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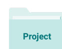
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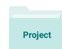
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# Calin From Hirudo Medicinalis, an Inhibitor of Platelet Adhesion to Collagen, Prevents Platelet-Rich Thrombosis in Hamsters

By Hans Deckmyn, Jean Marie Stassen, Ingrid Vreys, Elisabeth Van Houtte, Roy T. Sawyer, and Jozef Vermylen

**Interaction between exposed collagen and platelets and/or von Willebrand factor is believed to be one of the initiating events for thrombus formation at sites of damaged endothelium. Interference with this mechanism may provide an antithrombotic potential. Calin, a product from the saliva of the leech Hirudo medicinalis, was tested in vitro and for its in vivo activity in a thrombosis model in hamsters. Calin specifically and dose dependently (IC50: 6.5 to 13 μg/mL) inhibited human platelet aggregation induced by collagen. In addition, specific platelet adhesion onto microtiter wells coated with collagen and detected with a monoclonal antiglycoprotein IIb/IIIa antibody-conjugated with horseradish peroxidase, could be completely prevented with Calin (IC50: 22 μg/mL). A dose-response curve was constructed in groups of six hamsters in whom a standardized trauma was induced on the femoral vein. Thrombus formation was followed continuously using video recording and processing of the image obtained upon transillumination of the vessel. Intravenous Calin dose-dependently inhibited platelet-rich thrombus formation in this model with an ED50 of 0.07 mg/kg and complete inhibition with 0.2 mg/kg. No effects were seen on coagulation tests or bleeding times, whereas ex vivo aggregation induced by collagen was inhibited dose dependently. Local application of leech saliva, Calin, hirudin, or the combination of the latter two into the bleeding time wound of hamsters resulted in a mild prolongation of the bleeding time (twofold to threefold). A similar experiment in baboons did not cause any prolongation of the bleeding time. This is in sharp contrast with the long-lasting bleeding after a leech bite itself in both species. Calin from the leech Hirudo medicinalis is able, by binding to collagen, to effectively interfere with platelet-collagen interaction, which results in an antithrombotic effect observed in a platelet-rich thrombosis model in hamsters.**

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ACUTE CORONARY OCCLUSION after plaque rupture after successful coronary thrombolysis or angioplasty occurs in a significant number of patients and results in myocardial infarction despite therapeutic anticoagulation.[1-4] The primary role of platelet activation in coronary artery occlusion[5-7] as well as in some animal models of thrombosis[8-10] has been established. Prevention of platelet adhesion might contribute to a more efficient antithrombotic therapy.

Aspirin has only a moderate effect in preventing arterial reocclusion.[8,11,12] Many drugs, currently under investigation, were shown to be more potent than aspirin in maintaining arterial patency, and include GP IIb/IIIa receptor blockers,[13-15] selective thromboxane $A_2$/prostaglandin endoperoxide receptor antagonists,[16-20] selective thromboxane $A_2$ synthase inhibitors[21] and combined drugs.[22,23] Specific thrombin inhibitors, such as argatroban and r-hirudin, have also been found to inhibit platelet-rich thrombus formation in animal models.[24-26]

All these drugs interfere with the process of platelet aggregation; however, the formation of a platelet-rich thrombus on a damaged arterial wall is initiated by the adhesion of platelets to the exposed subendothelium, where collagen by itself or in association with von Willebrand factor is one of the more reactive substances. After this initial adhesion, the platelets undergo shape change and aggregate and secrete the contents of their granules.[27-30] Therefore, the inhibition of platelet adhesion might be of prime importance for the prevention of arterial occlusion.

So far, no pharmacologic agents are available that can effectively inhibit platelet adhesion to a damaged vessel wall in vivo, despite numerous reports on monoclonal antibodies, peptide protein fragments, or products derived from hematophagous animals that are active under various in vitro conditions.

One of these hematophagous animals, the medicinal leech (Hirudo Medicinalis) uses not only hirudin[31] to prevent thrombus formation in the bite, but also a fast and long-acting inhibitor of platelet adhesion with a direct action on collagen[32]; this substance might be responsible for the sustained bleeding in the apparent absence of hirudin.[33] Recently, an inhibitor of platelet aggregation to collagen was identified in the saliva of the medicinal leech, partially purified in small quantities and characterized as a protein with a molecular weight (Mr) of 65 kD under reducing conditions. This protein was named Calin and was found to be different from the leech collagenase. Calin interacts with collagen rather than with platelets as was evidenced by the findings that Calin causes flocculation of type I collagen fibril suspensions and, in addition, delays the autolysis of solubilized collagen.[32]

The purpose of the present study was to further evaluate the effects of Calin on human platelet aggregation and adhesion, and to evaluate its possible influence on the hemostasis, the ex vivo platelet aggregability and platelet-rich thrombus formation in hamsters.

