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Vascular anticoagulant : a new physiological anticoagulant mechanism

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VASCULAR ANTICOAGULANT
- A NEW PHYSIOLOGICAL ANTICOAGULANT MECHANISM -

Drukkerij Groenevelt b.v. — Landgraaf
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Vascular Anticoagulant - A New Physiological Anticoagulant Mechanism -

Proefschrift

ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit Limburg te Maastricht,
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volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen op vrijdag 19 juni 1987
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door

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—'existance conditions consciousness' is true for as long
as it takes consciousness to acquire the art of
estrangement; thereafter, consciousness is on its own
and can both condition and ignore existance.—

Joseph Brodsky

Ter herinnering aan mijn moeder
aan mijn vader
aan Patricia

Dit werk is het resultaat van een wisselwerking tussen U en mij,
Ik ben U daarvoor dankbaar.

Chris

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ABBREVIATIONS

Ab	Antibodies
BS	Buffered Saline; 110 mM NaCl, 20 mM $NaHCO_3$, 4 mM KCL, 14 mM glucose, 4 mg/ml bovine serum albumine, pH 7.4
BTP	Bovine Brain Thromboplastin
CBB	Coomassie Brilliant Blue
Da	Dalton
DAB	3,3-Diamino-benzidine-tetrachloride
DEAE	Diethylaminoethyl
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOPS	1,2-Dioleoyl-sn-glycero-3-phosphoserine
EDTA	Ethylenedinitro Tetraacetic Acid Disodium Salt
F.C.	Final Concentration
FPLC	Fast Protein Liquid Chromatography
GLA	-Carboxy Glutamic Acid
HRP	Horse Radish Peroxidase
HUVE	Human Umbilical Cord Venous Endothelial Cell
K-H	Krebs-Henseleit Buffer; 120 mM NaCl, 4.7 mM KCL, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11.6 mM glucose, 25 mM $NaHCO_3$, pH 7.4
MPT-test	Modified Prothrombin Time Test
M_r	Relative Molecular Mass
PFP	Platelet Free Plasma
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Polyacrylamide Gel Electrophoresis with SDS
S2238	D-Phenylalanyl-L-pipecolylarginine-p-nitroanilide dihydrochloride
S2337	N-Benzoyl-L-isoleucyl-L-arginine-p-nitroanilide hydrochloride
TBS	Tris Buffer; 100 mM NaCl, 50 mM Tris/HCl, pH 7.9
TBSA	TBS containing 0.5 mg/ml human serum albumine
TBSE	TBS containing 2 mM EDTA
VAC	Vascular Anticoagulant

CHAPTER 1

INTRODUCTION

INTRODUCTION

Blood coagulation comprises a complex series of reactions, which is initiated following blood vessel injury. A large number of proteins have been discovered to participate in the coagulation process. To our current knowledge fits best a conceptual model of a staged cascade, in which certain proteins form a distinct reaction stage (1). The separated reaction stage can heuristically be presented as shown in fig. 1. The functional entity is the enzyme substrate complex. The enzyme, an activated coagulation factor, cleaves a specific bond of the substrate, a coagulation zymogen, which, thereby, becomes enzymatically active (2). The rate of this enzymatic reaction is extremely low but can be greatly accelerated when enzyme and substrate interact with catalyzing compounds such as nonenzymatic protein cofactors and surfaces.
Three of the vitamin K-dependent coagulation factors (II, IX, and X), which posses specific γ-carboxyglutamic acid residues (GLA, 3), are involved in two important stages in the cascade, i.e. the intrinsic X-activation (IX_a and X) and prothrombinase (X_a and II). Both reactions require calcium ions and negatively charged phospholipids (4). The acidic phospholipids exert a high impact on the kinetic parameters of the enzymatic reactions (5,6). The precise mode of action of these phospholipids is still a subject of debate (7-9). It is generally accepted, however, that for this stimulatory effect of the phospholipids, the K-related proteins have to bind to the phospholipid surface. This binding has been extensively studied, using several techniques (10-16) and appaers to be governed by the GLA-residues and by calcium ions (17).
The nonenzymatic protein cofactors V_a and $VIII_a$, which lack GLA-residues, enhance the enzymatic conversion of II and X, respectively (5,6). These protein cofactors form a 1:1 stoichiometric complex with the activated coagulation factors X_a and IX_a, thereby facilitating the binding of these enzymes to the negatively charged phospholipids (12,18,19).
The coagulation cascade is an amplifying system: in the presence of the required accesory components, a molecule of an activated

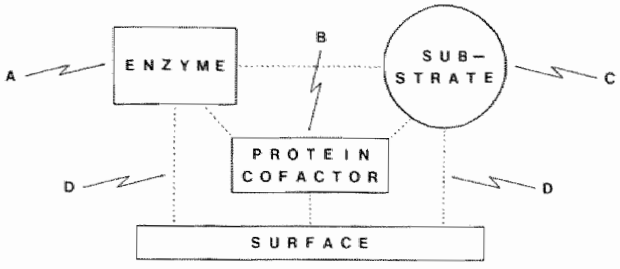

Fig. 1. Schematic representation of the structure of a reaction stage in the blood coagulation cascade.
The dotted lines represent the interactions between the different components of the complex. When the enzyme and the substrate are vitamin K-related proteins, the surface is formed by negatively charged phospholipids, and the protein cofactors are V_a (prothrombinase) and $VIII_a$ (intrinsic X-activation).
The arrows indicate the sites of action of 4 different anticoagulant mechanisms (A,B,C, and D), which are discussed in the text.

coagulation factor in the top of the cascade enables the formation of a large number of molecules of thrombin, the ultimate enzymatically active product of the cascade. In vivo, a minor activation of the coagulation cascade would, thus, result in a clotting of the entire blood. Anticoagulant mechanisms, however, strengthen the hair of this sword of Damocles.

On basis of their site of action, the anticoagulant mechanisms can be divided into 4 major groups (fig. 1).

A). This large group of anticoagulants comprises the plasma protease inhibitors. They bind apparently irreversibly in a time dependent process to an activated coagulation factor, which, thereby, becomes inactive (20).

Antithrombin III, the anticoagulant activity of which is enhanced by heparin (21), is a well studied member of this group. The vital role of antithrombin III as a physiological anticoagulant is indicated by the fact that patients with congenital antithrombin III deficiency, frequently develop thrombotic diseases (22-24).

B). With the isolation and characterization of the vitamin K-related protein C, a new anticoagulant mechanism was discovered (25). This pathway appeared to comprise a complex series of reactions, involving several plasma and tissue proteins. Protein C, which circulates in the plasma as a zymogen, is efficiently activated by thrombin in the presence of a cell surface-bound protein, thrombomodulin (26). The activated protein C rapidly cleaves the protein cofactors V_a (27) and $VIII_a$ (28) in the presence of protein S (29) and an, as yet undefined, compound on the endothelial cell surface (30). The cleaved protein cofactors are inactive in the coagulation process. The physiological significance of the protein C anticoagulant pathway is illustrated by the repeated thrombotic episodes occurring in several families with congenital reduction in the antigenic levels of protein C (31,32).

C). Vitamin K is a prerequisite for the posttranslational formation of GLA-residues in the K-related coagulation factors (33-35). A deficiency of this vitamin induces the synthesis of anomalies of these coagulation factors (36,37). These anomalies lack GLA-residues and are, therefore, poor substrates in the coagulation process (38). In contrast to the first two mentioned anticoagulant mechanisms, the vitamin K-deficiency is not a physiological mechanism, but is introduced in man by the administration of vitamin K-antagonists and serves the purpose of decreasing thrombotic tendency in patients (39).

D). This group of anticoagulants comprises compounds that interfere with the interaction of the clotting factor with a surface. On basis of the different types of interaction, this group can be divided into 4 subsets.

I. To this group belong some basic phospholipases that bind to a phospholipid bilayer in a calcium-independent way. They penetrate this bilayer and hydrolyze various of its components. The anticoagulant action of the phospholipases is explained by the occupation of the phospholipid surface and by changing the surface properties as a result of hydrolysis (40,41). The phospholipases investigated so far originate

from viper venoms. At the moment it is not clear whether this mechanism is of physiological importance in man.

II. This second subset comprises the lupus anticoagulant, which indicates a heterogenous group of acquired immunoglobulins, mostly of the IgG and IgM type, which may bind to procoagulant phospholipids, and which act as inhibitors of in vitro clotting tests (42). The binding of lupus anticoagulants to negatively charged phospholipids has recently been demonstrated (43,44). Moreover, this interaction inhibits the binding of II and X to these phospholipids (43). Lupus anticoagulant is rarely associated with hemorrhage (45) but has frequently been observed in an apparently paradoxical association with thrombosis (46-48). One of the explanations for this phenomenon is that the interaction between lupus anticoagulant with platelets and endothelial cells may result in a thrombosis predisposing situation (49). Another explanation says that the lupus anticoagulant inhibits other anticoagulant mechanisms, like the endothelial prostacyclin production (50) and the endothelial thrombomodulin activity (44).

III. GLA - containing fragments of II and X constitute this third subset of anticoagulants. These fragments have been shown to inhibit certain procoagulant reactions. Fragment 1 inhibits the tissue factor pathway (51) and in particular the X_a-catalyzed prothrombin activaton (52). The inhibitory effect is due to an interaction of fragment 1 with the phospholipid surface, that results in a competition with the II binding and X_a binding to this surface (52). Calcium and the fragment 1 GLA-residues are involved in this anticoagulant effect.

