Adhesion of Human Platelets to Immobilized Trimeric Collagen

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ABSTRACT   Human platelets adhere to trimeric Type I chick collagen that was covalently linked to plastic slides, providing the basis for a well-defined quantitative assay. The number of platelets that adhere is a function both of platelet concentration and of collagen density on the slides. In contrast with other in vitro assays using collagen that is not covalently linked to the substratum, we found no platelet-platelet aggregation. Adhesion was absolutely dependent on Mg^{2+}, whereas Ca^{2+} was ineffective. Native trimeric collagen conformation was required for adhesion, since platelets did not bind to slides containing heat-denatured collagen, or isolated alpha 1(I) or alpha 2(I) chains. Modifications of collagen oligosaccharides had no effect on adhesion. Adhesion was inhibited by cytochalasin D but was not affected by prostaglandin E_1, apyrase, acetylsalicylic acid, or theophylline. Because this assay measures platelet-collagen adhesion in the absence of platelet-platelet aggregation, it should facilitate identification of the platelet surface components that directly mediate this adhesion.

Human platelets are specialized for at least two types of adhesive reactions, platelet-collagen adhesion, and platelet-platelet aggregation. Each type of adhesion may involve a number of cellular and molecular events and is a critical part of hemostasis. Analysis of these adhesive reactions would be facilitated if they were separated. However, progress has been limited because assays of platelet-collagen adhesion often also measure extensive platelet aggregation. This is true both in physiological assays in which platelets are reacted with aortic subendothelium (1) and in assays in which platelets are reacted with purified collagen (see reference 2 for review).

Platelet aggregation is induced by fibrillar collagen, a polymeric form of the basic collagen trimer (3). To avoid inducing aggregation, some assays of platelet adhesion have, therefore, used trimeric collagen adsorbed to glass. However, adsorption does not prevent fibril formation, because the trimers are reversibly associated with the glass and can interact (4). The resultant fibrils induce platelet aggregation, which impedes study of pure platelet-collagen adhesion. For example, Meyer and Weisman (5) found that platelet-adhesion to trimeric collagen coated on glass slides was markedly contaminated with platelet aggregation, unless the temperature was reduced to 4°C. Similarly, Cazenave et al. (6) reported platelet aggregation in an assay in which trimeric collagen was coated on a glass probe and rotated in a suspension of washed platelets at 37°C, although aggregation could be minimized by reducing the temperature to 22°C.

To avoid these problems, we have covalently linked trimeric collagen to plastic slides for studying platelet-collagen adhesion. Irreversible immobilization under defined conditions pre-

vents the collagen from forming fibrils during derivatization, storage of the slides or during the assay. It also permits control of collagen density on the slides. With this assay, we can measure platelet-collagen adhesion at 37°C that is not contaminated by platelet aggregation. The adhesion we measure requires Mg^{2+} but not Ca^{2+} ions, which has not been observed previously. It is also specific for native collagen conformations. These properties will facilitate investigation of the specific adhesion mechanism.

MATERIALS AND METHODS

Collagen Purification and Immobilization

Type I collagen was purified from lathyritic chick skin by the method of Kang et al. (7). Briefly, chicks were killed after 2 wk of 0.1% β-aminoproprionitrile treatment, and collagen was extracted from the skin in neutral salt. Type I collagen was purified by repeated differential salt precipitations, and glycosaminoglycans were removed by chromatography on DEAE-Sephadex (8). Purity was assessed by PAGE in SDS.