To evaluate the latter, Stockmans et al[34] have developed a reproducible femoral vein thrombosis model in the rat that allows continuous and quantitative monitoring of mural platelet-dependent thrombus formation after endothelial damage. We have used this model in rats to study the effect of ridogrel, a combined thromboxane synthase and receptor

*From the Center for Molecular and Vascular Biology, University of Leuven, Leuven, Belgium; the Department of Orthopaedics and Hand Surgery, University of Umeå, Sweden; and Biopharm Ltd, Hendy, Dyfed, UK.*

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*Address reprint requests to Hans Deckmyn, PhD, Center for Molecular and Vascular Biology, Campus Gasthuisberg, O & N, Herestraat 49, B-3000 Leuven, Belgium.*

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blocker,[35] and in hamsters for the investigation of the antithrombotic effects of Arg-Gly-Asp–containing peptides,[36] heparin, r-hirudin, and Argatroban,[37] and for the evaluation of synergistic effects of Argatroban and a Arg-Gly-Asp–containing peptide and heparin and aspirin.[38]

## MATERIALS AND METHODS

Calin was partially purified as described.[32] The material used in this study was obtained by immersing starved leeches in 8% ethanol and leaving them to regurgitate. The thus-obtained saliva in ethanol was applied onto a CM-Sepharose FF-column (Pharmacia, Uppsala, Sweden), preequilibrated with 20 mmol/L TRIS-HCl, 10 mmol/L $CaCl_2$, 12% ethanol, pH 8.0. The Calin was eluted with a NaCl gradient and dialysed against the same buffer in the absence of NaCl. This Calin preparation is devoid of collagenase activity[32] and of any effects on coagulation tests (eg, less than 50 antithrombin U/mg). For in vitro adhesion experiments, lyophilized Calin was resuspended to obtain a stock solution of 1 mg/mL Calin in 92.3 mmol/L TRIS-HCl, 46 mmol/L $CaCl_2$, pH 8.0, 0.1% bovine serum albumin (BSA). Appropriate controls were obtained using the same final buffer. For in vitro aggregations and in vivo administration, lyophilized Calin was dissolved in 12% ethanol. Appropriate buffer controls were run in parallel.

Collagen from equine tendons was purchased from Hormon Chemie (München, Germany); adenosine diphosphate (ADP) and arachidonic acid sodium salt from Sigma Chemical Co (St Louis, MO); U 44069 (9,11-dideoxy-9$\alpha$,11$\alpha$-epoxymethano-PGF2$\alpha$) from Upjohn Co (Kalamazoo, MI); A23187 from Calbiochem Behring Corp (San Diego, CA); and thrombin from Roche (Brussels, Belgium).

### *Platelet Aggregation and Adhesion*

Platelet aggregation was followed using the optical method in an ELVI 840 dual channel aggregometer (Pabisch, Brussels, Belgium) in citrated platelet-rich plasma obtained through differential centrifugation of blood from drug-free healthy volunteers. Concentration of aggregation inducers was the minimal concentration (threshold concentration) needed to cause an aggregation starting within 1 minute after addition of the agonist and proceeding to an irreversible aggregation. Maximum velocity (millimeters/minute) and maximal amplitude (millimeter) of the aggregation tracings were measured and expressed as percentage of the value obtained in a parallel control experiment.

To determine platelet adhesion, polystyrene 96-well microtiter plates (Falcon, Lincoln Park, NJ) were coated with 150 $\mu$L/well 2.5 $\mu$g/mL equine tendon collagen in buffer supplied by the manufacturer or with 2.5 $\mu$g/mL gelatin in the same buffer for 1 hour at room temperature. Postcoating is done during 1 hour with 0.5% BSA in TRIS-HCl 5 mmol/L, NaCl 150 mmol/L, dextrose 5.5 mmol/L, $MgCl_2$ 2 mmol/L.

