Calin from the saliva of the medicinal leech, Hirudo medicinalis, is a potent inhibitor of collagen mediated platelet adhesion and activation. In addition to inhibition of the direct platelet-collagen interaction, we presently demonstrate that binding of von Willebrand to coated collagen can be prevented by Calin, both under static and flow conditions in agreement with the occurrence of binding of Calin to collagen, confirmed by Biospecific Interaction Analysis. To define whether Calin acted by inhibiting the platelet-collagen or the platelet-von Willebrand factor (vWF)-collagen-mediated thrombus formation, platelet adhesion to different types of collagens was studied in a parallel-plate flow chamber perfused with whole blood at different shear rates. Calin dose-dependently prevented platelet adhesion to the different collagens tested both at high- and low-shear stress. The concentration of Calin needed to cause 50% inhibition of platelet adhesion at high-shear stress was some fivefold lower than that needed for inhibition of vWF-binding under similar conditions, implying that at high-shear stress, the effect of Calin on the direct platelet-collagen interactions, suffices to prevent thrombus formation. Platelet adhesion to extracellular matrix (ECM) of cultured human umbilical vein endothelial cells was only partially prevented by Calin, and even less so at a high-shear rather than a low-shear rate, whereas the platelet binding to coated vWF and fibrinogen were minimally affected at both shear rates. Thus, Calin interferes with both the direct platelet-collagen interaction and the vWF-collagen binding. Both effects may contribute to the inhibition of platelet adhesion in flowing conditions, although the former seems to predominate.

Support was given by a grant from the National Science Foundation (FGWO 3.0030.90) and from the Belgian government (IUAP 35). Previous studies,30,31 suggested that Calin interacts with collagen rather than with platelets. Platelet binding to collagen can occur through a direct platelet-collagen interaction or be mediated via von Willebrand factor (vWF), forming a bridge between collagen and platelets. The former interaction is believed to be more important under conditions of low-shear stress, the latter being a prerequisite for platelet adhesion under high-shear stress. Indeed, under conditions of low-shear stress, the former interaction may be sufficient, but under high-shear stress the combination of both direct and vWF-mediated binding recently was shown to be essential.32

We report here on the effect of Calin on vWF-binding to collagen under both static and flow conditions, followed by experiments to determine the effect of Calin on platelet adhesion to collagen and other adhesive surfaces under high- and low-shear stress.

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MATERIALS AND METHODS

Materials

Unless otherwise stated, all reagents were obtained from VEL (Haasrode, Belgium) in analytic grade. Lysophosphatidyl Calin was obtained from BioPharm (UK) (Hendy, Dyfed, UK) and dissolved in 1 mL physiologic saline to obtain a solution of 200 μg/mL protein, 20 mmol/L Tris·HCl, 10 mmol/L CaCl₂ 150 mmol/L NaCl, pH 8. Tissue culture grade plastics and Thermofax coverslips were obtained from Gibco Europe (Merelbeke, Belgium).

Blood Samples

Blood was collected from the antecubital vein of medication-free donors in polypropylene tubes containing the anticoagulant, one-tenth volume 108 mmol/L trisodium citrate (citrate blood) or one-tenth volume 200 U/mL low molecular weight heparin (LMWH) (Clexane; Rhône-Poulenc Rorer, France) in 0.15 mol/L NaCl (LMWH blood). Platelet-poor plasma was prepared by centrifugation at 1,500g, for 15 minutes. A pool of seven healthy individuals were used in the vWF-binding studies. Blood or plasma were preincubated with Calin or the appropriate buffer for 20 minutes at room temperature before use.