A GLA-rich peptide, derived from the light chain of X, is also an inhibitor of the phospholipid dependent procoagulant reactions (53). The anticoagulant effect of this peptide is totally dependent on the presence of the GLA-residues.

IV. This final anticoagulant mechanism is presented and discussed in extenso in this thesis. This new anticoagulant mecha-

nism is introduced by the discovery of the anticoagulant protein VAC (vascular anticoagulant) in human umbilical cord arteries (chapter 2). VAC does not contain GLA-residues, but nevertheless shows a calcium dependent high affinity binding to negatively charged phospholipids. As a result of this binding VAC interferes with the coagulation factor - phospholipid interaction. Consequently, VAC inhibits the phospholipid dependent procoagulant reactions (chapters 3 and 4). VAC is demonstrated to be present in endothelial cells as an anticoagulant active moiety (chapter 5), which can inhibit the procoagulant activity of vessel wall structures (chapter 6). Hence, VAC provides an anticoagulant mechanism, which can control thrombin formation at the vessel wall lining. Its mechanism of action suggests, moreover, that VAC may be developed into a chemically relevant antithrombotic agent without the risk of bleeding.

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CHAPTER 2

ISOLATION AND PARTIAL PURIFICATION OF A NOVEL ANTICOAGULANT FROM ARTERIES OF HUMAN UMBILICAL CORD

ISOLATION AND PARTIAL PURIFICATION OF A NOVEL ANTICOAGULANT FROM ARTERIES OF HUMAN UMBILICAL CORD

Reutelingsperger, C.P.M., Hornstra, G., and Hemker, H.C.

SUMMARY

An anticoagulant fraction was isolated from the homogenate of human umbilical cord arteries, using Sephadex gel filtration and DEAE-Sephacel chromatography. Analysis with dodecyl sulfate/polyacrylamide gel electrophoresis and inactivation studies using proteolytic enzymes indicate that the anticoagulant activity is associated with a polypeptide with an apparent M_r of 32000. The anticoagulant inhibits thromboplastin as well as factor X_a induced clotting but does not affect thrombin initiated fibrin formation. The anticoagulant inhibits the activation of prothrombin by the complete prothrombinase complex, by phospholipid bound factor X_a but not by free factor X_a. The inhibition is instantaneous and independent of the incubation time over the whole range of concentrations tested. Therefore, the anticoagulant is unlikely to be a phospholipase or a protease. Its action does not resemble that of the plasma protease inhibitors, but it probably interferes with the phospholipid-clotting factor interactions.

INTRODUCTION

The coagulation cascade, wich has been studied extensively in the last decades, is now considered to be a multistage amplifying system of several linked proteolytic reactions in which an enzym converts a zymogen into its active form (for review see ref. 1). The rate of these reactions is considerably enhanced by the presence of phospholipids and protein cofactors (factor V_a, factor $VIII_a$) (2,3). In vivo, the procoagulant reactions are controlled by several inhibitory mechanism which prevent an explosive thrombotic event upon minor activation of the coagulation cascade.

Until now the three major anticoagulant mechanisms have been recognized (for review see ref. 4). (a) The serine protease factor X_a and thrombin are inactivated as a result of their binding to antithrombin III (5) or, more efficiently, to antithrombin III-heparin complex. Both prothrombin activation and fibrin formation can thus be inhibited. Apart from antithrombin III several other plasma protease inhibitors exist (α_2-macroglobulin, α_1-proteinase inhibitor (6)). They all act in a time-dependent manner. (b) The discovery of protein C (7) has lead to the understanding of another anticoagulant mechanism. Protein C, once activated, functions as an anticoagulant through the selective proteolysis of the protein cofactors, factor V_a (8) and factor $VIII_a$ (9), thus inactivating prothrombinase and the factor-X-converting enzyme. (c) Plasmin cleaves fibrin l monomer, a product of the action of thrombin on fibrinogen, thereby preventing the formation of insoluble fibrin (10).

In this paper we report the isolation of a protein fraction from human unbilical cord arteries that instantaneously inhibits the activation of prothrombin by prothrombinase, i.e. factor X_a, factor V_a, phospholipids and CA^{2+}. This inhibition leads to a prolongation of the prothrombin time. We demonstrate that the anticoagulant mechanism is unlike those mentioned in this introduction.

MATERIALS AND METHODS

The chromogenic substrate S2238 was purchased from AB Kabi Diagnostica, Stockholm, Sweden. DEAE-Sephacel, Sephadex G-100 and G-75 were products of Pharmacia. Chemicals for the analytical gel electrophoresis were from Bio-Rad. Human blood was collected by venepuncture in trisodium citrate (final concentration about 13 mM citrate) and centrifuged at 2000 x g for 10 min at room temperature. The resulting plasma was centrifuged at 10000 x g for 15 min in order to obtain platelet-free plasma. A standard pool of platelet-free plasma was prepared by mixing plasma obtained from several healthy donors.

Homogenization of human umbilical cord arteries - Human umbilical cords were obtained within 15 min after delivery. The arteries were immediately perfused with icecold TBS, subsequently prepared free from the Jelly of Warton and homogenized in TBS with a wirl mixer, Braun MX32. A 10% homogenate (w/v) was fractionated as described in Results.

Gel electrophoresis - Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (11) on slab gels containing 10% acrylamide, 0.27% N,N^3-methylene-bisacrylamide, and 0.1% sodium dodecyl sulfate. The gels were silver-stained according to the method of Merril et al.(12).

Modified prothrombin time test (MPT-test) - The modified prothrombin time test (MPT-test) was carried out as follows. In a siliconized glass cuvette 50 μl platelet-free plasma was stirred at 37 ^{o}C with 150 μl TBS, 25 μl of a standard human brain thromboplastin dilution, and 25 μl TBS (control) or 25 μl of a fraction of the arterial homogenate. After incubation for 3 min, coagulation was started at time zero with the addition of 250 μl Ca^{2+} buffer (80 mM NaCl, 20 mM $CaCl_2$ and 10 mM Tris/HCl pH 7.9). Fibrin formation was monitored optically with a Payton Dual Aggregation Module (13). When factor X_a was utilized to initiate

coagulation in the MPT-test, thromboplastin was omitted and 25 μl purified factor X_a was added together with the 250 μl Ca^{2+} buffer to the diluted platelet-free plasma.

Modified thrombin time test (MT-test) - This assay was carried out similarly to the X_a-initiated MPT-test described above, with the exception that the X_a preparation was replaced by 25 μl of purified thrombin.

Proteins - Protease type I and trypsin were obtained from Sigma. Thromboplastin was prepared from human brain as described by van Dam-Mieras et al. (14). Factor X_a, prothrombin and thrombin were purified from citrated bovine blood as described by Rosing et al. (2). Factor V was purified from bovine blood as described by Lindhout et al. (15). Factor V_a was obtained by incubation factor V with thrombin (15). Prothrombin concentrations were calculated using an M_r of 72,000 and $A_{280}^{1\%}$ of 9.6 (17). Factor X_a and thrombin concentrations were determined by active site titration (2). Other protein concentrations were determined as described by Lowry et al. (18).

Preparation of phospholipid and phospholipid vesicles - DOPS and DOPC were prepared as described by Rosing et al. (2). Single bilayer vesicles composed of DOPS/DOPC (molar ratio 20:80) were prepared by sonication (2). Phospholipid concentrations were determined by phosphate analysis according to the method of Boettcher et al. (19).

Measurement of prothrombin activation - The time course of prothrombin activation was examined at different concentrations of the anticoagulant. Mixtures of X_a with Ca^{2+}, X_a with phospholipids and Ca^{2+} or X_a with V_a, phospholipids and Ca^{2+} were stirred with different amounts of the anticoagulant at 37 ^{o}C in 50 mM Tris/HCl, 175mM NaCl, 0.5 mg/ml human serum albumin at pH 7.9. After 3 min, Prothrombin activation was started by the addition of prothrombin. At different time intervals, 25 μl was

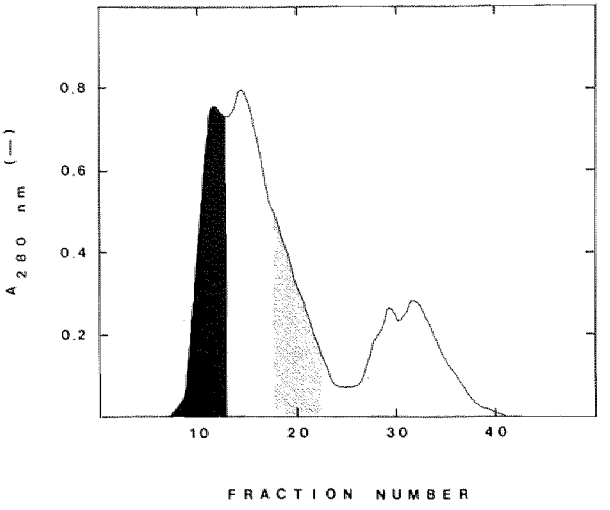

Fig. 1. Gel filtration of the 10,000 x g supernatant of an umbilical cord artery homogenate on Sephadex G-100. 2ml of the 10,000 x g supernatant was loaded on a Sephadex G-100 column (1.5 x 80 cm), which was preequilibrated with TBS. The column was eluted with TBS. Aliquots of the resulting fractions were tested in the MPT-test. Certain fractions (dark-shaded area) express a procoagulant activity and initiate coagulation in the MPT-test without the addition of thromboplastin, factor X_a, or thrombin. Other distinct fractions (light-shaded area) prolong the clotting time in the MPT-test, using thromboplastin to initiate coagulation. These fractions were pooled and further fractionated.

sampled from the reaction mixture into a cuvette (thermostated at 37 ^{o}C), containing TBS, 2mM EDTA and 0.23 mM S2238 (final volume 1 ml). From the absorption change at 405 nm, measured with a Kontron Spectrophotometer Uvikon 810, and a reference curve, made with known amounts of purified thrombin, the amount of thrombin formed was calculated at different concentrations of the anticoagulant. The phospholipids were added as vesicles composed of DOPS/DOPC with a molar ratio of 20:80.