Blood, collected on 1/10 vol 110 mmol/L citric acid, 15 mmol/L theophylline, 3.7 mmol/L adenosine, 0.198 mmol/L dipyridamole, pH 5.0 (CTAD), was centrifuged at 150*g* for 15 minutes. The platelet-rich plasma was applied onto a Sepharose 2B column, and eluted with modified Tyrode-HEPES buffer containing 10% CTAD. The thus-obtained gel-filtered platelets were pelleted by centrifugation and resuspended in modified Tyrode-HEPES, 10% CTAD, 2 mmol/L $MgCl_2$, 0.5% BSA, pH 7.4.

The microtiter plates were incubated with 50 $\mu$L of increasing concentrations of Calin for 30 minutes at room temperature, after which 150 $\mu$L of the platelet suspension was added to obtain a final concentration of $10^5$ pl/$\mu$L. Platelet adhesion was let to proceed for 2 hours at room temperature. After a washing step, 100 ng/mL of MoAb 28K7A7-conjugated with horse radish peroxidase was added. MoAb 28K7A7 is directed against glycoprotein IIb/IIIa and has no functional effects (unpublished). After a 1-hour incubation and a washing step, color development was done using 0.4 mg/mL 1,2-O-phenylenediamine and 0.003% $H_2O_2$, stopped with $H_2SO_4$ and the light absorbance was measured at 492 nm.

### *Ex Vivo Hamster Platelet Aggregation*

All in vivo experiments were performed according to the guidelines of the International Society on Thrombosis and Hemostasis.[39]

Male hamsters (Gold, proefdierencentrum [pfd]; Katholieke Universiteit [KU], Leuven, Belgium) with a body weight of 80 to 120 g were premedicated with 1.25 mg/kg atropin and anesthetized by intraperitoneal injection of 50 mg/kg Nembutal (CEVA, Brussels, Belgium); the jugular vein was exposed and a Portex blue (Portex, Hythe, UK) catheter was introduced for injection of either vehicle (0.2 mL 20 mmol/L TRIS, 10 mmol/L $CaCl_2$, 12% EtOH, pH 8.0) or 0.1, 0.2, or 0.3 mg/kg of Calin in vehicle. Two-millitliter blood samples were collected by an abdominal aorta puncture with a 24-g needle-mounted 3 mL syringe containing 0.3 mL 3.13% citrate (final concentration, 0.017 mol/L) 20 and 40 minutes after injection. Blood was also drawn from noninjected animals. Platelet-rich plasma was prepared by centrifugation for 10 minutes at 90*g* and platelet-poor plasma by centrifugation for 10 minutes at 1,000*g*. After a platelet count on the platelet-rich plasma, the platelet number was adjusted to 200 $\times$ $10^6$/mL with platelet-poor plasma. Platelet aggregation by collagen was performed within 1 hour after blood sampling. The maximal amplitude was expressed in percent of the difference in light transmission between platelet-rich plasma and platelet-poor plasma. A collagen dose (ED50) inducing a change in light transmission corresponding to 56% $\pm$ 5 % (mean $\pm$ SEM; n = 7) of the maximal amplitude was determined with the nontreated animals. This collagen concentration, 2 $\mu$L of 1 mg/mL per 200 $\mu$L platelet-rich plasma (final concentration, 0.01 mg/mL), was then used for all subsequent samples of the treated animals. Activated partial thromboplastin times (APTT) and thrombin times (TT) were determined by routine laboratory assays on frozen plasma samples.

### *Bleeding Times in Hamsters and Baboons*

Male hamsters were anesthetized and further treated as for the ex vivo platelet aggregation studies (see above). Bleeding times were measured before, 1, 10, 30, 45, and 60 minutes after bolus injection of vehicle, 0.1, 0.2, 0.3, 0.4, or 0.6 mg/kg Calin, using an automated spring-loaded device (Surgicutt, International Tesknidyne Corporation, Edison, NJ). The region of the incision site on the abdomen was shaved, washed, razed, and dried before performance of the bleeding times. The body temperature was maintained at 37°C with a thermostated electric heating pad.

Alternatively, vehicle, Calin, hirudin, a mixture of Calin and hirudin, or reconstituted lyophylized leech saliva were applied directly into the bleeding time wound of untreated anesthesized hamsters. Five microliters of each was instilled over a 30-second period. Bleeding times on the upper-arm were also measured in three baboons after similar local application experiments and, in addition, 2 minutes after subcutaneous injections of 100 $\mu$L of 10 times concentrated leech saliva under the bleeding time site. Finally, starved leeches were let to feed on the no-hair treated abdomen of hamsters and on the shaved calf, upper-arm and back of anesthesized baboons. Bleeding times were followed for up to 30 minutes and up to 4 hours, respectively.

