	0.82	5.38	3.26

Platelets from two volunteers were tested before and 4 hr after ingestion of 600 mg of aspirin. Platelets were eluted in the presence of PGI_2 (534 nM) as described for Table 2.

din synthesis. Thus the enhancement of coagulant activity by factor V would not be expected to be fully blocked by aspirin.

This study has attempted to define the subcellular localization and requirements for secretion of factor V in human platelets. The data show that factor V is localized to an α granule fraction in the unstimulated platelet and hence is not available to participate in the hemostatic process. Collagen stimulates the release of platelet factor V by a mechanism that requires metabolic energy but has only a partial dependence on the prostaglandin pathway. ADP and epinephrine release only about one-fifth as much factor V activity as collagen, and the process is inhibited by aspirin. Thus collagen, which may be exposed after endothelial injury, can accelerate the coagulation process by the release of factor V. The released platelet factor V bound to the platelet membrane might then be available for the binding of factor Xa (38). Factor V would facilitate prothrombin conversion to thrombin at the platelet surface, serving as an important accelerator of either physiologic hemostasis or pathologic thrombosis.

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