Purified collagen was covalently coupled to both sides of 100-mm² plastic cover slips (Fischer Scientific, Pittsburgh, PA) essentially by the procedure of Edelman et al. (9). Before coupling the collagen was dissolved overnight in 0.2 M NaCl, 0.01 M Tris, pH 7.0, and centrifuged to remove aggregates. Cover slips were prewashed in 0.1 N HCl. Then 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate, from Chemical Dynamics (South Plainfield, NJ) was reacted with cover slips for 1 min at 22°C, decanted, and the soluble collagen added immediately thereafter. Concentrations of the carbodiimide reagent varied from 0.1 to 2 mg/ml in 0.15 M NaCl and collagen concentrations varied from 0.1 to 1 mg/ml, depending on the immobilized collagen density desired. Coupling proceeded for 30 min on ice. This procedure resulted in slides that were derivatized with nonfibrillar trimers of collagen. Fibrils did not form during derivatization because of the pH and ionic strength of the buffer and the low temperature used. This was shown by monitoring the optical density at 430 nm of the collagen solutions before and after derivatization.

Control slides were reacted with bovine serum albumin (BSA) (RIA grade; Sigma Chemical Co., St. Louis, MO) in parallel incubations. After coupling, all slides were incubated in 4 mg/ml BSA for 15 min and then rinsed. Collagen density was estimated by adding measured amounts of ^{125}I-collagen to parallel reaction mixtures and determination of the amount of ^{125}I-collagen bound. The ^{125}I-collagen was prepared by the chloramine-T procedure (10).

In experiments where the collagen structure was varied, slides were prepared as above immediately after an incubation step that altered the collagen conformation. One collagen preparation was used for all couplings, which were done simultaneously under identical conditions. Fibrillar collagen was prepared by preincubation of the collagen stock at 37°C for 15 min in 0.2 N NaCl, 0.01 M Tris, pH 7.0. Collagen polymerization was monitored by increase in optical density at 430 nm. Denatured collagen was prepared by incubation of the collagen stock at 55°C for 15 min just before the coupling reaction. Isolated collagen alpha 1(I) and alpha 2(I) chains were prepared by fractionation on carboxymethyl cellulose (11) (Whatman Inc., Clinton, NJ) and coupled to slides as above.

Modification of Collagen Structure

Collagen oligosaccharides were modified after collagen was coupled to slides. Galactose oxidase (Sigma Chemical Co.) was added to derivatized slides at 10 U/ml of PBS, pH 7.2. Slides were incubated overnight at 25°C, then washed extensively in buffered saline. Two types of control slides were used: slides incubated without enzyme and enzyme-treated slides that were postincubated in 0.02 M NaBH₄, 0.1 M sodium phosphate, pH 7.8, to restore the native state of the terminal galactose.

Periodate oxidation was done by incubating derivatized slides in 0.01 M sodium metaperiodate, 0.05 M sodium acetate, pH 4.5, at 4°C overnight. Oxidation controls were either incubated without periodate or postincubated with 0.02 M NaBH₄, 0.1 M sodium phosphate buffer, pH 7.8. Incorporation of tritiated NaBH₄ was used to show that oxidation had been successful. All slides were extensively washed in assay buffer.

Some slides were digested with 12 U/ml bacterial collagenase (Sigma Chemical Co.) in an 0.05 M Tris, 0.2 M NaCl, 1.5 mM CaCl₂ buffer, pH 7.4, for 2 h at 25°C on a shaker. Controls were incubated in Tris buffer without enzyme.

Preparation of Assay Buffers

Standard assay buffer was a modified Tyrode's buffer which contained in 1 l: 80 g NaCl, 2.13 g MgCl₂·6H₂O, 10.15 g NaHCO₃, 1.95 g KCl, 1 g glucose, and 2 g BSA; and contained no calcium. The pH was adjusted to 7.4 for assays and to 6.5 for platelet preparation. Calcium- and magnesium-free assay buffer was prepared by treatment with Chelex 100 (12), (Bio-Rad Laboratories, Richmond, CA), of a stock solution of the standard assay buffer prepared without MgCl₂ or BSA. A solution of 60 mg/ml RIA grade BSA in 1 mM EDTA was dialyzed extensively against dialyzed water to remove bound divalent cations, then added to the Chelex-treated solution, and the pH was adjusted to 7.4. Platelets were prepared, assayed, and washed in this buffer, and cations were added as required. Mg^{2+} concentration was 1 mM unless otherwise indicated.