RESULTS

Isolation - Fractionation of the supernatant of a 10,000 x g spin

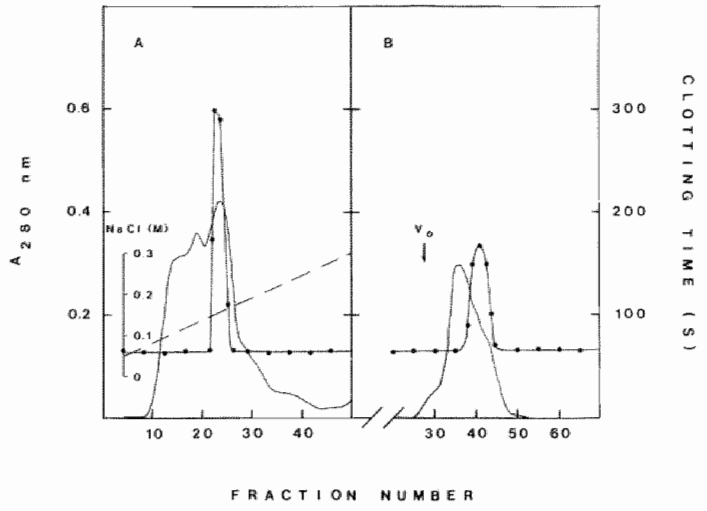

Fig. 2. Chromatography of the anticoagulant on DEAE-Sephacel (A) and Sephadex G-75 (B).
The pool containing the anticoagulant from the Sephadex G-100 column was applied to the DEAE-Sephacel. Elution was performed with a 200 ml linear gradient of 50-300 mM NaCl (----). Fractions (4ml) were collected and assayed for A_{280} (——) and for anticoagulant activity in the MPT-test, using thromboplastin (final concentration 95 μg protein/ml) as initiator of coagulation (●). The fractions with anticoagulant activity were pooled, concentrated and subsequently applied to Sephadex G-75 (B). Fractions (2ml) were collected and assayed for A_{280} (——) and for anticoagulant activity (●). V_o represents the void volume of the column.

of an umbilical cord artery homogenate on Sephadex G-100 results in a reproducible specific elution profile (Fig.1).
The fractions affecting the coagulation system as measured with the MPT-test (see Materials and Methods) are indicated in Fig. 1. A procoagulant activity eluted with the void volume. This activity can only be detected in the presence of factor VII in the MPT-test, as indicated by experiments in which human congenital factor VII-deficient plasma was used (data not shown). Therefore this procoagulant must be considered to be tissue thromboplastin. Certain fractions expressed a distinct anticoagulant activity.

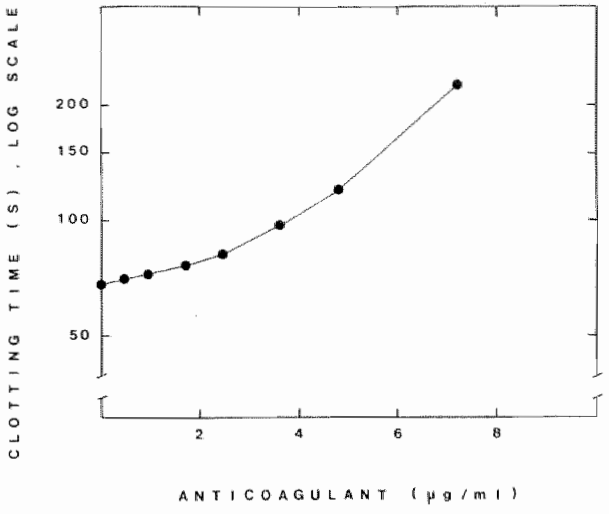

Fig. 3. Dose response of the anticoagulant in the MPT-test. Varying amounts of the anticoagulant were added to the MPT-test. Coagulation was initiated with thromboplastin (final concentration 95 μg protein/ml). The control clotting time was 65 s.

These fractions were pooled and further purified with DEAE-Sephacel chromatography (Fig.2A).

The anticoagulant appeared to bind to the DEAE-Sephacel at 50 mM NaCl and 50 mM Tris/HCl pH 7.9. Elution of the activity with a linear gradient of NaCl at pH 7.9 was achieved at 150-160 mM NaCl. The DEAE-Sephacel fractions expressing anticoagulant activity were pooled and submitted to Sephadex G-75 gel filtration (Fig. 2B). The column (1.5 x 50cm) was equilibrated with TBS. The activity appeared in the fractions which correspond to molecular masses of 30-60 kDa.

The MPT-test is used as a quantitative assay for the determination of the amount of anticoagulant activity (Fig.3). We define one unit as the anticoagulant activity that prolongs clotting time in the MPT-test, using thromboplastin (final concentration 95 μg protein/ml) as initiator of coagulation, from its control value of 65 s to 100 s. With this assay it is calculated that from 10 g wet arterial tissue 2 mg protein with approximately

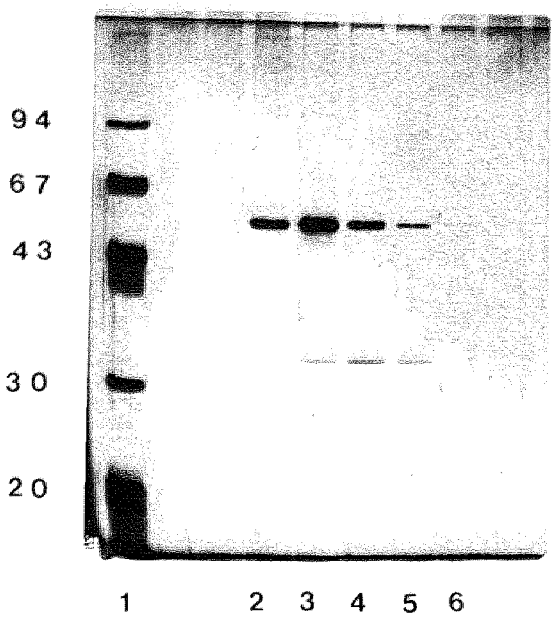

Fig. 4. Gel electrophoresis of several fractions of the Sephadex G-75 eluent.
A fixed amount of several fractions of the G-75 eluent were submitted to gel electrophoresis as described in Materials and Methods. The gels were silver-stained according to Merril et al. (12). Lane 1; reduced low molecular mass standards (in kDa); lane 2-6 unreduced aliquots of the G-75 fractions with numbers 35, 39, 41, 43 and 50 respectively.

1200 units anticoagulant activity are isolated.

Characterization - Several fractions of the Sephadex G-75 chromatography were tested in the MPT-test and analyzed with SDS-PAGE. The results (Fig.4) suggested that the anticoagulant has a molecular mass of approximately 32 kDa. The association between the anticoagulant activity and the 32-kDa band was confirmed by slicing the polyacrylamide gel and subsequent elution of the proteins out of the slices with TBS, containing 0.5 mg/ml bovine serum albumin. An anticoagulant activity is present only in the eluent of the slice that corresponds to the 32-kDa band. Furthermore this activity is thermolabile at 56 ^{o}C and demonstrates a similar dose-response relationship in the MPT-test as the starting material.

The G-75 fractions, containing the peak anticoagulant activities were pooled and used for further characterization of the anticoagulant. Incubation of the anticoagulant at 56 ^{o}C rapidly decreases its activity until after 2 min no activity can be measured. The anticoagulant loses its activity completely within 2 h upon incubation at 37 ^{o}C with protease type I, whereas trypsin hardly inactivates the anticoagulant after an incubation period of 3 h (Fig.5). The concentrations of protease type I and trypsin, used

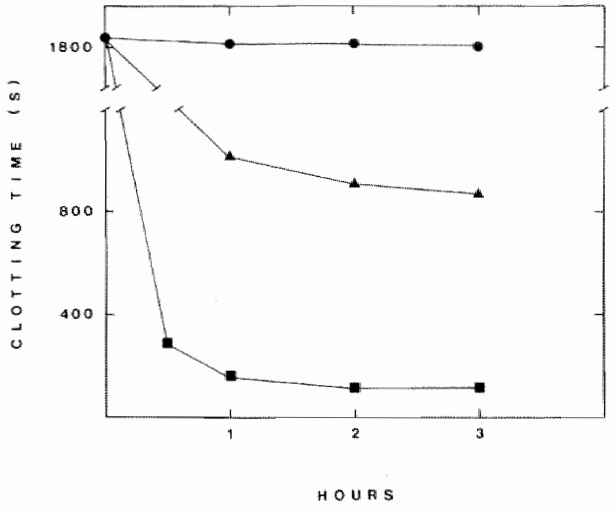

Fig. 5. The effect of proteolytic enzymes on the activity of the anticoagulant.
The anticoagulant was incubated at 37 °C with protease type I (■ , final concentration 0.11 units/ml), trypsin (▲ , final concentration 88 BAEE units/ml) and without proteolytic enzymes (●). At the time points indicated, 5 μl, containing 6 μg protein of the anticoagulant, was removed from the reaction mixture and added to a MPT-test. Clotting was initiated with thromboplastin (final concentration 18 μg protein/ml). Control clotting time was 110 s. The units given in this legend for the proteolytic enzymes are calculated from the values supplied by the manufacturer.

in these experiments, completely inactivate 2.5 nM thrombin in 15 min (data not shown). The amounts of protease type I and trypsin, carried over from the reaction mixtures to the MPT-test, have no effect on the control clotting time.

