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Characteristics of collagen-induced fibrinogen binding to human platelets

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Polymerized type I calf skin collagen induced a time-dependent specific binding of ^{125}I-fibrinogen to washed human platelets. Binding occurred more rapidly in a shaken rather than in an unstirred system. It was linear in the range 0.05–0.3 μM added fibrinogen and was saturated at higher fibrinogen concentrations (more than 0.8 μM). Scatchard analysis showed a single population of binding sites (16530 $\pm$ 5410 per platelet) with a K_d = 0.53 $\pm$ 0.23 μM. Collagen-induced ^{125}I-fibrinogen binding to platelets was completely inhibited by ADP antagonists such as creatine phosphate/creatine phosphokinase and AMP, and partially inhibited by pretreatment of the platelets with aspirin. With both normal and aspirin-treated platelets a close correlation was observed between the amount of ^{125}I-fibrinogen bound and the extent of dense granule secretion. Our results confirm that fibrinogen becomes bound to platelet surface receptors during collagen-induced platelet aggregation and suggest that secreted ADP is an essential cofactor in this process.

Introduction

Fibrinogen is a cofactor for the aggregation of washed human platelets by ADP (for a review see Ref. 1) and the ability of ADP to expose fibrinogen receptors on the platelet surface has been well documented [1–6]. More recently, binding of fibrinogen has also been demonstrated following platelet stimulation by thrombin [7,8], arachidonic acid [9,10], prostaglandin endoperoxides and thromboxanes [11,12], and epinephrine [3,13,14]. Conflicting evidence was obtained regarding the role of ADP in fibrinogen receptor expression induced by these agonists. The binding of fibrinogen to thrombin-stimulated platelets was entirely attributed to the release of intracellular ADP [7]. But, whereas arachidonic acid induced fibrinogen binding to washed human platelets was found to be ADP-dependent [10], a normal binding occurred to thrombin-degranulated rabbit platelets [9]. Furthermore, while prostaglandin H_2 has been claimed to expose directly fibrinogen receptors on human platelets [11], results obtained with two stable prostaglandin endoperoxide analogues (9,11 azo PGH_2 and U 46 619) demonstrated an effect mediated through the release of platelet ADP [12].

One of the most physiologically important inducers of platelet aggregation is collagen. Recently a commercial preparation of equine collagen was found to induce also fibrinogen binding to platelets [10]. This binding was partially inhibited by apyrase, an enzyme which degrades ADP to AMP. However, it was not established whether collagen-induced fibrinogen binding was dependent on the release of ADP from dense granules or whether

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

small amounts of exogenous ADP were synergistically potentiating the stimulation of platelets. The latter mechanism has been previously proposed for collagen-induced platelet aggregation [15,16].

In the present study, we have used polymerized calf skin type I collagen (i) to investigate whether collagen-induced fibrinogen binding to platelets correlated with dense body secretion, and (ii) to define more precisely the mechanism whereby collagen induces fibrinogen receptor expression. With this aim, the role of ADP was assessed using apyrase, creatine phosphate/creatine phosphokinase, ATP and AMP, all substances which have been previously shown to inhibit ADP-induced platelet aggregation and fibrinogen binding [1,4,6].

Materials and Methods

Commercial materials

Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylene diamine (TEMED), ammonium persulfate, sodium dodecyl sulphate (SDS) and 2-mercaptoethanol were from BioRad (Richmond, CA, USA). Apyrase (grade I), bovine serum albumin (fraction V), Hepes, ATP (disodium salt), ADP (sodium salt, grade I), AMP (sodium salt type III) and prostaglandin E_1 were from Sigma Chemicals Co. (St. Louis, MO, USA). Human fibrinogen (grade L) was from Kabi (Stockholm, Sweden); it was further purified by gel filtration on gelatin-ultrogel purchased from LKB (Bromma, Sweden). Creatine phosphate and creatine phosphokinase were from Boehringer (Mannheim, F.R.G.). Chlorimipramine was from Ciba Geigy (Basel, Switzerland). Sodium acetylsalicylate was from Theraplix (Paris, France). 5-hydroxy [^{14}C]tryptamine creatinine sulphate (50 mCi/mmol) was from the Radiochemical Center (Amersham, UK). Sodium chromate (^{51}Cr, 114 mCi/mg) was from the Commissariat à l'Energie Atomique (Orsay, France). Unisolve was from Koch Light Laboratories Ltd. (Colnbrooks, Berks., UK). Phthalatedibutylester and phthalate-bis(2-ethylhexylester) were obtained form Aldrich-Chemie (Steinheim, F.R.G.), Other chemicals used were of analytical grade.