Platelet Preparation and Labeling

Gel-filtered human platelets from normal, aspirin-free donors were prepared by the method of Ginsberg et al. (13). The platelet button was resuspended at 10^{10} platelets/ml in assay buffer, pH 6.5, and 0.1 vol of ^{51}Cr (ICN Nutritional Biochemicals, Cleveland, OH; 343 mCi/mg Cr, 5 mCi/ml) was added. After 1 h at 37°C platelets were filtered on a column of Sepharose 2B equilibrated with assay buffer. Labeling with ^{14}C-5-hydroxytryptamine (^{14}C-5HT (56 mCi/mmol, 50 μCi/ml; Amersham-Searle, Arlington Heights, IL), when necessary, was done by adding 0.2 vol of ^{14}C-5HT, with the ^{51}Cr. All assays were completed within 4 h of the time blood was drawn.

Adhesion Assays

The collagen cover slips were placed in 35-mm plastic Linbro tissue culture wells (Flow Laboratories, Rockville, MD) containing 0.35 ml of assay buffer, and 0.05 ml of platelet suspension containing ~2 × 10⁷ labeled platelets was added. In drug inhibition studies, the platelets were preincubated with drugs for 15 min at 37°C, then transferred to assay wells. Assay incubations were at 37°C, 150 rpm on a gyratory shaker (model G24; New Brunswick Scientific Co., Edison, NJ) and proceeded for 10 min unless otherwise indicated.

To wash the slides, the wells were gently dipped three times into serial beakers containing assay buffers maintained at 37°C. This provided a thorough, uniform rinse at low shear. Slides were then removed with a forceps and ^{51}Cr was measured with a gamma counter. When used, ^{14}C-5HT was measured with a liquid scintillation counter.

Each data point was done in triplicate with triplicate matching BSA-derivatized slides as blanks. Specific ^{51}Cr-label bound to collagen was calculated by subtracting the mean counts bound to the corresponding BSA control slides from the mean counts bound to collagen. Identical 10-min incubations under standard assay conditions were performed at the beginning and end of each experiment to ensure that platelet properties were not changing with time. All data are reported as triplicate means ± standard error.

Poly-L-hydroxyproline was tested as a possible hapten inhibitor of platelet collagen adhesion. The polymer, with molecular weights in the range of 10,000–30,000 (Sigma Chemical Co.) was dissolved in assay buffer and added to platelets to a final concentration of 100 μg/ml. Platelets were incubated for 15 min at 37°C before collagen adhesion and secretion of ^{14}C-5HT were assayed. Under these conditions, some trimers of poly-L-hydroxyproline can form in solution (5).

Scanning Electron Microscopy

For scanning electron microscopy (SEM) studies, slides were fixed after the last assay wash in 1% glutaraldehyde, 0.03 M sodium phosphate, pH 7.4, for 3–6 h. After ethanol dehydration, they were critical-point-dried in Freon, sputter-coated with gold/palladium (150 Å thickness) and viewed at 27 kV, 45° tilt, on a Cambridge S4 SEM.

RESULTS

Properties of Adhesion

Washed platelets adhered to trimeric collagen, reaching a steady state within ~20 min (Fig. 1). Assays were routinely done with 10-min incubations because this facilitated multiple determinations and allowed the evaluation of the effects of inhibitors on the initial binding reaction. BSA controls showed relatively little ^{51}Cr binding (Fig. 1). Platelets attached as single particles, as assessed by both light microscopy and SEM. Most SEM fields showed only a single platelet, but occasionally there were many in a field, some of which had spread extensively (Fig. 2). Platelet lysis was minimal since <0.1% of the ^{51}Cr added was found in the medium after incubation.