vWF Binding to Collagen Under Static Conditions

Polystyrene 96-well microtiter plates (Costar, Kruibeke, Belgium) were coated with 100 μL/well 50 μg/mL collagen type I calf skin (Sigma Chemical Co, St Louis, MO). Collagen was dissolved in 0.1 mol/L acetic acid followed by extensive dialysis against 67 mmol/L phosphate buffer, pH 7.2. Plates were postcoated with 100 μL 1% bovine serum albumin (BSA), and washed and incubated with Calin for the indicated times at 37°C. Unbound material was either left in the well or removed by washing. As a source of vWF, either normal human plasma diluted 1/80 in phosphate buffered saline, 1% propylene diamine in citrate-phosphate buffer, pH 5.0 and 0.003% HZO₂ stopped with HzSO₄ and the light absorbance was measured at 492/690 nm.

vWF Binding and Platelet Adhesion Under Flow Conditions

Preparation of coverslips. Human collagen types I and III (Sigma) solubilized at 1mg/mL in 50 mmol/L acetic acid, or collagen type I from calf skin, treated as above and fibrillar equine tendon collagen (Collagen Reagent Horm, Hormon Chemie, Munich, Germany) were coated on glass or plastic coverslips using a retouching air brush (Badger Model 100, Franklin Park, IL) at a nitrogen operating pressure at 1 atm. 0.3 Spraying 97 μL of collagen dispersion per glass (18 × 18 mm) and 119 μL per plastic (18 × 22 mm) coverslip, respectively, resulted in a collagen density of 20 to 30 μg/cm². The coverslips were stored at 22°C for about 16 hours before use in perfusion experiments. Other proteins were coated on the coverslip by adhesion: coverslips were layered on a 100 μL drop of fibrinogen (500 μg/mL in 20 mmol/L HEPES, 150 mmol/L NaCl buffer pH 7.35, HEPES buffered saline [HBS]) or vWF (125 μg/mL in HBS) for 1 hour at room temperature or overnight at 4°C, then rinsed with and stored in HBS at room temperature until the analysis. Purification of fibrinogen was carried out according to Blomback et al. and aliquots of 10 mg/mL fibrinogen solution in 0.3 mol/L NaCl were stored at -30°C. Human vWF, purified as before was stored as a 2,500 μg/mL stock solution at -30°C. Human endothelial cells derived from umbilical veins were isolated and cultured as described. Second to fourth passage cells were grown on glass or plastic coverslips coated with gelatin fixed with glutaraldehyde. To isolate the extracellular matrix, cells grown to confluence were exposed to 0.1 mol/L ammonia for the shortest time needed to remove the cell layer completely (2 to 5 minutes). The extracellular matrix was washed three times with HBS and used the same day.

Perfusion studies. Perfs were performed in a parallel-plate perfusion chamber with two coverslip holders, a flow slit of 0.6 or 1.0 mm height and 10 mm width under pulsatile blood flow conditions. Using a roller pump with two rollers (Watson Marlow 503S; VEL, Leuven, Belgium). Average flow rates of 36 and 45 mL/min were maintained for 5 minutes resulting in shear rates of approximately 300 and 1,300 s⁻¹, respectively. The perfusate was either 10 mL of normal pooled plasma diluted 1/10, or anticoagulated whole blood to which varying concentrations of Calin were added. Single platelet disappearance was measured by counting platelets before and after perfusion with a Cell-Dyn 610 (Sequana-Turner Corp, Mountain View, CA) according to Falcon et al. Evaluation of coverslips. After perfusion, the coverslips were removed, rinsed with HBS, then treated for quantitation of collagen bound vWF or platelet deposition. For the detection of vWF, coverslips were fixed in methanol for at least 10 minutes. Then, 5 mm diameter disks were punched out and the surface-bound vWF was detected by an enzyme-linked immunosorbent assay (ELISA)-like method (in preparation) using the same reagents and antibody as under static conditions. Platelet deposition was visualized by May-Grünwald Giemsa staining or by immunostaining for GPIIb-IIIa after coverslips were fixed with formaldehyde solution, neutralized with 0.1 mol/L glycine for 10 minutes and rinsed again with HBS. For immunostaining, 2 μg/mL solution of a peroxidase labeled monoclinal antibody (MA-28K;A) and freshly prepared substrate (5% w/vjol 3.3′ Diaminobenzidine-tetrahydrochloride dihydrate, UCB, Brussels, Belgium, with 1% vol/vol H₂O₂ in HBS) were used. MA-28K-A, a monoclonal antibody raised against human platelets with specificity for GPIIb-IIIa, was conjugated with horse radish peroxidase as described. Platelet adhesion was evaluated on coverslips with a light microscope connected to an image analyzer, at an 800-fold magnification on the monitor. An average of 30 fields per coverslip were analyzed. Platelet adhesion was expressed as percent of total surface covered with platelets. The relative surface coverage (%) was defined as the surface covered at each Calin concentration normalized to values obtained in the presence of the appropriate buffer in the absence of inhibitor.