Extraction and purification of type I collagen

Type I calf skin collagen was extracted with 0.1 M acetic acid (pH 3.5) and purified as previously described [17]. Its purity was assessed as being at least 95% by amino acid analysis, by polyacrylamide gel electrophoresis using the conditions of Furthmayr and Timpl [18] and by agarose chromatography [19]. In a typical experiment, 1 mg of purified lyophilized material was dissolved in 0.1 ml of 0.1 M acetic acid, the solution was then diluted 1 : 10 in 0.01 M phosphate/0.15 M NaCl buffer (pH 7.4). The pH of the suspension was adjusted to 7.4 with NaOH. The collagen was polymerized into fibrils by heating the suspension at 33°C for 5 min just before use.

Preparation of platelet suspensions

Blood was collected by venipuncture from healthy drug-free volunteer donors into acid-citrate-dextrose (0.8% w/v citric acid/2.2% w/v trisodium citrate/2.45% w/v dextrose) at a ratio of 1 vol. anticoagulant: 6 vol. blood. The platelet rich plasma was obtained by centrifuging the blood at 120 g for 15 min at 20°C and immediately mixed with 25 μg/ml apyrase (10 units/mg protein). In some initial experiments the platelets were then incubated for 30 min at room temperature with 100 μCi of ^{51}Cr per ml plasma. In most studies the platelet-rich plasma was incubated with 0.6 μM [^{14}C]5-hydroxytryptamine for 30 min at 37°C. Occasionally, this incubation was performed in the additional presence of 0.5 mM sodium acetylsalicylate. Prior to platelet sedimentation, the platelet rich plasma was acidified to pH 6.5 with acide-citrate-dextrose and 100 nM prostaglandin E_1 was added. Centrifugation was performed at $1200 \times g$ for 15 min. The resulting pellet was resuspended in washing buffer which was slightly modified from that of Patscheke [20] and which consisted of 36 mM citric acid/103 mM NaCl/5 mM glucose/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/25 μg/ml apyrase/100 nM prostaglandin E_1 and 3.5 mg per ml bovine serum albumin (pH 6.5). The platelets were washed three times. Centrifugation was performed at $1200 \times g$ for 15 min at 20°C. The washed platelets were finally resuspended at $5 \cdot 10^8$ platelets/ml in Tyrode buffer [21] containing 5 mM Hepes/2 mM $CaCl_2$/1 mM $MgCl_2$/3.5 mg per ml bovine serum albumin (pH 7.4) (Tyrode-albumin). To eliminate the effect of the prostaglandin E_1, the washed

platelets were left at room temperature for 10 min before use (see Results). When the release reaction was studied, chlorimipramine was added to a final concentration of $3 \cdot 10^{-6}$ M to prevent reuptake of the released serotonin. Release was estimated in samples (0.2 ml) mixed with 0.04 ml ice-cold 0.1 M EDTA and immediately centrifuged at $12000 \times g$ for 1 min in an Eppendorf centrifuge (Model 5414, Brinkman Instruments Inc., Wesbury, NY, U.S.A.). The supernatants (0.2 ml) were mixed with 4 ml Unisolve, and counted for ^{14}C radioactivity in an Intertechnique SL 4000 liquid scintillation spectrometer (Kontron, France).

Platelet aggregation

Platelet aggregation was followed spectrophotometrically either at 20°C in a Labintec aggregometer (Montpellier, France) or at 37°C in a Lumi-aggregometer (Chrono Log Corp., Havertown, PA, U.S.A.). The instruments were adjusted for 0 and 100% light transmission with the platelet suspension and the Tyrode-albumin buffer, respectively. For ADP-induced platelet aggregation, aliquots (0.4 ml) of washed platelets at $2.5 \cdot 10^8$ platelets/ml were stirred at room temperature in a siliconized glass cuvette. After 1 min, 20 μl of fibrinogen (4 mg/ml) was added, and this was immediately followed by the ADP. With collagen, aggregation was studied at 37°C without added fibrinogen. Changes in light transmission were continuously recorded. The release of serotonin was measured 3 min after the addition of ADP and 5 min after the addition of collagen.