Platelet adhesion was linear with platelet concentration over a wide range (Fig. 3). Over this range, ~2% of the platelets added to the well adhered to the collagen during a 10-min incubation. Since only a very small fraction of the platelets bound, we considered the possibility that the bound platelets were a nonrepresentative subpopulation. To test this, we collected the nonadherent platelets after a routine incubation and reacted them with fresh cover slips. Again, 2% of the added platelets bound. With a further repetition a similar result was
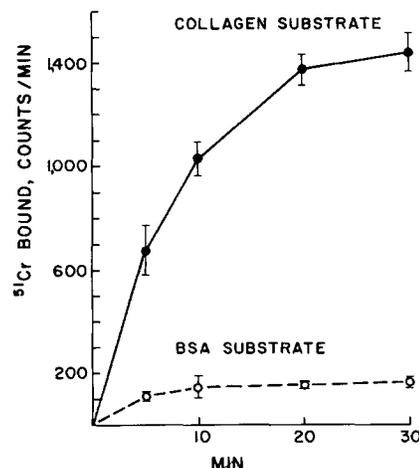

FIGURE 1 Time course of platelet adhesion on trimeric collagen and on BSA. Platelets, at 5.8 × 10⁷/ml, were incubated on collagen-derivatized slides (●) of density 0.04 μg/mm² or on matching BSA-derivatized slides (○). Specific activity of platelets was 2.2 × 10⁴ cpm ^{51}Cr/10⁷ platelets.
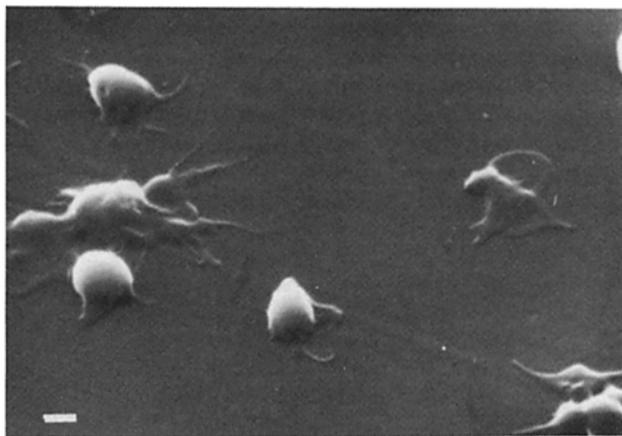
FIGURE 2 SEM of platelets bound to a collagen-derivatized plastic slide under standard assay conditions. A field containing an unusually high platelet density is shown. Bar, 1 μm. × 4,000.

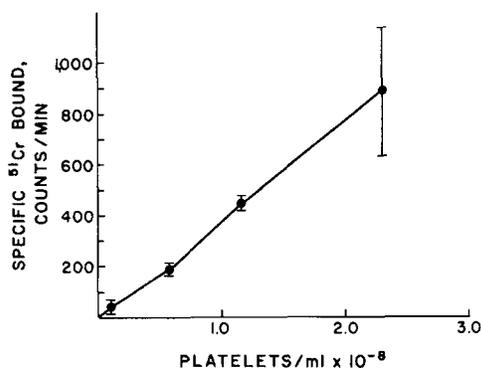date did not change the amount or time course of platelet binding. This suggests that the collagen oligosaccharides are not critical for the adhesion we measure, although it remains possible that all the saccharide residues were not modified by these treatments. Bacterial collagenase digestion, in contrast, completely destroyed the collagen-specific adhesion. Because of evidence that trimers of poly-L-hydroxyproline block platelet adhesion to collagen coated glass slides (5), we tested the effect of this polymer in our assay. It had no effect when added to assay buffer at concentrations from 10-100 μg/ml, further showing a high specificity for collagen.