Calin binding to collagen. The presence in the Calin extract of collagen binding proteins was verified by Biospecific Interaction Analysis (BIA), using a BIAcore instrument (Phamica Biosensor, Uppsala, Sweden). Human collagen type III (20 μg/mL) was coupled to the carboxylated dextran matrix of a sensor chip in 0.1 mol/L acetate buffer pH 5.0, and the resonance signal was recorded continuously upon passage of the Calin solution (30 to 250 μg/mL) at a flow rate of 3 μL/min for 10 minutes. Rate constants were calculated using the BIAlogue software provided by the manufacturer.

Statistical analysis. The IC₅₀, the Calin concentration, which caused 50% reduction of the initial vWF binding or of the surface coverage, was calculated by exponential smoothing and significance was checked by a paired t-test using Microsoft Excel software (Brussels, Belgium).
RESULTS

Inhibition of vWF Binding to Collagen Under Static Conditions

Binding of both plasma vWF (Fig 1A) or purified vWF (Fig 1B) to calf skin collagen type I could be prevented by Calin in a dose-dependent manner. When diluted plasma was used, 3.6 to 3.9 μg/mL Calin was needed to produce 50% of the maximal effect irrespective of whether nonbound proteins present in the Calin preparation were still present or not. Using purified vWF, 3 μg/mL Calin is required to yield 50% of the maximal inhibition, provided the unbound Calin proteins were still present; when removed by washing, collagen had to be preincubated with ~19.5 μg/mL Calin to produce a similar effect. These experiments detail that Calin binding is not irreversible, but more importantly, indicate that inhibition of the vWF binding results from binding of a Calin component to the binding site on collagen. This conclusion was substantiated from the study of the real-time interaction between Calin and insolubilized collagen III by BIA.

As shown in Fig 2, Calin dose-dependently bound to the sensor chip, reaching equilibrium for the highest concentration (0.25 mg/mL) tested. Analysis of the washing phase (beyond 700 s) showed a monophasic dissociation of collagen-bound Calin described by a dissociation constant $K_{diss} = 8.8 \pm 0.3 \times 10^{-4}$ s$^{-1}$ (Fig 2, inset).

Inhibition of vWF-binding to collagen by Calin was already maximal after a 10-minute preincubation period (Fig 3).

Inhibition of vWF Binding to Collagen Under Flow Conditions

Binding of plasma vWF to calf skin collagen type I under flow conditions was inhibited by Calin in a shear stress-dependent manner (Fig 4). When diluted plasma was supplemented with Calin, 16 to 19 μg/mL Calin reduced binding of vWF to 50% at low-shear stress (300 s$^{-1}$), but the same inhibition at high-shear stress (1,300 s$^{-1}$) was achieved only at 75 to 83 μg/mL Calin.