Fibrinogen binding experiments

^{125}I-labeled fibrinogen was obtained from the Service des Radioisotopes of the Centre National de Transfusion Sanguine (Paris, France). The fibrinogen, purified by the method of Jaques [22], had been radiolabeled using a modification of the chloramine T procedure [23]. The radioactivity in a typical ^{125}I-fibrinogen preparation was at least 96% precipitable in 20% wt./vol trichloracetic acid and the protein was at least 94% clottable by thrombin. The specific activity of different batches varied between 200 and 300 μCi/mg protein. The labeled fibrinogen was diluted 5-fold in Tyrode-albumin buffer and stored in small aliquots at −80°C as a 0.4 mg/ml solution. The fibrinogen was thawed at 37°C immediately before use and centrifuged 10 min at $12000 \times g$ as a precaution to eliminate any aggregates. The purity of each ^{125}I-fibrinogen preparation was verified by SDS-polyacrylamide gel electrophoresis using 7–12% gradient acrylamide slab gels [24]. Samples were electrophoresed after reduction with 5% (vol/vol) 2-mercaptoethanol. Autoradiography of the dried gel showed three bands corresponding to the Aα, Bβ and γ chains of fibrinogen, and no bands in the positions of reduced Von Willebrand factor and cold insoluble globulin.

Unlabeled Kabi fibrinogen was dissolved at 20 mg/ml, dialyzed overnight against 0.05 M Tris/0.25 M NaCl (pH 7.2) and clarified by centrifugation at $12000 \times g$ for 15 min. Protein concentration was measured spectrophotometrically using an extinction coefficient $E_{0.1\%}^{280\,nm}$ of 1.51. The fibrinogen was stored in small aliquots at −80°C. Coagulability of these preparations by purified α-thrombin was at least 95%. In some experiments, Kabi fibrinogen was further purified by chromatography on gelatin Sepharose. ^{125}I-fibrinogen and unlabeled fibrinogen supported ADP-induced platelet aggregation to the same extent and behaved identically in binding studies as judged by competitive inhibition experiments.

In the fibrinogen-binding assay, platelet suspensions (0.5 ml, $2.5 \cdot 10^8$ platelets) were incubated at room temperature with 0.03 ml ^{125}I-fibrinogen (0.05–1.2 μM), 0.03 ml Tyrode-albumin buffer and 0.03 ml ADP or collagen at the concentrations stated in the text and figure legends. The incubations were performed in a metabolic water bath (Laboratory Thermal Equipment, Greenfield Oldham, U.K.) either without shaking or at 60 rotations per min. When used, ATP, AMP or creatine phosphate/creatine phosphokinase were added 1 min before the collagen in place of the Tyrode-albumin buffer. At different time intervals, triplicate samples (0.12 ml) were each layered onto 0.5 ml of a 1.25 : 1 (vol/vol) mixture (d = 1.014) of phthalatedibutylester and phthalate-bis-(2-ethylhexylester) and immediately centrifuged at $12000 \times g$ for 2 min in an Eppendorf centrifuge. As verified using platelets labeled with ^{51}Cr, these conditions allowed a 95% recovery of the platelets at the bottom of the tubes. This applied to unstimulated as well as ADP- or collagen-activated

platelets. After sedimentation, the supernatants were removed by aspiration and the ^{125}I-radioactivity associated with the platelet pellets was measured in a Gamma 7000 counter (Beckman Instr. Inc., Fullerton, CA, U.S.A.). Controls were also performed with collagen and ^{125}I-fibrinogen in the absence of platelets. Non-specific binding to platelets was defined as that observed in the presence of a 50-fold excess of unlabeled fibrinogen. It was usually the same as that observed with ^{125}I-fibrinogen in the absence of ADP or collagen and represented less than 15% of total binding. Agonist-induced specific binding of ^{125}I-fibrinogen was calculated by subtracting the nonspecific binding from the total value and was expressed as microgram fibrinogen bound by $1 \cdot 10^9$ platelets or molecules of fibrinogen bound per platelet.

Analysis by SDS-polyacrylamide gel electrophoresis and autoradiography of platelet associated ^{125}I after ADP- or collagen-induced ^{125}I-fibrinogen binding to platelets, revealed that the platelet-bound fibrinogen retained the same profile as the added ^{125}I-fibrinogen.