### *Hamster Platelet-Rich Femoral Vein Thrombosis Model*

A platelet-rich mural thrombus was produced in the femoral vein of hamsters as previously described.[34-38] Briefly the femoral vein

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was exposed and mounted on a transilluminator, the thrombus was produced by vessel clamp damage with a modified Acland traumatic vessel clamp (#ST-RD-S, S & t Micro Lab AG, Neuhausen am Rheinfall, Switzerland) and the thrombus formation was continuously monitored over 40 minutes under a Zeiss OPT1-1 operating microscope at × 30 magnification (Zeiss, Oberkochen, Germany), with video-tape recording, using a beam splitter, a black-and-white camera (RCA CCD TC104x) and a VHS video recorder (Panasonic NVG21ha; Matsushita Electric Trading, Osaka, Japan). The change over time of the light transmission through the blood vessel at the site of the trauma, which correlates highly with the platelet accumulation,[34] was continuously recorded on video-tape. Calin or vehicle were given as a bolus injection via a jugular vein canulla 5 minutes before induction of the trauma. At the end of the 40-minute observation period, a 1-mL blood sample was drawn from the abdominal aorta into citrate (final concentration, 0.017 mol/L) and the plasma was frozen for determination of the APTT and TT by routine laboratory assays.

### *Data Analysis of the Thrombosis Model*

The analytical procedure as described elsewhere[34,38] was modified to increase the performance capacity of the model, allowing on-line data analysis. The experiments are analyzed on line using a dedicated, adaptive, automated image analysis system based on an arithmetic frame grabber (DT-2862, Datatranslation, Marlboro, MA) connected to a personal computer (486/30 MHz, 8HsRAM; Laser, Zouterwoude, the Netherlands), using image processing software (TCL-Image, Multihouse TSI, Amsterdam, The Netherlands) applying an expanded routine for this specific application (Rood, Rijswijck, The Netherlands). At 10-second intervals, images are digitized on a 128- × 128-pixel matrix (indicating the thrombus surface) with a resolution of 256 gray values (indicating the thrombus volume).

The total area under the light intensity–versus–time curve was determined and the drug effect is expressed as a fraction of the one obtained in the absence of drugs.

### *Statistics*

All data are represented as mean ± SEM. The significance of the drug effects versus the vehicle were determined using the Student's *t*-test for paired or unpaired values, as appropriate.

## RESULTS

### *Inhibition of Human Platelet Aggregation and Adhesion by Calin*

Calin dose-dependently inhibited human platelet aggregation induced by threshold concentrations of equine tendon collagen (Fig 1) with an IC50 of 6.5 and 13 μg/mL when maximum velocity or amplitude is used as a measure of platelet aggregation, respectively. Calin was specific for collagen-induced aggregation because aggregations induced by threshold concentrations of A23187, ADP, arachidonic acid, and U 44069 were not significantly inhibited by the highest concentration of Calin tested (50 μg/mL) (Fig 2). Platelet adhesion studies were performed in the presence of adenosine, theophylline, and dipyridamole (see Materials and Methods) to prevent any platelet aggregation. Calin inhibited platelet adhesion to collagen dose dependently with an estimated IC50 of 22 μg/mL (Fig 3).