Inhibition of Platelet Adhesion

In a series of in vitro whole blood perfusion experiments, the interference of Calin with platelet adhesion to different types of collagen, extracellular matrix (ECM), vWF, or fibrinogen was studied. Calin caused a concentration-dependent reduction of platelet deposition on equine tendon collagen at 1,300 s$^{-1}$ wall shear rate (Fig 5A). The relative surface coverage was reduced by 50% at 19 ± 3.5 μg/mL final concentration (IC$_{50}$ ± SEM). As equine tendon collagen is a mixture of different types of collagen, in a further series of experiments coverslips were coated with either type I or III human fibrillar collagen. A decreasing platelet deposition was found with increasing Calin concentrations on type I collagen both at high- (1,300 s$^{-1}$) and low- (300 s$^{-1}$) shear
Fig 3. Time-dependent inhibition by Calin of plasma vWF or purified vWF binding to collagen under static conditions. Collagen-coated wells were preincubated with 50 (○) or 195 (●) μg/mL Calin for the indicated times, after which unbound material was washed away before plasma (○) or purified vWF (●) was applied. Detection was as in Fig 1 (n = 3, mean ± SEM).

Fig 4. Dose-dependent inhibition by Calin of plasma vWF binding to collagen under flow conditions. Platelet adhesion to type III collagen was nearly completely inhibited at both shear rates (Fig 5C). The IC_{50}^- were approximately 5.1 ± 2.1 μg/mL at high and 3.0 ± 1.5 μg/mL at low-shear rate.

The dose-response curve for the inhibition by Calin of platelet adhesion to ECM of cultured human umbilical vein endothelial cells showed only a partial reduction of surface coverage by platelets. The reduction caused by 18 μg/mL Calin (highest concentration tested) at high-shear rate was 32% ± 24% (no significant decrease) and the IC_{50} was 12.1 ± 4.2 μg/mL at low-shear rate (Fig 5D). Calin had a negligible effect on platelet adhesion to coated vWF at low-shear rate, whereas at high-shear rate 12 μg/mL Calin resulted in 32% ± 18% inhibition (no significant decrease) (Fig 5E). Platelet adhesion to purified fibrinogen was not significantly affected by Calin either at high- or low-shear rates (Fig 5F).

Anticoagulation of blood with citrate or LMWH did not alter the above results (data not shown), except with type I collagen, where good platelet adhesion could only be obtained by using heparinized blood.

DISCUSSION

Earlier studies have shown that Calin suppresses collagen-induced platelet aggregation and adhesion of platelets to collagen coated on beads or on microtiter wells. This effect was specific because aggregations induced by ADP, A23187, arachidonic acid, or U44069 were hardly affected. Furthermore, Calin inhibited platelet rich thrombus formation in a hamster thrombosis model.

In addition, we show here that Calin also inhibits the binding of vWF to collagen dose-dependently. However, the inhibition, under static conditions, was not complete, which could be due to only a partial shielding of the vWF-binding sites on collagen by Calin. Because vWF has at least two collagen binding domains, it is possible that the binding of only one of these domains is blocked by Calin. It is noteworthy that not only Calin, but also rLAPP inhibits both the direct platelet collagen and the vWF-collagen-interaction, which opens the intriguing possibility that vWF and platelets may interact with the same sequence in immobilized collagen. Platelets interact with collagen mainly through glycoprotein IIa/IIb, with the I-domain of GPIa being the putative ligand binding site. Three such I-domains (A_1, A_2, A_3) are also present in vWF and the A_1 and A_2 domains are involved in collagen binding. However, at present no direct evidence for a common binding site exists. Calin interacts directly with collagen as evidenced by the observation that collagen preincubated with Calin was no longer able to support vWF binding, an effect that was maximal after a preincubation period of as little as 10 minutes. Binding studies by means of Biospecific Interaction Analysis provided direct proof that...
Calin, indeed, interacts with collagen. Furthermore, in view of the slow monophasic dissociation phase of the bound material, the active component in the Calin preparation primarily consists of a single protein.

Inhibition of vWF-binding to collagen by Calin not only occurred under static conditions, but also under conditions of flow. We were not able to obtain evidence for an effect of shear stress on vWF binding to collagen, so our data essentially show that the effect of Calin is shear-stress-dependent: at high-shear stress (1,300 s⁻¹) a fivefold higher concentration of Calin was needed to result in an effect similar to the one obtained at 300 s⁻¹. Shear-stress, however, is important in determining the mechanisms by which platelets will adhere to collagen: at high-shear stress platelets essentially bind to collagen both through collagen-bound vWF⁴⁶ and by GPIa/IIa,³⁹ in contrast to low-shear stress where the collagen receptors of platelets are believed to play a predominant role.⁵⁰ Because Calin apparently acts at both levels, it was interesting to investigate what its influence would be on platelet deposition under various flow conditions and on different substrates.