Similar material could be obtained from human, bovine, rabbit and rat aorta and from a strongly vascularized tissue like lung. It could not be obtained from a poorly vascularized tissue like human diaphragm.

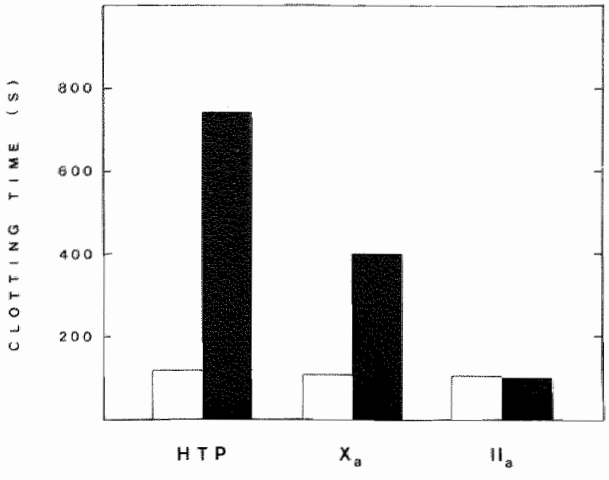

Fig. 6. The effect of the vascular anticoagulant on the clotting times, induced in the MPT-test by either thromboplastin, factor X_a or thrombin.
The concentrations of the initiators of coagulation (thromboplastin 18 μg protein/ml, 1.5 nM factor X_a or 0.4 nM thrombin) were chosen such to give control clotting times of about 110 s (open bars). When factor Xa was used, phospholipids vesicles (final concentration 10 μM), composed of DOPS/DOPC (molar ratio, 20:80) were added to the reaction mixture. Clotting times induced by the indicated agents in the presence of 3.5 μg protein of the anticoagulant are presented by the black bars.

Mode of action - The MPT-test is prolonged in the presence of the anticoagulant (Fig.6) both when triggered with thromboplastin and when started with factor X_a. Thrombin-induced coagulation, however, is not inhibited.
Because of these findings we investigated the effect of the anticoagulant on the conversion of prothrombin to thrombin by factor X_a, factor V_a, phospholipids and Ca^{2+}. Under the experimental conditions mentioned, thrombin formation is inhibited by the anticoagulant in a dose-dependent way (Fig. 7A). The activation of prothrombin by factor X_a, phospholipids and Ca^{2+} in the absence of factor V_a can also be inhibited by the anticoagulant (fig. 7B). However this inhibition is not observed if the

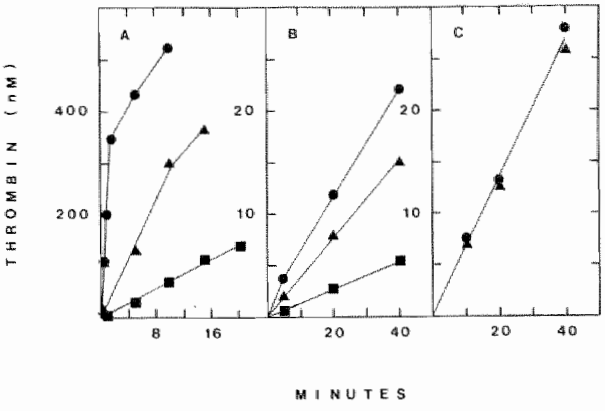

Fig. 7. The effect of the anticoagulant on prothrombin activation by X_a with V_a, phospholipids and Ca^{2+}; X_a with phospholipids and Ca^{2+} and X_a with Ca^{2+}.
The reaction mixtures contained: (A), 1 μM prothrombin, 0.3 nM X_a, 0.6 nM V_a, 0.5 μM phospholipids and 10 mM $CaCl_2$ with 12.0 μg/ml anticoagulant (■), 4.8 μg/ml anticoagulant (▲) and without anticoagulant (●). (B) 1 μM prothrombin, 10 nM X_a, 0.5 μM phospholipids and 10 mM $CaCl_2$ with 2.4 μg/ml anticoagulant (■), 0.48 μg/ml anticoagulant (▲) and without anticoagulant (●). (C) 1 μM prothrombin, 75 nM X_a and 10 nM $CaCl_2$ with 120 μg/ml anticoagulant (▲) and without anticoagulant (●). At the times indicated samples were removed and thrombin was determined as described in materials and methods.

activation takes place in the absence of phospholipids (Fig. 7C).

DISCUSSION

In this paper we describe the isolation procedure of a novel anticoagulant from a homogenate of human umbilical cord arteries. The anticoagulant has been discovered by its ability to prolong the clotting time in a prothrombin time test. The anticoagulant activity became measurable after Sephadex G-100 fractionation of the arterial homogenate. From further isolation procedures we assume this activity to be associated with a water-soluble substance(s), that carries an overall negative charge at pH 7.9.

Analysis of the Sephadex G-75 fractions (the final purification step we performed so far) with gel electrophoresis shows a positive correlation between the intensity of the 32-kDa band and the prolongation of the clotting time as measured with the MPT-test. The identity of the 32-kDa band with the anticoagulant activity is demonstrated unambiguously since the anticoagulant activity can only be eluted from the site of the 32-kDa band on the polyacrylamide gel. From these findings and the fact that the anticoagulant rapidly loses its activity upon incubation at 56 ^{o}C and proteolytic enzymes can destroy its activity, we assume the anticoagulant activity to be expressed by a single protein with an apparent molecular mass of 32 kDa.

Trypsin, in contrast to protease type I, is a poor inactivator of the anticoagulant. This suggests that the anticoagulant possesses a small number of lysine and arginine residues that are accessible for trypsin.

We studied the nature of the anticoagulant activity by initiating coagulation in different ways. Clotting, induced by either the vascular procoagulant, thromboplastin, or factor X_a, is inhibited by the anticoagulant; thrombin-induced clotting, on the other hand, is not. From these findings we conclude that the anticoagulant interferes with thrombin formation, not with thrombin action.

To study the anticoagulant mechanism of the compound further we have used prothrombinase reconstituted from purified coagulation factors and phospholipids (2). Under the experimental conditions mentioned, the anticoagulant can inhibit the activation of prothrombin by complete prothrombinase (factor X_a, factor V_a, phospholipids, Ca^{2+}) but not by free factor X_a (factor X_a, Ca^{2+}). The time courses of prothrombin activation in the presence of the anticoagulant indicate an instantaneous inhibition of prothrombin activation, that remains constant in time. Therefore, we conclude that the anticoagulant does not act by phospholipase or by proteolytic activity. The fact that the activation of prothrombin by factor X_a and Ca^{2+} is not affected by the anticoagulant at all strongly indicates that the anticoagulant mechanism of the

vascular compound differs from that of the well known plasma protease inhibitors such as antithrombin III. Since Walker at al. (8) have demonstrated that activated protein C does not inhibit prothrombin activation by factor X_a, Ca^{2+} and phospholipids, it can also be concluded that our compound is not protein C either. Preliminary binding studies indicate that the vascular anticoagulant probably interferes with the lipid binding of factor X_a and/or prothrombin. Whether the ability of the anticoagulant to inhibit prothrombin activation completely accounts for its prolonging effect on the prothrombin time remains to be established. The fact that we could find this inhibitor in various types of arteries but not in a poorly vascularized tissue may indicate that we have found a physiological modulator of hemostasis and thrombosis, active at the vascular level.

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CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF A NOVEL PROTEIN FROM BOVINE AORTA, THAT INHIBITS COAGULATION

PURIFICATION AND CHARACTERIZATION OF A NOVEL PROTEIN FROM BOVINE AORTA, THAT INHIBITS COAGULATION

Reutelingsperger, C.P.M., Kop, J.M.M., Hornstra, G., and Hemker, H.C.