Results

In agreement with the fact that the elevation of platelet cyclic AMP by prostaglandin E_1 during platelet isolation may be rapidly and totally reversed [25], the sensitivity of the platelets to aggregating agents was fully recovered within 10 min after their resuspension in Tyrode-albumin buffer. Fig. 1 illustrates the aggregation response of the washed platelets to low dose ADP in the presence of added fibrinogen and to polymerized type I collagen added alone. Both ADP-induced platelet aggregation and subsequent fibrinogen-binding experiments were performed at room temperature. Collagen-induced platelet aggregation was associated with extensive dense granule release and was studied at 37°C. ADP-induced platelet aggregation occurred without granule release. The characteristics of ADP-induced ^{125}I-fibrinogen binding to platelets prepared as in the present study have been recently described [6]. Binding reached saturation in a nonstirred system within 20 min (the $T_{1/2}$ was between 2 and 5 min). After 30 min, a single population of binding sites ($18\,120 \pm 6850$ sites per platelet) with a $K_d = 0.49 \pm 0.17$ μM were occupied (Table I).

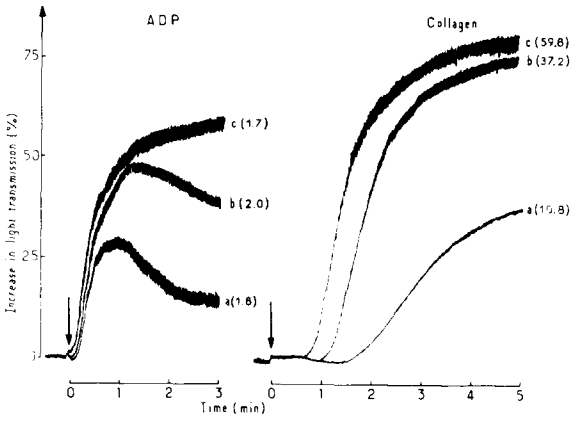

Fig. 1. Aggregation of the washed platelet preparations by ADP and collagen. For ADP-induced platelet aggregation, platelet samples ($2.5 \cdot 10^8$/ml) were stirred (1100 rpm) at room temperature in the presence of 0.2 mg/ml fibrinogen. ADP was added at a final concentration of (a) 1 μM, (b) 2 μM and (c) 5 μM. Changes in light transmission were continuously recorded. [^{14}C]serotonin release was measured after 3 min (values in parentheses). Collagen-induced platelet aggregation was measured at 37°C without the addition of fibrinogen. Collagen was added at final concentrations of (a) 6.25 μg/ml, (b) 12.5 μg/ml and (c) 50 μg/ml. [^{14}C]serotonin release was measured after 5 min (values in parentheses). The results of a typical experiment are shown.

As shown in Fig. 2, collagen also induced a time-dependent association of ^{125}I-fibrinogen to the washed platelets. However, binding was much slower than with ADP and it was maximal and reached saturation within the 30 min time scale of the experiment only when the suspensions were shaken. Shaking was performed at 60 rotations per min in a rotating water bath, conditions that did not lead to aggregate formation as verified by phase contrast microscopy (not illustrated). Controls performed in the absence of platelets showed that if collagen-^{125}I-fibrinogen complexes were formed, they did not penetrate the layer of inert oil used to separate the platelets from the reaction medium. When platelets were stimulated by collagen in the presence of ^{3}H-inulin, only 0.14% of the ^{3}H radioactivity cosedimented with the platelets. This was approx. 20 times less than the radioactivity associated with platelets stimulated in the presence of ^{125}I-fibrinogen. The nonspecific binding estimated in the presence of ^{125}I-fibrinogen and a 50-fold excess of unlabeled fibrinogen al-