Effect of Agents That Influence Platelet Secretion

Platelets that adhered to collagen in this assay secreted 58% of their ^{14}C-5HT (Table I). In contrast, secretion by nonadherent platelets, as measured by ^{14}C-5HT in the medium after completion of the assay, was <3%. To investigate the role of

FIGURE 3 Relationship of platelet concentration to platelet-collagen adhesion. Specific binding to collagen-derivatized slides, density 0.06 μg trimeric collagen/mm^2, was determined after 10 min incubations under standard assay conditions. Platelets had specific ^{51}Cr activity of 8.9 × 10^4 cpm/10^7 platelets.

obtained. Therefore, the population binding in the initial assay is not unique. We also considered that the fraction bound may have been activated during collection or processing. To minimize this, we included 100 nM PGE$_1$ in all solutions in one experiment. Platelets prepared in this way adhered exactly like controls.

At very high platelet concentrations some aggregation was observed, as indicated by a marked increase in the percentage of the platelets associated with the slide. For routine assays we did not exceed concentrations of 10^8/ml, which never resulted in aggregation. Adhesion was also dependent on collagen density, saturating at high collagen density (Fig. 4).

Role of Collagen Conformation

Platelets adhered to native collagen which was in a trimeric or fibrillar form, but did not bind to heat denatured collagen (Fig. 5). Trimeric collagen was a more effective substrate than fibrillar collagen on a weight basis. Equivalent amounts of isolated alpha 1(I) or alpha 2(I) subunits of type I collagen coupled to cover slips did not support platelet adhesion (data not shown).

Modifications of collagen oligosaccharides, which can influence collagen-mediated platelet aggregation (2, 11), had no effect on platelet adhesion (data not shown). Digestion of slides before assays with galactose oxidase or oxidation with perio-
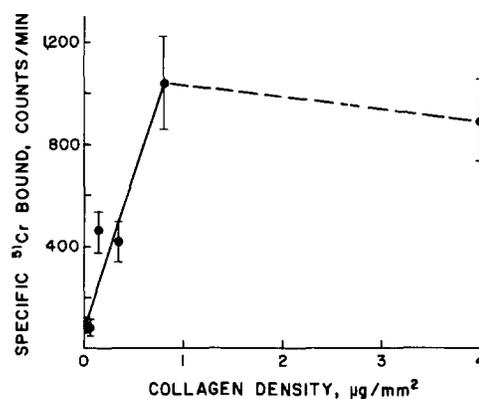

FIGURE 4 Relationship of collagen density to platelet adhesion. Trimeric collagen was coupled to plastic cover slips at various densities. The density achieved was determined in parallel incubations that included ^{125}I-collagen in the coupling solution. Platelet concentration in this experiment was 1.3 × 10^8/ml, and specific ^{51}Cr activity was 1.6 × 10^5 cpm/10^7 platelets. Incubation was for 10 min under standard assay conditions.
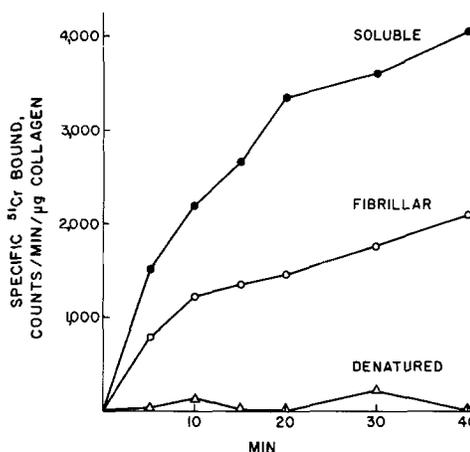

FIGURE 5 Platelet adhesion requires native collagen conformations. Time courses are shown for the adhesion of platelets, 9.4 × 10^7/ml, to trimeric collagen (●), fibrillar collagen (O), and heat denatured collagen (△). Specific adhesion was normalized for the collagen density on the slides; trimeric collagen (0.02 μg/mm^2), fibrillar collagen (0.06 μg/mm^2), denatured collagen (0.02 μg/mm^2). Platelets had a specific activity of 5.5 × 10^4 cpm ^{51}Cr/10^7 platelets.