SUMMARY

A novel inhibitor of blood coagulation has been isolated from the intima of bovine aorta. The inhibitor (VAC) is purified to an active fraction, that contains two Coomassie Blue staining bands (M_r=34,000 and M_r=32,000, as judged by sodium dodecyl sulfate polyacrylamide electrophoresis). Both bands are single chain proteins, having no glycoprotein features. Furthermore, they do not contain any detectable γ-carboxyglutamic acid residues. Both proteins have an identical isoelectric pH of approximately 4.5.
VAC binds in the presence of calcium-ions to a bilayer consisting of 20% dioleoyl phosphatidylserine and 80% dioleoyl phosphatidylcholine with a $K_d=6x10^{-9}$ M. The binding is depended on the calcium concentration: half saturation of binding occurs at a calcium concentration of 0.8 mM. The binding is completely reversible with EDTA. Furthermore the phospholipid to VAC ratio at saturation was n=112 and n=32 moles /mole for 0.5 mM Ca^{2+} and 2 mM Ca^{2+}, respectively. Binding does not occur between VAC and pure dioleoyl phosphatidylcholine.
In a system with purified coagulation factors VAC inhibits the activation of prothrombin by factor X_a and calcium only in the presence of negatively charged phospholipids. VAC decreases the V_{max} and increases the K_m of the factor X_a-catalyzed prothrombin activation. Based on these results we conclude that we have purified from bovine aortic intima an anticoagulant protein, which exerts its activity through a calcium dependent binding to negatively charged phospholipids, and thus interfering with the formation of prothrombinase.

INTRODUCTION

The enzymes, active in the process of blood coagulation, belong to the class of serine-proteases, and circulate in the blood as nonenzymatic precursors, the zymogens (1-3). The activation of such a zymogen proceeds in a proteolytic process, in which an already activated coagulation factor cleaves a specific bond of its substrate, a zymogen (4). The rate of this reaction is extremely low, but can be greatly enhanced by the presence of certain nonenzymatic cofactors, which can be divided into protein cofactors and negatively charged surfaces. The factor X_a-catalyzed activation of prothrombin, for example, which is a well documented reaction in blood coagulation, is accelerated by the protein cofactor V_a and by negatively charged phospholipids (5-7).

The procoagulant reactions are regulated by physiological anticoagulants, which can be classified, on basis of their mechanisms of action, into:

1) Inhibitors of the serine-proteases. Antithrombin III, α_2-macroglobulin, and α_1-antitrypsin can be assigned to this group of anticoagulants (8-10).

2) Inhibitors of the nonenzymatic cofactors. Activated protein C proteolytically cleaves the protein cofactors V_a and $VIII_a$, and thereby inhibits the prothrombinase complex and the intrinsic factor X activation, respectively (11,12). Some phospholipases A_2 and some Lupus anticoagulant species, which show a calcium independent binding to negatively charged phospholipids, have been described as anticoagulants, inhibiting the phospholipid dependent procoagulant reactions (13,14). However, at the moment, activated protein C is the only physiologically relevant anticoagulant, able to be classified into this second group.

Recently, we have discovered in human umbilical cord arteries a protein (Mr=32,000), with an anticoagulant activity, which suggested a classification of this protein into the above mentioned second group (15). A similar protein has now been found in bovine aortic intima. This paper reports on the purification of

this protein, which we have termed VAC (vascular anticoagulant). It is shown that VAC binds reversibly in the presence of calcium ions to negatively charged phospholipids, and thereby inhibits the phospholipid dependent activation of prothrombin by factor X_a. Furthermore, VAC has neither a phospholipase- nor a proteolytic activity. Based on these findings, we conclude that VAC is a novel type of anticoagulant, which can be assigned to the above mentioned second group of anticoagulants. Part of this work has been presented earlier (16).

MATERIALS AND METHODS

Bovine aorta's were removed from the animals within half an hour after they had been slaughtered. Bovine blood was collected in trisodium citrate (final concentration 0,38%) and centrifuged at 2,000 x g for 10 minutes at room temperature. The platelet poor plasma was recentrifuged for 15 minutes at 10,000 x g, to obtain PFP. Hydroxylapatite and the chemicals for the analytical SDS-PAGE were from Bio-Rad (Richmond, California). Sephadex G-75, DEAE-Sephacel, and the Low Molecular Weight calibration kit were purchased from Pharmacia (Uppsala, Sweden). The chromogenic substrates S2238 and S2337 were from Kabi Vitrum. Diaflo PM-10 ultrafiltration devices and membranes were obtained from Amicon.

Purification of VAC - After the aorta's had been removed from the animals, they were rinsed thorougly with TBS. The intima was dissected from the aorta and homogenized with a whirl mixer, Braun MX32, in TBSE, containing soybean trypsin inhibitor (16 mg/l) and benzamidine (1.57 g/l). The 20% (w/v) homogenate obtained from 8 aorta's was centrifuged at 100,000 x g for 60 minutes. The supernatant was brought to 35% saturation with solid ammonium sulfate, stirred for 30 minutes, and centrifuged for 20 minutes at 12,000 x g. This procedure was repeated with the resulting supernatant at 90% ammonium sulfate saturation. The pellet was suspended in a small volume of TBS and dialyzed against TBS, containing benzamidine (1.57 g/l). The dialyzed

fraction was applied to a hydroxylapatite column (3x5 cm), which had been equilibrated with TBS. After washing, VAC was eluted from the column with a 200 ml linear gradient of 0-500 mM sodium phosphate buffer (pH 7.5). The VAC containing fractions were pooled and dialyzed against 50 mM NaCl, 20 mM Tris/HCl, and pH 7.5, which buffer was also used to equilibrate a 3x5 cm DEAE-Sephacel column. The dialyzed VAC-pool was applied to the DEAE-column. VAC, bound to this column, could be eluted with a 200 ml linear gradient of 50-300 mM NaCl in 20 mM Tris/HCl, pH 7.5. The eluted VAC was concentrated in an Amicon concentration device, using a PM-10 ultrafiltration membrane. Finally, the VAC-concentrate was submitted to gel filtration on a 3x160 cm Sephadex G-75 column, which had been equilibrated with TBS. The eluent was collected in fractions of 2 ml and stored at -70 °C. All purification procedures were carried out at 0-4 °C.

Assay of VAC activity - A modified prothrombin time test (MPT-test) was used to assay VAC-activity throughout the purification procedure. The test is essentially similar to that described for the human anticoagulant (15), the only difference being the use of bovine PFP and bovine thromboplastin (the BTP batch contained 3.6 mg protein/ml and 3.4 mM phospholipid). For quantification of VAC-activity we arbitarily defined one VAC-unit as the amount of VAC that prolongs the clotting time in the MPT-test, using BTP diluted 1:75, from the control value of 65 s to 100 s.

Measurement of rate of prothrombin activation - Purified bovine factor X_a, $CaCl_2$, phospholipid vesicles, composed of 80% DOPC and 20% DOPS were stirred in TBSA for 3 minutes at 37 °C. The activation was started with the addition of prothrombin (final concentrations are indicated in the legends to the figures). At different time points 50 µl aliquots were sampled from the mixtures and added to plastic cuvettes thermostated at 37 °C and containing 950 µl TBSE with 0.23 mM S2238. From the absorbance change at 405 nm, recorded with an Aminco DW-2 Spectrophotometer, the amount of activated prothrombin in the reaction mixture as a

function of the time was determined, using a calibration curve made with known amounts of purified bovine thrombin. The rate of prothrombin activation was calculated from samples taken after different time intervals.

Proteins - The vitamin K-dependent factors prothrombin and factor X_2 were purified from bovine-citrated plasma as described by Stenflo (17). After barium citrate adsorption and elution, ammonium sulphate fractionation and DEAE-Sephadex chromatography, two protein pools were obtained, containing a prothrombin-factor IX mixture and factor X respectivily. Factor X was obtained by the method of Fujikawa et al. (18) and activated, using RVV-X (19). Prothrombin was separated from factor IX by heparin agarose affinity chromatography (Fujikawa et al. 20). The prothrombin containing fractions from the heparin-agarose column were pooled and further purified using the method of Owen et al (21). Prothrombin and factor X_a concentrations were determined as described by Rosing et al. (7).
BTP was prepared using a standard method as described by van Dam-Mieras et al. (22). Protein concentrations, except for prothrombin and factor X_a, were determined according to Lowry et al. (23).

Phospholipid and phospholipid (large volume) vesicle preparation and phospholipid bilayer preparation on silicon slides - DOPC and DOPS were prepared as described by Rosing et al. (7). Single bilayer vesicles of DOPC and DOPS were prepared by sonication as described in (7). Phospholipid large volume vesicles, composed of 20% DOPS/80% DOPC were prepared according to the method described by van de Waart et al. (24). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (25). Phospholipid bilayers were stacked on silicon slides in a preparative Langmuir trough (Landa, type FW-1) at a constant surface pressure of 40 dynes/cm and at a dipping speed of 2 mm/min. The subphase consisted of 50 μM Ca^{2+} in filtered and deionized water (Millipore). The temperature was kept at 18°C.

Further details of this technique have been published (26).

Protein binding determination - The binding of VAC to a phospholipid bilayer was examined with an ellipsometric technique, which has been described in detail earlier (26). In brief, monochromatic light (Spectra-Physics Laser model, λ = 632.8 nm) was reflected against a silicon slide covered with a deposited or adsorbed layer. Before and after reflection, two polarizing prisms were passed and automatically rotated, such that the light intensity reaching the photodiode is kept minimal. From the changes in the position of both polarizers, the refractive index, n, and the thickeness, d, of the layer can be calculated. The mass of the substance deposited on the slide (Γ, the two - dimensional concentration) can be calculated from the values of n and d by using a modified Lorentz - Lorenz relation:

$$\Gamma = 3d\ (\ n^2 - n_b{}^2\ )/\{(\ n^2 + 2\ )(\ r\ (\ n_b{}^2 - 1\ ) - v\ (\ n_b{}^2 - 1\ )\}$$

where r and v are the specific refractivity and the partial specific volume of the deposited substance, respectively. n_b is the refractive index of the buffer. Equilibrium binding data were analyzed from double-reciprocal plots according to the following equation:

$$1\ /\ \Gamma\ =\ (\ K_d\ /\ \Gamma_{max}\ )(\ 1\ /\ c\ )\ +\ 1\ /\ \Gamma_{max}$$

where c is the concentration of VAC in the solution. The dissociation constant K_d and the maximal binding capacity Γ_{max} were calculated from the horizontal and vertical intercepts of a double reciprocal plot, respectively.