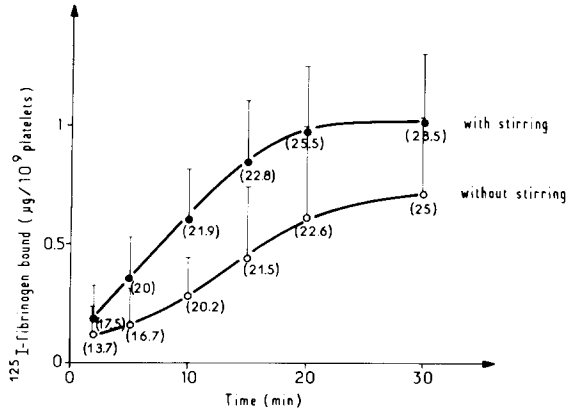

Fig. 2. Time-course of collagen-induced ^{125}I-fibrinogen binding to platelets. Platelet samples ($5 \cdot 10^8$/ml) were incubated at room temperature with 20 μg/ml ^{125}I-fibrinogen and 50 μg/ml collagen without (○) or with (●) mild agitation (60 rpm). At the indicated times, triplicate samples were taken and the platelets sedimented through a layer of inert oil (see Methods). The amounts of ^{125}I in each pellet was measured. Specific binding curves are shown and these were derived by subtracting the binding observed in the presence of a 50-fold excess of unlabeled fibrinogen from the total ^{125}I-fibrinogen binding. Results are the mean values with the half standard deviation (vertical bars) of six separate experiments. The percentage of [^{14}C]serotonin release (values in parentheses) was determined in parallel using separate samples processed identically but using unlabeled fibrinogen. Here, the mean value only is given.

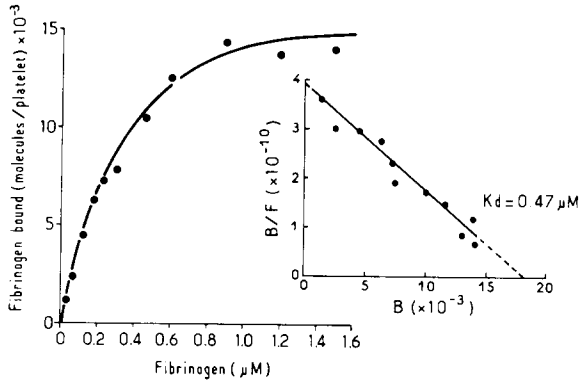

Fig. 3. Equilibrium binding of fibrinogen to platelets stimulated by collagen. Platelet samples ($5 \cdot 10^8$/ml) were incubated at room temperature with mild agitation in the presence of 50 μg/ml collagen, ^{125}I-fibrinogen and increasing concentrations of unlabeled fibrinogen. Fibrinogen binding was assessed after 30 min as described in Fig. 2. The specific binding curve obtained from a typical experiment is shown and the Scatchard plot of the data is inserted. The data were analyzed by linear regression, and the K_d value was determined from the slope of the line.

ways represented less than 15% of the total binding.

Features of collagen as compared to ADP-induced ^{125}I-fibrinogen binding to platelets were (i) the presence of a lag phase, especially when the suspension was not agitated and (ii) the fact that it was always accompanied by [^{14}C]serotonin release (Fig. 2) *. At equilibrium, with added fibrinogen concentrations in the range 0.05–0.3 μM, collagen-induced fibrinogen binding was linear and receptors were saturated at the higher concentrations (Fig. 3). Scatchard analysis showed the presence of a single population of binding sites (16 530 $\pm$ 5410 per platelet) with a $K_d = 0.53 \pm 0.23$ μM. These values were similar to those obtained with the same platelet preparations stimulated by ADP (Table I).

Fig. 4 shows how collagen-induced ^{125}I-fibrinogen binding was inhibited by creatine phosphate/creatine phosphokinase and AMP at con-

TABLE I

FIBRINOGEN-BINDING TO ADP- AND COLLAGEN-STIMULATED PLATELETS

Platelets from each donor were stimulated with ADP (10 μM) or collagen (50 μg/ml) in the presence of 0.12 μM ^{125}I-fibrinogen and increasing concentrations of unlabeled fibrinogen. Data were derived from the Scatchard analysis of binding isotherms performed. Results are the mean $\pm$ S.D. of those obtained for seven different platelet preparations.

Agonist	Number of receptors	Dissociation constant K_d (μM)
ADP (10 μM)	18 120 $\pm$ 6850	0.49 $\pm$ 0.17
Collagen (50 μg/ml)	16 530 $\pm$ 5410	0.53 $\pm$ 0.23

* Although α-granule secretion would result in the appearance of platelet fibrinogen in the medium the amounts secreted under our experimental conditions (see Discussion) would be insufficient to interfere with the estimation of the number of fibrinogen binding sites as measured with much higher concentrations of ^{125}I-fibrinogen. In agreement with this conclusion we have obtained identical results to those presented in Fig. 2 in studies on platelets of patients lacking α-granule proteins [46].