TABLE I

Effect of Agents That Influence Platelet Secretion

Addition	% Secretion by collagen-adherent platelets		Specific adhesion (% of control)		ADP-induced platelet secretion
None	58 ± 2	(6)	100	(6)	30%
PGE₁ (10 μM)	48 ± 4	(6)	109 ± 9	(6)	3%
PGE₁ (1 μM) + apyrase (20 μg/ml) + acetylsalicylate (1 mM) + theophylline (50 μg/ml)	51 ± 3	(2)	101 ± 2	(2)	Not done
Cytochalasin D (0.5 μg/ml)	14	(1)	55	(1)	Not done

All agents were tested for effects on platelet-collagen adhesion and on secretion of ¹⁴C-5HT by collagen adherent platelets. The latter was determined by comparing ratios of ¹⁴C-5HT to ⁵¹Cr in collagen-bound platelets and in the starting platelet population. To measure ADP-induced platelet secretion, platelets were incubated with shaking for 10 min at 37°C in a test tube in assay buffer containing 1 μM ADP and 50 μg/ml fibrinogen, lightly fixed with 0.5% paraformaldehyde in 0.03 M phosphate, pH 7.4, then applied to 0.22 μm Millipore filters. The secreted ¹⁴C-5HT was determined by counting the filtrate and the percentage of total platelet ¹⁴C-5HT found in the filtrate is shown.

platelet secretion in this assay, we tested several agents that inhibit collagen-induced platelet secretion in other systems. Prostaglandin E₁, apyrase, acetylsalicylic acid, and theophylline, either alone or in combination, had no effect on platelet adhesion to collagen (Table I). These agents also had no effect on secretion of ¹⁴C-5HT by collagen-adherent platelets (Table I). Since prostaglandin E₁ is the most potent and most labile of these reagents, its activity was checked in a secretion assay. When prostaglandin E₁ was added to ¹⁴C-5HT-labeled platelets it inhibited ADP-stimulated secretion of ¹⁴C-5HT (Table I). Therefore, the failure of PGE₁ to inhibit platelet adhesion or secretion by adherent platelets is not due to use of a degraded sample.

The only agent that did affect platelet adhesion was cytochalasin D. It blocked about half of platelet adhesion at 0.5 μg/ml and also inhibited secretion by adherent platelets (Table I). Concentrations of up to 16 μg/ml were no more effective in blocking adhesion. SEM showed that none of the cytochalasin D-treated platelets were attached in the spread morphology commonly seen in the absence of the drug. Many of the adherent platelets were associated with the substratum by single pseudopodia and were rounded.

Ionic Requirements of Platelet Adhesion

Adhesion in this assay was absolutely dependent on Mg^{2+} (Fig. 6). In contrast, Ca^{2+} could not replace Mg^{2+} in supporting platelet-collagen adhesion (Fig. 6). Indeed, platelet binding in the presence of 1 mM Mg^{2+} was inhibited by 0.5–3 mM Ca^{2+} (Fig. 6).

This finding did not exclude the possibility that small amounts of external calcium ion released by the platelets were required for adhesion. To test this, the effects of EDTA and EGTA were studied. In normal assay buffer, 1 mM EDTA or 1 mM EGTA inhibited specific adhesion by ~90% (Table II). The fact that EGTA was effective raised the possibility that calcium ion was required. However, increasing the concentration of Mg^{2+} without addition of Ca^{2+} counteracted the effect of EGTA on adhesion (Table II). This indicates that EGTA inhibition is due to chelation of Mg^{2+}, an effect that has been

observed by Hovig (14). It cannot be due to displacement of chelated Ca^{2+} by the added Mg^{2+}, because the affinity of EGTA for Ca^{2+} exceeds its affinity for Mg^{2+} by 5.6 orders of magnitude at pH 7.4 (14). Thus, there is no direct or indirect evidence for a Ca^{2+} requirement in this adhesion assay. It should be noted that adding 3–5 mM Mg^{2+} to standard buffer plus 1 mM EGTA, although very effective, does not completely restore adhesion (Table II). This may be explained, at least in part, by the fact that these combinations may result in a nonoptimal true Mg^{2+} concentration, as observed in Fig. 6.