Binding of prothrombin and factor X_a to large volume vesicles was assessed as described by van de Waart et al. (24). Mixtures of large volume vesicles, $CaCl_2$, prothrombin or factor X_a, and varying amounts of VAC were incubated for 5 minutes at 37°C.

Prior to and after centrifugation for 30 minutes at 30,000 x g and 20°C, 25 μl samples were taken to determine total protein and

unbound protein, respectively. The phospholipid concentration in the supernatant was less than 0.1% of the total amount added. Prothrombin concentration was determined with Echis Carinatus venom as described by van Dieijen et al. (27). These protein assays were not influenced by phospholipid carried over from the reaction mixture prior to centrifugation, nor by the carry over of VAC, when present in the reaction mixture. Prothrombin binding and factor X_a binding to the large volume vesicles was reversible , as shown by the addition of EDTA. Determination of bound protein by resuspending the pelleted vesicles in EDTA and an second spin down, established the recovery of total protein to be virtually 100%. All binding experiments were carried out in a buffer containing 50 mM Tris/HCl, 100 mM NaCl, 0.5 mg ovalbumin/ml, and 3 mM $CaCl_2$, pH 7.5.

SDS-PAGE - Slab gel electrophoresis in the presence of SDS was carried out as described by Laemmli (28) on the gels containing 10% acrylamide, 0.27% N/N^3-methylene bisacrylamide and 0.1% SDS. 5% β-mercaptoethanol was present in disulfide bond reduced gel samples. The gels were stained with 0.25% Coomassie Blue R-250 in 50% ethanol, 15% acetic acid and destained in 10% ethanol, 10% acetic acid. Gels were also stained using Schiff's reagent prepared from basic fuchsin (Merck) according to the method of Segrest (29).

Electrofocusing - Isoelectric pH determination of proteins was performed with ready-prepared thin-layer polyacrylamide gels containing Ampholine carrier ampholytes (PAGplates, LKB) in a pH range between 3.5-9.5, according to the manual of the manufacturer. The pH gradient in the gel was determined immediately after the termination of the electrofocusing by slicing a strip of the gel along the line between the anode and the cathode. The ampholytes were eluted from each slice with distilled water and the pH of the water was measured.

γ-Carboxy glutamic acid determination - GLA-determination was

Table I

Summary of the purification of VAC from bovine aortic intima

Purification step	Protein[a] mg	VAC[b] Units	Specific activity Units/mg	Yield %	Purification fold
35% $(NH_4)_2SO_4$ supernatant	630	19,500	31.0	100	1.0
90% $(NH_4)_2SO_4$ pellet	470	19,000	40.4	97	1.3
Hydroxylapatite-pool	206	17,300	84.0	89	2.7
DEAE-pool	35.8	13,900	388	71	12.5
Sephadex G-75	0.45	666	1480	3.4	47.7

[a]Protein was determined according to Lowry et al. (23).
[b]VAC-units were determined by testing several dilutions of the sample in the MPT-test, using BTP diluted 1:75. One unit of VAC-activity arbitrarily prolongs the clotting time from the control time of 65 seconds to 100 seconds.

performed with high pressure liquid chromatography using a nucleosil 5SB column (Chrompack) according to the method of Kuwada and Katayama (30).

RESULTS

Isolation and purification of VAC - Using the methods employed for the isolation of the human anticoagulant (15), it appeared that the water-soluble part of a bovine aortic homogenate also contains an anticoagulant activity with properties very similar to those of the human anticoagulant. For purification of this bovine anticoagulant, however, an extension of the isolation procedure was required. The procedure together with its results is summarized in table 1. The presence of a procoagulant activity in the homogenate and in the 100,000 x g supernatant prevents quantification of VAC-activity in these fractions. The procoagulant activity precipitates with ammonium sulfate at 35% saturation, while VAC remains in solution. The 35% ammonium sulfate supernatant is chosen arbitrarily to contain 100% of VAC-activity. The anticoagulant, that binds to a hydroxylapatite column, elutes from this column at approximately 150 mM phosphate and pH 7.5. At low ionic strength VAC binds to DEAE-Sephacel and elutes from the DEAE at 150-180 mM NaCl and pH 7.5. The final purification step involves a Sephadex G-75 gel filtration. VAC elutes from the Sephadex column in a fraction that corresponds with the molecular weight range of 30,000-40,000 daltons, as assessed with globular proteins with known molecular weights. The recovery of VAC-activity in the purified fraction, is 3.4%. VAC stored at $-70^{o}C$ is stable for at least six months.

Chemical characterization of VAC - From analytical SDS-PAGE it appeares that two polypeptides (M_r=32,000 and M_r=34,000) copurify with the VAC-activity (fig. 1). The association of the VAC-activity with the 32-k DA band and/or 34-k Da band is established by eluting the bands from the gel. Only from the sites corresponding with the 32-k Da bands and 34-k Da bands the VAC-activity could

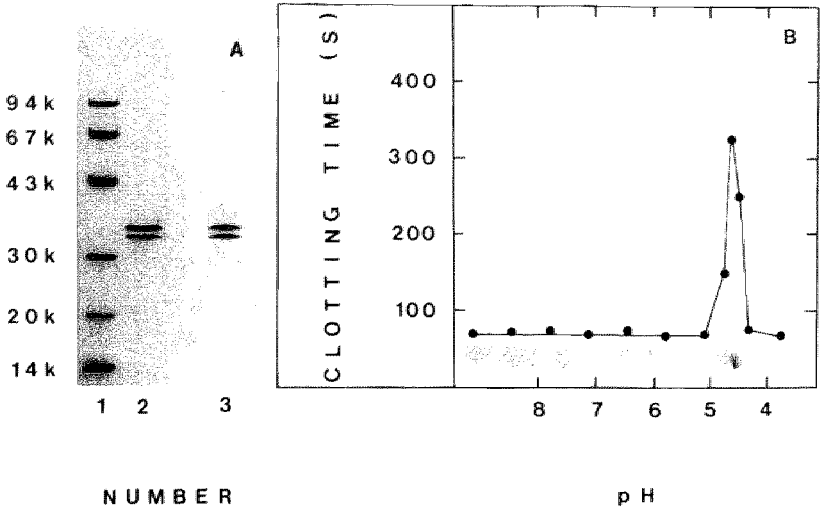

Fig. 1. Analytical SDS-PAGE (A) and isoelectric pH determination (B) of (VAC).
SDS-PAGE with slab gels containing 10% acrylamide, 0.27% N,N^3-methylenebisacrylamide, and 0.1% SDS was carried out according to Laemmli (28). The gel is as follows: 1) Reduced molecular weight standards (in Da). 2) 25 μg reduced purified VAC. 3) 25 μg unreduced purified VAC. The gel was stained with Coomassie Brilliant Blue and destained as described in materials and methods. 5 μg of VAC was submitted to isoelectric focussing (B). After which, the gel was either fixed for 30 minutes in 0.7% trichloric acid and subsequently stained with Coomassie Brilliant Blue, or cut into slices, which were eluted overnight at 4^o C with TBSA. The eluents were tested in the MPT-test, using BTP (f.c. 54 μg protein/ml and 51 μM phospholipid).

be recovered. However, due to their close proximity on the gel, we do not know whether the VAC-activity is associated with only one band or with both bands.

Both polypeptides appear to have the same isoelectric pH of approximately 4.5 (fig. 1). This is confirmed by elution of the 4.5-band out of the PAGplate and subsequent analysis of the eluent with SDS-PAGE.

Furthermore, both polypeptides are single chain proteins, which have no glycoprotein features, e.g. they do not stain with a

Schiff's reagent and they do not bind to concanavalin A. Basic hydrolysis of the polypeptides and subsequent analysis of the amino acid contents on HPLC, using a nucleosil 5SB column, demonstrates no detectable GLA-residues to be present.
Similar to the human anticoagulant (15), VAC-activity is abolished upon incubation at 56 ^{o}C for two minutes, and destructed in a time dependent way upon proteolytic digestion with protease type I.