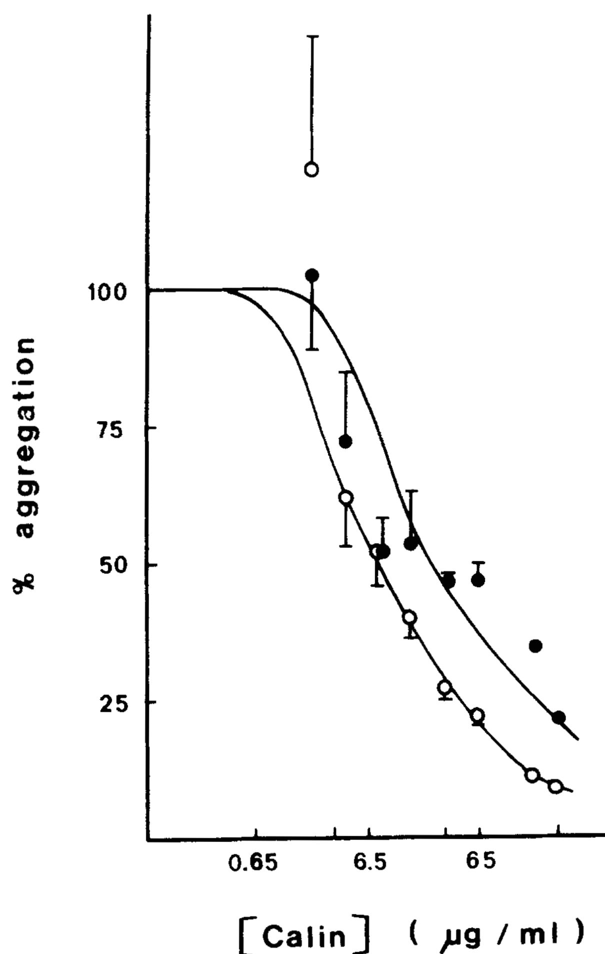


**Fig 1. Dose-dependent inhibition by Calin of collagen-induced human platelet aggregation in platelet-rich plasma. Platelet aggregation was measured as maximum velocity (○) or as maximum amplitude (●) and expressed as percentage of the respective control aggregations (n = 3 donors, mean ± SEM).**

### *Effect of Calin Administration to Hamsters on Ex Vivo Tests*

The results of the ex vivo platelet aggregations in hamsters are summarized in Table 1. Platelet aggregation 20 minutes after bolus injection of 0.1, 0.2, or 0.3 mg/kg Calin was dose dependently inhibited with 0.3 mg/kg Calin still resulting in complete inhibition of ex vivo collagen-induced platelet aggregation when measured 40 minutes after administration, implying that sufficient Calin was still present in the blood. Furthermore, only minor, nonsignificant changes in both the APTT and TT were observed (Table 1). At present, no data are available on the pharmacokinetics of Calin in hamsters.

### *Effect of Calin Administration to Hamsters on Hemostasis and Thrombus Formation*

*Bleeding times.* Intravenous bolus administration of vehicle (n = 4), 0.1 (n = 2), 0.2 (n = 3), 0.3 (n = 4), 0.4 (n = 4), or 0.6 (n = 2) mg/kg Calin to hamsters did not influence the bleeding time measured after 1, 10, 30, 45, or 60 minutes.

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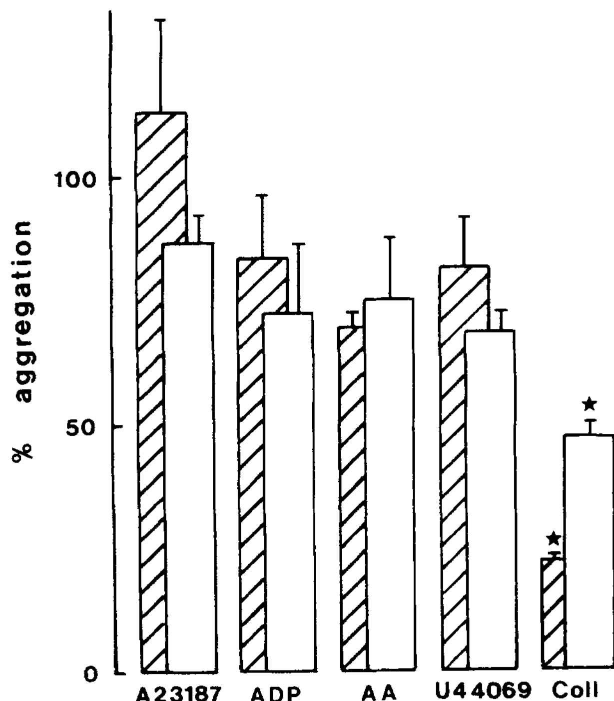


**Fig 2. Effect of 50 μg/mL Calin on platelet aggregation in human platelet-rich plasma induced by A23187, ADP, arachidonic acid, U 44069, or collagen. Platelet aggregation was measured as maximum velocity (■) or as maximum amplitude (□) and expressed as percentage of the respective control aggregations (n = 3 donors, mean ± SEM, *P < .025 v control).**