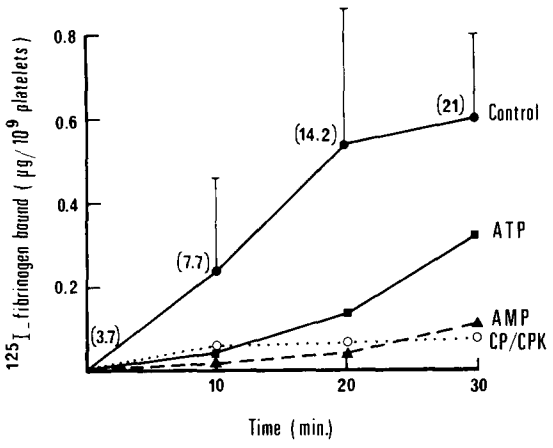

Fig. 4. Inhibition of collagen-induced ^{125}I-fibrinogen binding to platelets. Platelet samples ($5 \cdot 10^8$/ml) were agitated at room temperature with 20 μg/ml ^{125}I-fibrinogen and 50 μg/ml collagen. The specific ^{125}I-fibrinogen binding to platelets and [^{14}C]serotonin release were determined as described in the legend to Fig. 2. The substances indicated on the figure were added one minute before the collagen. Results are the mean values of eight separate experiments. The half standard deviation (vertical bars) is given for the control experiments. ○, 10 mM creatine phosphate/1.5 units per ml creatine phosphokinase; ■, 0.5 mM ATP; ▲, 1 mM AMP.

centrations previously found to totally inhibit platelet aggregation and fibrinogen binding induced by 5 μM ADP [1,4,6,26]. Identical results were obtained with 0.1 mg/ml apyrase (data not shown). No inhibition was observed with creatine phosphate or creatine phosphokinase alone. When 0.5 mM ATP was used, inhibition was less than was observed with the other substances. This was not observed in previous experiments performed with ADP [6] and may be due to an increased degradation of ATP by platelets in the presence of collagen (see Discussion). Overall, these results suggest an ADP-dependency for fibrinogen binding induced by collagen.

On varying the concentration of collagen in the medium, a close relationship was observed between the amounts of ^{125}I-fibrinogen bound to the platelets and the extent of [^{14}C]serotonin release (Fig. 5). When platelets had been pretreated with aspirin both collagen-induced fibrinogen binding and serotonin release were partially inhibited (Table II). At all collagen concentrations tested, the apparent relationship between the extent of dense granule secretion and ^{125}I-fibrinogen binding was maintained (Fig. 5). As also shown in Table II, the

TABLE II

COLLAGEN-INDUCED [^{125}I]FIBRINOGEN BINDING AND [^{14}C]SEROTONIN RELEASE FROM HUMAN PLATELETS

Platelets ($5 \cdot 10^8$/ml) were incubated with agitation at room temperature with 20 μg/ml [^{125}I]fibrinogen and various concentrations of collagen in the presence or absence of 10 mM creatine phosphate/1.5 units per ml creatine phosphokinase (CP/CPK). Fibrinogen binding was measured after 30 min as illustrated in Fig. 2. [^{14}C]Serotonin release was determined under identical conditions in separate samples using unlabeled fibrinogen in place of [^{125}I]fibrinogen. Results are the mean values (±S.D.) determined from seven different experiments.

Collagen concentrations (μg/ml)	CP/CPK in the medium	Control platelets		Aspirin-treated platelets	
		[^{125}I]fibrinogen bound (μg/10^9 platelets)	[^{14}C]serotonin released (%)	[^{125}I]fibrinogen bound (μg/10^9 platelets)	[^{14}C]serotonin released (%)
6.25	−	0.26±0.22	5.2±2.2	0.20±0.15	3.3±1.8
	+	0.06±0.05	3.6±1.7	0.08±0.07	3.0±1.3
12.5	−	0.42±0.24	9.9±3.7	0.36±0.28	5.4±2.1
	+	0.14±0.09	8.8±3.4	0.10±0.08	5.4±1.7
25	−	0.76±0.40	15.6±4.3	0.42±0.30	10.6±1.7
	+	0.20±0.13	11.7±3.6	0.14±0.10	9.3±1.9
50	−	1.08±0.60	26.7±7.7	0.58±0.34	15.6±3.1
	+	0.28±0.14	19.4±5.1	0.22±0.12	12.8±2.7