The interaction of VAC with phospholipids - Preliminary experiments, in which binding of VAC to negatively charged large volume phospholipid vesicles was studied qualitatively, have indicated a reversible binding of VAC to these phospholipids. A quantitative determination of the phospholipid binding of VAC was performed with the automated ellipsometer. This approach has already been described in a study of the phospholipid binding of prothrombin (26). Equilibrium binding of VAC to phospholipids was studied with a bilayer of 20% DOPS and 80% DOPC, stacked on a silicon slide. Fig. 2 shows a double reciprocal plot obtained from the equilibrium values of Γ as a function of the amount of VAC at two calcium concentrations. From these plots a K_d is calculated of $6x10^{-9}$ M with a Γ_{max}=0.25 μg VAC/cm^2 and Γ_{max}=0.07 μg VAC/cm^2 for 2 mM Ca^{2+} and 0.5 mM Ca^{2+}, respectively. The binding curves are monophasic as revealed after stepwise addition of VAC.
Binding of VAC is more then 95% reversible as followed from the addition of excess EDTA. No detectable adsorption is observed in the absence of calcium nor on pure DOPC.
There appears to excist a calcium dependence of the binding of VAC to negatively charged phospholipids. Fig. 3 shows the effect of varying calcium concentrations on the binding of VAC to a phospholipid bilayer, consisting of 20% DOPS and 80% DOPC. Excess VAC (ten times the K_d) was present in these experiments. Equilibrium values of Γ are shown in this figure as reached after stepwise addition of $CaCl_2$. The half saturation Ca^{2+}-concentration on 20% DOPS/80% DOPC is 0.8 mM.
Since prothrombin and factor X_a bind to negatively charged large

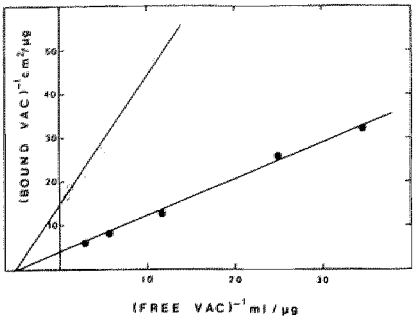

Fig. 2 Double-reciprocal plots of adsorbed mass of VAC to a bilayer of 20% DOPS/80% DOPC as a function of the free VAC concentration.
Binding of VAC to a bilayer of 20% DOPS/80% DOPC, stacked on a silicon slide, was estimated with an automated ellipsometer. Equilibrium values of adsorbed VAC are plotted reciprocal against the reciprocal values of the free VAC concentrations. Binding was studied at 0.5 mM (○) and 2.0 mM calcium (●).

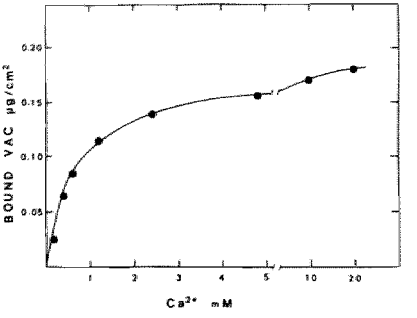

Fig. 3. Effect of the calcium concentration on the amount of VAC, adsorbed to a 20% DOPS/80% DOPC bilayer.
The calcium dependent binding of VAC to a bilayer of 20% DOPS/80% DOPC was measured with an automated ellipsometer. Equilibrium values are given as reached after stepwise addition of calcium. The VAC concentration was 2 µg/ml.

volume phospholipid vesicles, both with a K_d of about 2×10^{-7} M (24), we have examined the effect of VAC on the phospholipid binding of prothrombin and factor X_a to large volume vesicles, composed of 20% DOPS/80% DOPC. Fig. 4 shows that VAC inhibits dose-dependently the binding of prothrombin and factor X_a to these vesicles. At 20 µM phospholipids, the binding of 100 nM prothrombin and 10 nM factor X_a is reduced with 50% at 20 nM VAC and 60 nM VAC, respectively.

The effect of VAC on the factor X_a-catalyzed activation of prothrombin - VAC prolongs the clotting time in the MPT-test in a dose-dependent manner when coagulation is initiated with bovine thromboplastin (fig. 5).

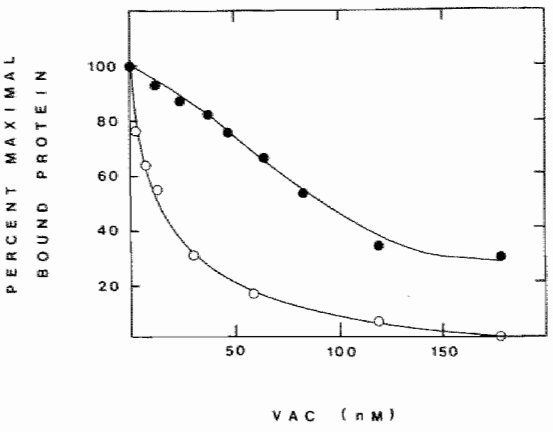

Fig. 4. Effect of VAC on the binding of prothrombin and factor X_a to large volume phospholipid vesicles. Mixtures of 20 μM large volume vesicles (20% DOPS/80% DOPC), 100 nM prothrombin (○) or 10 nM factor X_a (●), 3 mM $CaCl_2$ and varying amounts of VAC were incubated for 5 minutes at 37°C. After centrifugation of the mixtures, the resulting supernatants were assayed for prothrombin and factor X_a concentration as described in materials and methods. From these values the amounts of bound prothrombin and factor X_a were calculated, as percentage of maximal bound prothrombin and factor X_a, respectively.

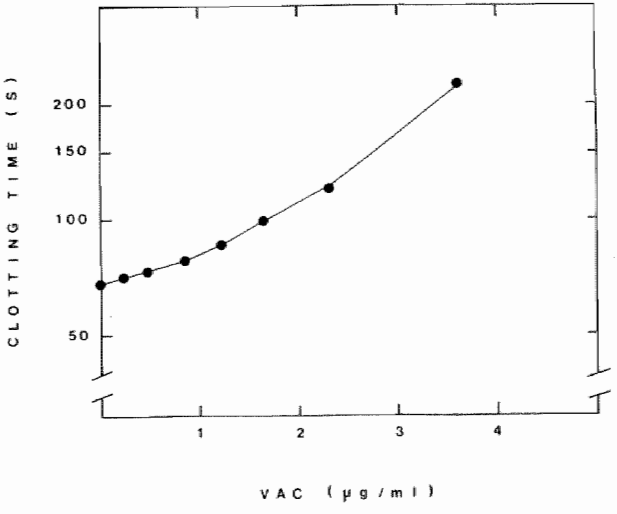

Fig. 5. Dose-response of purified VAC in the MPT-test. The MPT-test was performed with BTP diluted 1:75. The blank clotting time in the absence of VAC was 65 seconds.

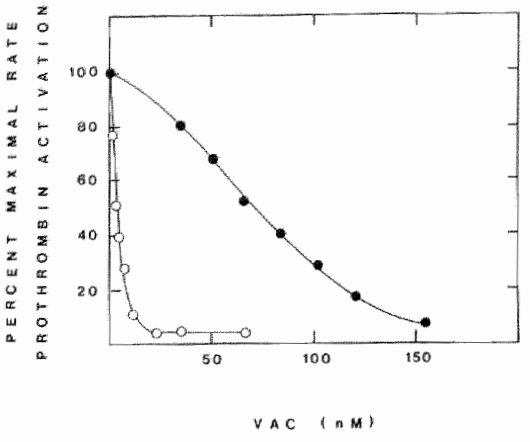

Fig. 6. Effect of various amounts of VAC on the rate of prothrombin activation. 10 nM factor X_a, 0.5 μM (○) or 5 μM 20% DOPS/80% DOPC vesicles (●), 10 mM $CaCl_2$, and various amounts of VAC, were stirred in TBSA at 37°C for 3 minutes. Prothrombin activation was started with the addition of 1 μM prothrombin. The rate of prothrombin activation was determined at different VAC concentrations and is expressed as percentage of the rate of prothrombin activation in the absence of VAC. The latter rates were 3.3 nM IIa/min (0.5 μM phospholipid), and 10.9 nM IIa/min (5 μM phospholipid).

Since VAC inhibits factor X_a-induced clotting, but not thrombin-induced clotting of plasma, and since the amidolytic activities of factor X_a as well as of thrombin are not affected by VAC, it is very unlikely that VAC should be an inhibitor directed against the active sites of these proteins.

In order to gain further insight in the mechanism through which VAC inhibits coagulation, we have explored the effects of VAC on the factor X_a-catalyzed activation of prothrombin in a purified system. In the absence of phospholipids, VAC, up to 5 μM, does not inhibit the activation of 1 μM prothrombin by 10 nM factor X_a. Hence, VAC does not interfere with the catalytic activity of factor X_a on prothrombin per se. If activation of 1 μM prothrombin by 10 nM factor Xa proceeds in the presence of phospholipids, a strong VAC-induced inhibition is observed (fig. 6). In the presence of 0.5 μM and 5 μM phospholipids, a 50% inhibition of the activation is accomplished with 3 nM and 65 nM VAC, respectively. Examination of the time courses of prothrombin activation by factor X_a in the presence of phospholipids, revealed that the VAC-induced inhibition, takes place immediately following the addition of VAC to the prothrombin activating complex, and is

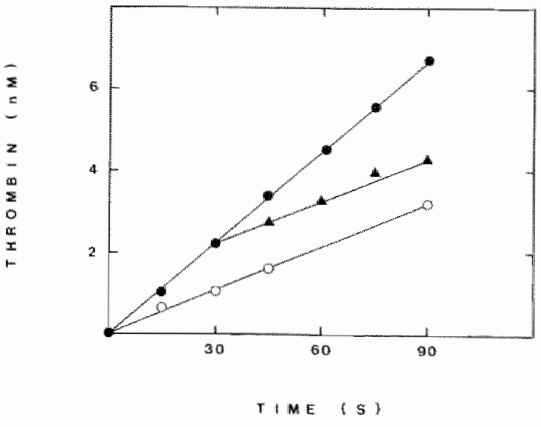

Fig. 7. Effect of the moment of addition of VAC to the prothrombinase, on prothrombin activation. 10 nM factor X_a, 20 μM large volume phospholipid vesicles (20% DOPS /80% DOPC), and 3 mM $CaCl_2$ were stirred in TBSA for 3 minutes. Prothrombin activation was started with the addition of 100 nM prothrombin. At the indicated time points samples were withdrawn, and tested for the amount of activated prothrombin (●). 65 nM VAC was added to the reaction mixture either 15 s before (○), or 30 s (▲) after the activation was started.

linear in time (fig. 7). This linearity excludes a time-dependent action of VAC, like a proteolytic and a phospholipase activity. Further characterization of the type of inhibition was assessed by a kinetic approach. The activation of prothrombin by 10 nM factor X_a and 20 μM phospholipids, was measured at varying prothrombin and VAC concentrations. The activation data are converted into Lineweaver-Burke plots (fig. 8). VAC appears to be a mixed-type inhibitor, which increases the K_m and decreases the V_{max} of the reaction. In addition to the other findings, these results strongly indicate an interference of VAC with the prothrombin activation through an interaction with the phospholipid surface.