DISCUSSION

We describe a quantitative assay for platelet adhesion to trimeric collagen which is covalently bound to plastic cover slips. Because the collagen is irreversibly linked to the substrate, it cannot form fibrils, which are potent stimulators of platelet aggregation. This feature, in addition to the use of washed platelets and low platelet concentrations, resulted in an assay of platelet-collagen adhesion that shows no significant contamination by aggregation.

The assay differs from those reported previously in several of its properties, especially the lack of sensitivity of adhesion to certain drugs, dependence on Mg^{2+}, and specificity for native collagen conformations. Although prostaglandin E₁, apyrase, acetylsalicylic acid, and theophylline have been reported to
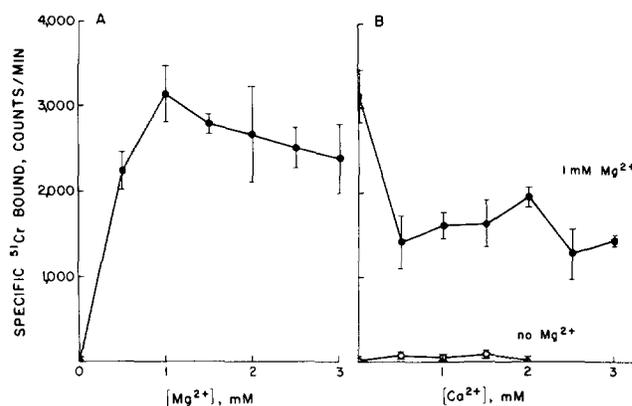

FIGURE 6 Effect of Mg^{2+} concentration (A) and Ca^{2+} concentration (B) on platelet adhesion. Platelets of specific activity 7 × 10⁵ cpm ⁵¹Cr/10⁷ platelets, at 1.3 × 10⁸ platelets/ml, were incubated on slides derivatized with 0.04 μg trimeric collagen/mm² under standard assay conditions. In A there was no Ca^{2+} and the Mg^{2+} concentration is indicated. In B Ca^{2+} concentrations were added in the presence (●) or absence (○) of 1 mM Mg^{2+}.

TABLE II

Effect of EGTA on Platelet Adhesion Is Blocked by Mg^{2+}

Addition	Adhesion, % of control
None	100 ± 1
1 mM EGTA	9 ± 3
1 mM EGTA + 1 mM Mg²⁺	37 ± 3
1 mM EGTA + 3 mM Mg²⁺	69 ± 3
1 mM EGTA + 5 mM Mg²⁺	84 ± 3

Platelets were incubated for 10 min in standard assay buffer containing the indicated additions. Specific adhesion to collagen is expressed in comparison with adhesion in standard assay buffer. Data are mean ± SE of three determinations.

inhibit collagen-induced secretion as well as platelet-collagen adhesion in other assays (6, 15), in our assay they affected neither adhesion nor secretion. A possible explanation for this difference is that other assays had a component of platelet aggregation that was inhibited by these drugs. The failure of these agents to influence our assay is consistent with the hypothesis that we are measuring only platelet-collagen adhesion.

The absolute dependence of platelet-collagen adhesion on Mg^{2+} has not been previously observed, and the role of this divalent cation is not known. Mg^{2+} may be required to bind a secreted platelet surface ligand to collagen, to bind a ligand to the platelet surface itself, or for other events associated with adhesion. It is not essential for binding of fibrinogen or fibronectin to platelets, since Ca^{2+} suffices for this purpose (16, 17). The fact that purified fibronectin binds to denatured as well as native collagen (18) also suggests that it does not mediate adhesion in our assay, which requires native collagen.

Because this assay measures platelet-collagen adhesion without aggregation, it should be useful for identification of the platelet constituents that mediate adhesion to collagen. It should also be useful for investigating the mechanism of certain platelet disorders.

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