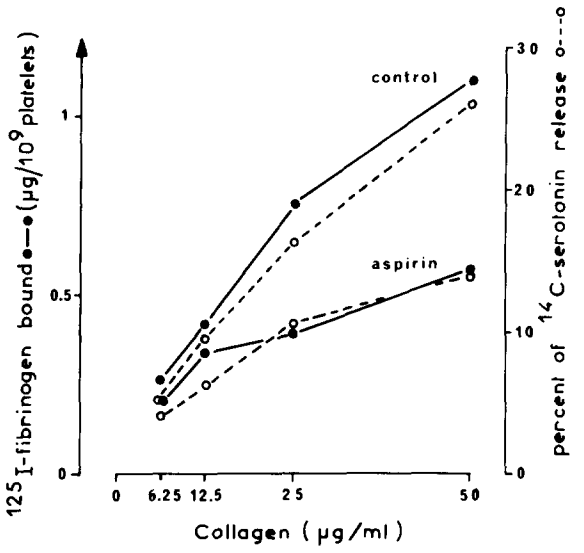

Fig. 5. Correlation between collagen-induced ^{125}I-fibrinogen binding (●) and [^{14}C]serotonin release (○). Platelet samples ($5 \cdot 10^8$/ml) were agitated at room temperature with 20 μg/ml ^{125}I-fibrinogen and various concentrations (6.25–50 μg/ml) of collagen. Binding of ^{125}I-fibrinogen was assessed after 30 min and [^{14}C]serotonin release was estimated in parallel samples as described in the legend to Fig. 2. Experiments were also performed with control and aspirin-treated platelets (see Methods). Results are the mean of six different experiments.

fibrinogen binding which occurred to aspirin-treated platelets was also markedly reduced in the presence of creatine phosphate/creatine phosphokinase. An important result was that the enzyme system did not significantly affect collagen-induced [^{14}C]serotonin release implying that it was not interfering with the stimulation of the platelets by collagen.

Discussion

Platelet adhesion to collagen fibrils during haemostasis is a multistep process involving an initial platelet contact or attachment, that is rapidly followed by morphologic changes associated with pseudopod formation and platelet spreading on the collagen [27]. The latter events are accompanied by the secretion of granule contents and the expression of cohesive properties at the platelet surface. These lead to platelet aggregation and thrombus formation. Our present study suggests that the expression of fibrinogen receptors by collagen-stimulated platelets is an important step in the mechanism of collagen-induced platelet aggregation confirming the previous studies of Di Minno et al. [10] and a preliminary report by Plow and Marguerie [28]. Fibrinogen receptor expression by collagen-stimulated platelets differed from that induced by ADP [6] in that agitation was required for maximal fibrinogen binding (Fig. 2). Although the mechanism by which fibrinogen receptors are expressed is unknown, a role for platelet-platelet contact interactions in this process is suggested. This may occur by enhancing the release reaction and therefore the amount of ADP in the medium (see below). Alternatively, we cannot discount that continuous agitation may be required to ensure the optimal interaction of collagen fibrils with the platelets. A feature of both collagen-induced platelet aggregation [15,16,27] and fibrinogen receptor expression is the presence of a lag phase. This suggests that fibrinogen binding correlates with the onset of aggregation, although a direct comparison is difficult owing to the different experimental procedures that are used. This is a result of the need to study fibrinogen binding under conditions where no aggregates were formed to avoid nonspecific trapping of the ^{125}I-fibrinogen.

Collagen-induced fibrinogen binding to platelets was extensively inhibited by creatine phosphate/creatine phosphokinase, apyrase, ATP and AMP, all of which antagonize the effects of ADP on platelets [1,4,6,26]. In these experiments, a time-dependent loss of the inhibitory effect of ATP was observed (Fig. 4). This may be due to a degradation of the ATP by the platelets. For example, collagen has been reported to stimulate a platelet pyrophosphatase activity [29]. Alternatively, the amount of ATP in the platelet suspension may also depend on its rate of metabolism by platelet ecto-ATPase [30]. Our results with ADP antagonists differ in degree to those reported by Di Minno et al. [10] who found that apyrase inhibited collagen-induced fibrinogen binding by a maximum of 50%. The reason for this difference is not known, but could be due to (i) the different collagen preparations used in the two studies, or (ii) the fact that platelet suspensions as studied by Di Minno et al. were washed in the presence of EDTA, a substance normally thought to interfere

with the platelet aggregation mechanism [31] or (iii) the different cation composition of the platelet resuspension buffers which have been previously shown to affect the number of fibrinogen binding sites on platelets [3].

The number of fibrinogen binding sites estimated in the present study after ADP stimulation (18 100 ± 6800) is close to that found by Peerschke et al. [4] or Harfenist et al. [9], but is less than has been measured by other authors [2,3,5,8,10,11]. This variation may result from the different techniques used to isolate the platelets.