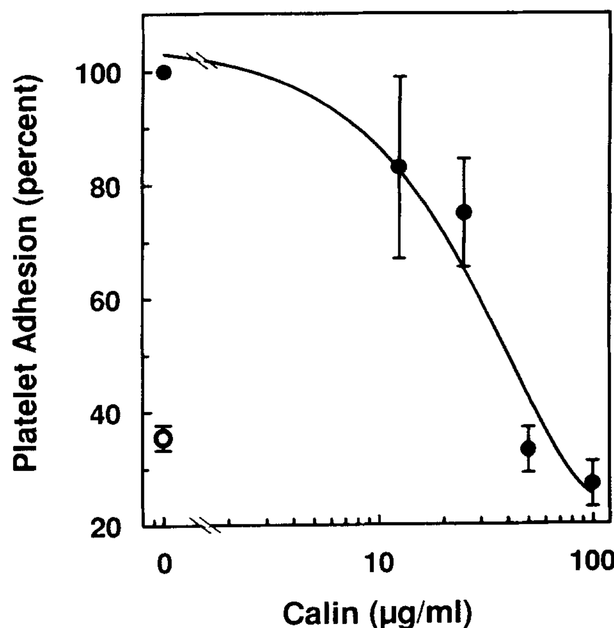


**Fig 3. Dose-dependent inhibition by Calin of human platelet adhesion to collagen. Collagen (●)- or gelatin (○)-coated wells were incubated with Calin for 30 minutes after which platelets, in the presence of platelet activation inhibitors, were added. After 2 hours, bound platelets were quantified using a peroxidase-conjugated anti-GPIIb/IIIa monoclonal antibody. Results are expressed as percentage of platelet adhesion in the absence of Calin (n = 2 to 4, mean ± SEM).**

To further study this unexpected finding, we checked whether leeches were able to cause a severely prolonged bleeding time in hamsters. When starved, leeches were left to feed on hamsters until saturated (increase in body weight from 1.1 ± 0.2 g (n = 3) before feeding to 3.1 ± 0.5 g (n = 3) after feeding with an average feeding time of 100 ± 20 minutes), bleeding from the bite wound invariably lasted for at least 30 minutes. On the other hand, local application of 5 μL reconstituted lyophylized leech saliva, 5 μL 1 mg/mL Calin, 5 μL 10 mg/mL hirudin, or the combination of the latter two did result in a significant prolongation of the bleeding time (Table 2), which, however, did not compare to the one seen after a leech bite.

Comparable findings were obtained with baboons; bleeding after a leech bite lasted for at least 4 hours (increase in leech body weight from 1.5 ± 0.1 g to 9.9 ± 0.8 g after feeding with an average feeding time of 49 ± 6 minutes [n = 9]) independently of the site of application, whereas treatment with saliva, Calin, hirudin, or the combination did not provoke a significant change whether applied into the bleeding time wound or whether injected subcutaneously at the bleeding time site on the upper arm (Table 2).

*Platelet-rich thrombus formation.* A vascular damage applied 5 minutes after intravenous bolus administration of vehicle to hamsters resulted in thrombus formation with a total light intensity of 110 × $10^6$ ± 19 × $10^6$ arbitrary units (n = 6). This was not significantly different from the results obtained in 67 animals receiving saline for which a total light intensity of 100 × $10^6$ ± 19 ×$10^6$ was found.

Calin, on the other hand, dose dependently reduced thrombus formation: administration of 0.05, 0.1, 0.15, and 0.2 mg/

**Table 1. Influence of Bolus Intravenous Administration on Ex Vivo Hemostasis Tests**

| Result | Agent | Dose (mg/kg) | Minutes After Injection 20 | 40 |
|---|---|---|---|---|
| Platelet aggregation | Vehicle | — | 42 ± 6 (5) | 32 ± 3 (3) |
| | Calin | 0.1 | 51 ± 25 (3) | 56 ± 10 (2) |
| | | 0.2 | 19 ± 3 (3)* | 39 ± 17 (2) |
| | | 0.3 | 0 + 0 (2)* | 0 + 0 (2)* |
| APTT | Vehicle | — | 34 ± 2 (7) | 41 ± 6 (3) |
| | Calin | 0.1 | 33 ± 3 (2) | 42 ± 5 (2) |
| | | 0.2 | 38 ± 3 (2) | 38 ± 1 (2) |
| | | 0.3 | 39 ± 5 (4) | 36 ± 5 (3) |
| TT | Vehicle | — | 33 ± 5 (7) | 35 ± 3 (3) |
| | Calin | 0.1 | 37 ± 2 (3) | 36 ± 1 (2) |
| | | 0.2 | 34 ± 2 (2) | 35 ± 5 (2) |
| | | 0.3 | 29 ± 1 (2) | 25 ± 9 (3) |

Platelet aggregation was induced by 10 μg/mL collagen and is expressed as percentage of the difference between the signal from PRP and PPP. APTT and TT are in seconds. Data represent mean ± SEM (n).