Platelet adhesion to different types of collagen was inhibited by Calin dose-dependently. Under high-shear stress, lower concentrations of Calin were needed to prevent platelet adhesion than to inhibit vWF-binding to collagen I. Although the conditions of the measurement were not fully comparable (eg, diluted plasma, absence of blood cells), this could indicate that the Calin-mediated inhibition of platelet adhesion is not primarily caused by its effect on vWF-binding, but rather by interference with the direct platelet-collagen interaction. Indeed, Saelman et al⁵² recently showed that under high-shear conditions, platelet GPIa/IIa-collagen interaction is absolutely required to obtain adequate adhesion. The picture that emerges from their work is that platelets rely on a simultaneous interaction to bind to collagen at high-shear stress, both through GP Ib-vWF and GPIa/IIa. Interfering at each level of interaction results in loss of adhesion.

Five times less Calin was needed to induce 50% inhibition of platelet adhesion to type I or III collagen, as compared with equine tendon collagen, which may indicate that Calin does not equally interact with different types of collagen, or that this equine tendon collagen may contain noncollagen type adhesive or activator components (eg, facilitating thrombin-dependent aggregate formation). Equine tendon collagen is a mixture of different types of collagens and initiates large aggregate formation on its surface more actively than any other type of isolated collagen. In comparison, rLAPP also inhibits the collagen-induced platelet aggregation and platelet adhesion to type I collagen in situic conditions.⁵⁸,⁵⁹ Under flow conditions, the platelet adhesion to type I, III, and IV was completely inhibited by rLAPP and to type VI partially inhibited by rLAPP, whereas adhesion to fibronectin or vWF was not affected by rLAPP.⁶⁰ The experiments under dynamic conditions showed complete inhibition of platelet adhesion by 3 μmol/L rLAPP to those collagens, at least at high-shear rate. At low-shear rate, at least 10 times more rLAPP was required to obtain the same effect. This study also showed that LAPP caused a pronounced inhibition of platelet adhesion on cross sections of atherosclerotic coronary arteries.

Calin showed a moderate inhibition of platelet adhesion to a more complex substrate, such as the ECM of cultured endothelial cells. At low-shear rate, where direct adhesion to collagen is dominant,⁵¹,⁵² there is a concentration dependent inhibition, but at high-shear rate, where in addition vWF mediates adhesion, thirty the inhibition is not significant despite
the previous finding that Calin also prevents vWF binding to isolated collagen. This may be explained by the fact that ECM from endothelial cells already contains bound vWF, so that uptake of vWF from the flowing blood, inhibited by Calin, is no longer required. Alternatively, Calin, similarly to rLAPP, may be less active on, eg, collagen VI, which is present in the extracellular matrix and, which ultimately may represent the more important vWF attachment site. Despite this we could show a beneficial effect of Calin in an animal thrombosis model. In this model, standardized damage is induced on the femoral vein of the hamster. Due to the nature of the damage, a platelet-rich thrombus is formed. Because low-shear stresses prevail in the venous system, it is possible that Calin here mainly prevents the direct platelet-collagen interaction. Because the damage is more severe than a superficial removal of endothelium, under these conditions additional collagens more susceptible to Calin inhibition, may become exposed. Further work is needed to clarify this issue.

In conclusion, we could show that Calin, apart from preventing the direct platelet-collagen interaction, also interferes with vWF-binding to collagen, resulting in a pronounced effect on platelet adhesion under both high- and low-shear stresses. We believe that Calin could be a very useful tool to study the contribution of platelet adhesion to collagen in different thrombosis models.

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CALIN INHIBITS VWF-COLLAGEN INTERACTION