Also, platelets in different media may respond in different ways. For example, in Tyrode-albumin buffer containing 2 mM calcium and 1 mM magnesium, i.e., in physiological divalent ion concentration, ADP does not induce the release reaction [32] and fibrinogen binding to platelets is not affected by the presence of aspirin (Legrand, C., unpublished data). In buffers containing 1 mM or less calcium, or containing magnesium in place of calcium, ADP may induce the release reaction at least when aggregates are formed [2,10,32]. Under these conditions ADP-induced platelet aggregation and fibrinogen binding are partially inhibited by aspirin [10,11]. In the present study, under collagen stimulation, pretreatment of the platelets with aspirin resulted in a decreased binding of ^{125}I-fibrinogen. As arachidonic acid metabolites have been shown to influence fibrinogen receptor expression directly [9–11], this result may suggest a role for thromboxanes and/or endoperoxides in collagen-induced fibrinogen receptor expression as has been previously proposed [10]. However, in all of our studies, there was a strict correlation between fibrinogen binding and the amount of dense granule secretion (see Fig. 5). Thus, an alternative explanation for the effect of aspirin pretreatment is that by inhibiting collagen-induced secretion, it was influencing the amount of ADP available to support fibrinogen binding.

Making the assumption that 35 μmol of ADP are present in the dense granules of $1 \cdot 10^{12}$ platelets [33] and that adenine nucleotides stored in the dense bodies are secreted simultaneously with serotonin [34], the stimulation of platelets with 6.25–50 μg/ml collagen would result in the appearance of 1–5 μM ADP in the medium. This concentration is enough to support fibrinogen binding [6]. Indeed, platelets shaken in the presence of 50 μg/ml collagen express the same number of fibrinogen receptors as do nonstirred platelets in the presence of similar concentrations of exogenously added ADP (Table I). Furthermore, fibrinogen binding occurs with much the same affinity. Our results imply a role for secreted ADP in fibrinogen receptor expression induced by collagen. That these results may be of physiologic importance is suggested by the fact that in haemostasis collagen interaction with platelets occurs in a moving blood system, and that a decreased platelet aggregation with collagen is observed with platelets from patients with inherited disorders characterized by deficiencies of storage pools of dense granule nucleotides [35].

Although emphasizing a possible function for secreted ADP, our results do not exclude a role for ADP from other sources in collagen-induced fibrinogen binding. For example, it has been shown that creatine phosphate/creatine phosphokinase may act by decreasing the potentiation of platelets by removing extracellular membrane-bound ADP present prior to the addition of other aggregation-inducing stimuli [36]. It was significant, however, that this enzymatic system only slightly affected collagen-induced dense granule secretion in our study (Table II) while the amount of ^{125}I-fibrinogen bound to platelets was correlated with the extent of dense granule secretion. This latter result strongly suggests a major role for secreted ADP.

It has long been postulated that certain agonists may aggregate platelets at least in part through the release of ADP and fibrinogen [37]. Assuming a value of granule-located fibrinogen around 0.1 mg/10^9 platelets [37–39] and a platelet count of $2.5 \cdot 10^8$ cells/ml in the aggregometer cuvette, the platelets carry a load of intracellular fibrinogen which would correspond to approximately 0.025 mg/ml (75 pmol/ml). Thus, if platelet fibrinogen plays a role in collagen-induced platelet aggregation, it does so at lower concentrations than are required for exogenous fibrinogen supporting ADP-induced platelet aggregation. According to Marguerie et al. [3] the extent of platelet aggregation induced by 6 μM ADP gradually increased with the concentration of added fibrinogen from 0.016 mg/ml (50 pmol/ml) to 0.1 mg/ml (300 pmol/ml). Fibrinogen present in the alpha-gran-

ules of resting platelets is redistributed to the plasma membrane after thrombin stimulation [40]. However, the proportion of bound fibrinogen as compared to the total amount of secreted protein is not known. Other platelet granule proteins such as thrombospondin [41], fibronectin [42] and the Von Willebrand factor [43] appear on the platelet surface after thrombin stimulation. George and Onofre [43] have suggested that the membrane surface could provide a mechanism by which the platelet may concentrate and organize its secreted proteins for subsequent physiologic reactions. The fact that alpha-granule deficient platelets show an abnormal aggregatory response to thrombin and collagen [44,45] argues for a role for these proteins in platelet function.

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