* $P < .05$ v vehicle.

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**Table 2. Bleeding Times in Hamsters and Baboons After a Leech Bite or After In Situ Application of Calin, Hirudin, or the Combination**

| Agent | Hamsters | Baboons |
|---|---|---|
| Control | 29 ± 2 (12) | 260 ± 90 (3) |
| Leech bite | >1,800 (3) | >15,000 (3) |
| Saliva | 53 ± 2 (15) | 190 ± 61 (3) |
| Saliva subcutaneously | — | 240 ± 30 (3) |
| Vehicle | 40 ± 2 (15) | — |
| Calin | 86 ± 6 (15)* | 205 ± 14 (3) |
| Hirudin | 72 ± 6 (9)† | 294 ± 96 (3) |
| Hirudin + Calin | 76 ± 7 (9)* | 312 ± 76 (3) |

Bleeding times (sec) were measured from the leech bite site or after application of 5 μL saline, vehicle, saliva, 1 mg/mL Calin, 10 mg/mL hirudin, or the combination into the bleeding-time wound or subcutaneously. Data are mean ± SEM (n). Data are obtained from multiple experiments on three hamsters per group or from three baboons on which four bleeding-time measurements were performed or three leeches were applied.

*$P < .001$ *v* vehicle.

†$P < .001$ *v* control.

kg resulted in 29% ± 22%, 76% ± 13%, 89% ± 5%, and 99% ± 1% (n = 6) inhibition when compared with the thrombus formation in animals receiving saline (Fig 4). The ED50 was estimated to be 0.07 mg/kg.

## DISCUSSION

In this study, we extended on previous in vitro findings on Calin, a protein isolated from the leech Hirudo medicinalis.[32] Calin interacts with collagen[32,40] and by this inhibits not only platelet aggregation induced by collagen, but also platelet adhesion to collagen. Because adhesion experiments were performed in the absence of plasma proteins, this implies that Calin blocked the binding site for the collagen receptors on platelets. However, Calin also dose-dependently prevented the binding of vWF and of platelets in plasma to collagen, but not to vWF or to fibrinogen under both static and flow conditions.[40] vWF bound to collagen is competent to bind to platelets, a process that is relevant predominantly in conditions of high-shear stress.[41] The action of Calin on both the direct and on the vWF-mediated binding of platelets to collagen can explain why Calin was effective in preventing platelet adhesion under both low- and high-shear stresses to different types of collagen mounted in a flow chamber.[40] However, although recent evidence would indicate that the main binding site might be collagen type VI,[43] there is still debate as to whether collagen is the major subendothelial attachment site for vWF.[42] The effects of Calin on platelet and vWF interaction with collagen may both be beneficial in the prevention of platelet deposition onto a damaged vessel wall.

To check this, we studied the effects of Calin administration on the formation of platelet-rich thromboses in a hamster model. Ex vivo aggregation of hamster platelets induced by collagen was dose-dependently inhibited by intravenous administration of Calin, whereas no effects were seen on clotting times. More importantly, Calin was able to strongly inhibit platelet-rich thrombus formation. Calin was administered as a single bolus injection, which seemed to be adequate because, in the present model, thrombus formation occurs maximally between 10 and 25 minutes after drug injection. These short-exposure times most probably also preclude any collagen digestion because collagen treatment with Calin in vitro for up to 24 hours at 37°C did not result in any noticeable degradation of collagen.[32] A complete inhibition of detectable platelet accumulation was obtained with 0.2 mg/kg, with an estimated ED50 of 0.07 mg/kg.

The different dose-response curves obtained for the antithrombotic effect versus the ex vivo antiaggregating action may have several causes: first, Calin may be cleared rapidly such that more Calin is available when thrombus formation is determined (maximum thrombus size occurs around 10 minutes) than at the times (20 or 40 minutes) blood was sampled. Alternatively, part of the Calin may become bound to collagenous structures in vivo, reducing the plasma levels, resulting in a weaker ex vivo action.

Despite the profound antithrombotic effects, no prolongation of the bleeding time was obtained after intravenous administration, in contrast to what is seen with all known antiplatelet drugs. It is possible that collagen-platelet and/or collagen-vWF interaction is only of minor importance in the arrest of bleeding, in contrast with other, eg, platelet-platelet interactions via the fibrinogen receptor. So far, evidence for the contribution of collagen-platelet binding in the bleeding time is anecdotal: patients with platelets with a selective defective response to collagen caused by either a deficiency of or to the presence of an inhibitory antibody against one of the putative collagen receptors: GPIa/IIa,[44-46] GPVI/62 kD,[47-49] an 88-kD protein[50] tend to have anything from mild to severe bleeding problems.

On the other hand, local application of Calin into the

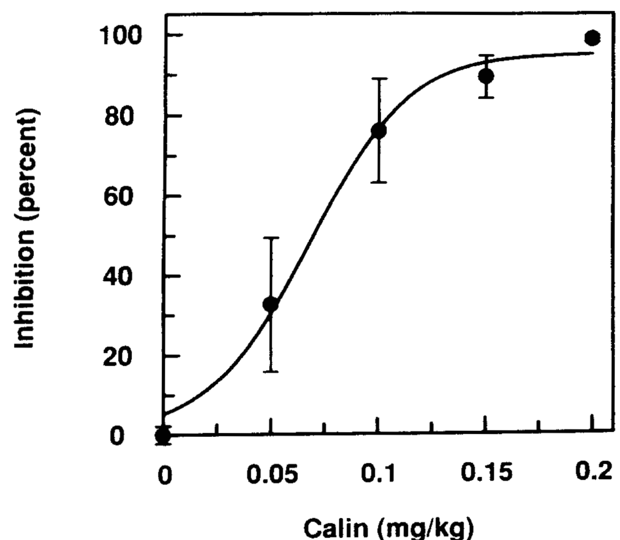


**Fig 4. Platelet-rich thrombus formation in damaged femoral veins of hamsters treated with a bolus intravenous injection of increasing doses of Calin (n = 6), expressed as percentage of the thrombus size obtained in a pool of 67 untreated hamsters.**

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bleeding time wound, resulting in a markedly higher local concentration and perhaps better mimicking a leech bite, did induce a twofold to threefold prolongation of the bleeding time. Also hirudin, and even the combination of Calin and hirudin, only gave this rather mild prolongation in hamsters. A similar experiment using identical amounts of agents in baboons failed to show any significant prolongation of the bleeding time. This is in sharp contrast with the long-lasting bleeding that was obtained when leeches were allowed to feed on either hamsters or baboons, indicating that perhaps still other components not present in the saliva of Hirudo medicinalis are required to explain the observed bleeding.[32]

Products such as Calin nevertheless should allow to differentiate between collagen-dependent and -independent platelet functions in vivo. So far, this has not been possible because inhibitory monoclonal antibodies against GPIa/IIa may not recognize platelets from nonhuman species.[51] A collagen-derived tetrapeptide was described that blocks platelet adhesion to collagen[52]; however, the utility of this peptide in other conditions remains to be determined. On the other hand, no reports are available on the in vivo effects of antibodies that interfere with the binding of vWF to collagen.[42] Furthermore, as mentioned above, because both a direct and indirect vWF-mediated binding of platelets to collagen may occur, the previous approaches, only directed at one target interaction, may be insufficient. Therefore, a compound such as the one studied here, interacting with collagen and preventing both platelet and vWF-binding, might indeed be more powerful.

Several other natural inhibitors of collagen-induced platelet aggregation and adhesion have been described such as a 50-kD protein from Bothrops atrox snake venom,[53] leech antiplatelet protein (LAPP; 16 kD) from the Haementeria officinalis leech saliva,[54,55] moubatin (17 kD) from the soft tick Ornithodoros moubata[56,57] and pallidipin (18 kD) from the saliva of Triatoma pallidipennis or assassin bug.[58] From the cloned sequences,[55,57,58] no evidence was obtained that these products are members of the same family.

In contrast with the antithrombotic effect obtained with Calin in a hamster model, in vivo administration of LAPP to baboons was unable to prevent thrombus formation on a collagen-coated graft,[59] the reason for this being unclear. However, as mentioned above, because the Calin preparation was only partially purified, we cannot completely exclude that yet another active principle may be present that would result in the antithrombotic effect.

In conclusion, we here for the first time provide evidence that products such as Calin that interfere with platelet-collagen and vWF-collagen interactions, have an antithrombotic effect on mural platelet-dependent thrombus formation at sites of endothelial damage. Because adhesion of platelets to collagen directly or through von Willebrand factor is one of the first steps of in vivo platelet activation, interference at this level may have profound effects not only on thrombus development, as shown here, but also on other platelet-dependent effects including (re)stenosis after endothelial damage, which is dependent on platelet-secretion products such as platelet-derived growth factor to some extent.[60,61]

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