DISCUSSION

Recently we reported the discovery of a novel anticoagulant protein in human umbilical cord arteries (15). This anticoagulant was shown to inhibit factor X_a-catalyzed activation of prothrombin only, when the reaction proceeds in the presence of negati-

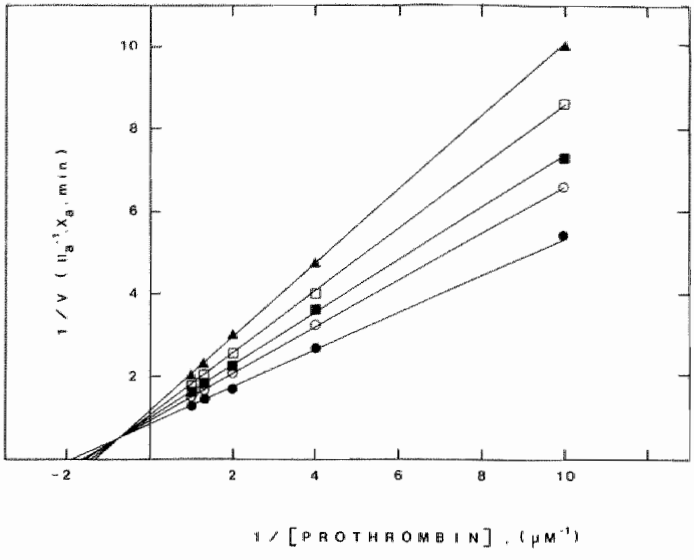

fig 8. Double-reciprocal plots of the rates of prothrombin activation as a function of the prothrombin concentration. Effect of the VAC concentration.
10 nM factor X_a, 20 μM 20% DOPS/80% DOPC, 3 mM $CaCl_2$, and varying amounts of VAC were stirred in TBSA for 3 minutes. Prothrombin activation was started by the addition of prothrombin. The rate of the prothrombin activation was determined as described in the materials and methods. VAC concentrations were, 0 (●) , 5 nM (○) , 10 nM (■), 25 nM (□), and 50 nM (▲).

vely charged phospholipids. We then already suggested that the mechanism through which this protein inhibits coagulation, differs fundamentally from those of the well known anticoagulants (8-12), and enclaves negatively charged phospholipids as a key part.

In this paper we have demonstrated the presence of an anticoagulant protein(s) in bovine aortic intima. From the chemical as well as the biochemical features, described for the anticoagulants in this paper and in (15), it is concluded that bovine VAC is very similar to the human umbilical cord anticoagulant.

Therefore, we will address these anticoagulants with bovine VAC

and human VAC, from hereon. We also presented here a purification procedure for bovine VAC, as well as experiments, which elucidate the mechanism through which VAC inhibits the factor X_a-catalyzed conversion of prothrombin.

Our purification data suggest that bovine VAC is only a minor component of the aortic intima. 1 g of intima contains a minimum of 2.5 μg of VAC. The exact concentration could not be determined because of a possible loss of material in the first purification steps. Upon analysis with gel electrophoresis, it appears that the purified bovine VAC contains two single chain proteins with M_r=32,000, and M_r=34,000. These proteins are no glycoproteins and have identical pI-values of about 4.5, indicating that they are acidic at physiological pH. Furthermore, with a very sensitive method, we could exclude the presence of GLA-residues in bovine VAC. The importance of this observation is discussed below.

The association of the anticoagulant activity with these two proteins has been confirmed by elution experiments. At the moment, however, it remains unknown whether the anticoagulant activity is associated with either one of these proteins or with both. Due to their similarities in chemical properties, we failed, as yet, to separate these proteins from each other.

Experiments with negatively charged, large-volume phospholipid vesicles demonstrated that both proteins bind to these vesicles in the presence of calcium only. With the use of an automated ellipsometer (26) this observation was confirmed, and, moreover, the binding data could be quantified. Binding of bovine VAC to a phospholipid surface requires the presence of both calcium and negatively charged phospholipids. Unlike the vitamin K-dependent coagulation factors, the calcium-dependent binding of which to negatively charged phospholipids is explained by the presence of GLA-residues (33-35), bovine VAC lacks these structures. Hence, bovine VAC is a phospholipid binding protein, the binding of which depends on the presence of calcium, although GLA-residues are absent. The mechanism of this calcium-dependent binding remains to be resolved.

The monophasic behavior observed for the binding of bovine VAC to

a 20% DOPS/80% DOPC surface, indicates that both proteins (M_r=32,000, and M_r=34,000) show similar binding kinetics. Moreover, this surface contains a single class of binding sites for bovine VAC with a K_d of $6x10^{-9}$M. Binding parameters as measured with the ellipsometer (26) are comparable with those obtained with the employment of other methods (24,31,32). Thus, extrapolation of the determined binding parameters of bovine VAC to the large volume vesicle system (24), indicates that bovine VAC has about a 30 times higher affinity for a 20% DOPS/80% DOPC surface as compared to prothrombin and factor X_a (24). In concert to this, it is observed that VAC interferes with the binding of prothrombin and factor X_a to such a phospholipid surface.

Bovine VAC strongly inhibits the factor X_a-catalyzed conversion of prothrombin in the presence of phospholipids. It is now well established that prothrombin activation in the presence of phospholipids is a surface bound process (7,36-38), in which, in the absence of factor V_a, phospholipid-bound prothrombin is converted by phospholipid-bound factor X_a. Our data indicate that VAC interferes with both prothrombin and factor X_a binding on the phospholipid surface, and thereby interferes with the membrane-bound process of prothrombin activation. The type of inhibition is not purely competitive but appears to be a mixed-type. Bovine VAC not only increases the K_m of the prothrombin activation but it decreases the V_{max} of this reaction, as well. The increase of the K_m is readily explained from the interference of bovine VAC with prothrombin binding to the phospholipids. Whereas the decreased V_{max} likely originates from a interference with factor X_a binding to the phospholipids. A similar type of inhibition of the prothrombinase complex has been described for fragment 1 and fragment 1.2 (39), which are GLA-containing activation peptides of prothrombin.

In conclusion we can state that we have discovered a protein(s) (VAC), present in human and bovine arterial tissues, that can be classified to the extrinsic membrane proteins. Through its nonenzymatically interaction with negatively charged phospholi-

prothrombin, and probably also other phospholipid dependent procoagulant reactions. On basis of this mechanism of anticoagulation, VAC can be assigned to the group of anticoagulants, which interfere with the accessory components of procoagulant complexes. To this class already belong activated protein C (11,12), some phospholipases A_2 (13), and some Lupus anticoagualant species (14). Among them VAC is unique in these respects, that VAC binds calcium-dependently and reversibly to negatively charged phospholipids, and anticoagulates without proteolytic action.

Whether VAC has a physiological function as an anticoagulant is unknown, as yet. Its location in the intima of a vessel wall, however, may indicate a role for VAC as an anticoagulant after vessel wall injury, when negatively charged phospholipid structures become exposed to plasma constituents.

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CHAPTER 4

RAPID PURIFICATION AND FURTHER CHARACTERIZATION OF THE VASCULAR ANTICOAGULANT (VAC).

RAPID PURIFICATION AND FURTHER CHARACTERIZATION OF THE VASCULAR ANTICOAGULANT (VAC).

Reutelingsperger, C.P.M., Maroofi, K., Kop, J.M.M., Hornstra, G., and Hemker, H.C.

SUMMARY

Vascular anticoagulant (VAC), a single chain anticoagulant protein first discovered in human umbilical cord arteries, is present in human placenta. A rapid and convenient purification procedure of placenta-VAC (P-VAC) is presented. The molecular weight and the amino acid composition of P-VAC appear to be identical with those of umbilical cord VAC (U-VAC). Both proteins affect in a similar way the factor X_a-catalyzed prothrombin activation. It is, moreover, shown that P-VAC inhibits the phospholipid dependent factor X-activation.

P-VAC binds with a $K_d=7.8x10^{-9}$ M to a phospholipid bilayer, consisting of 20% dioleoyl phosphatidylserine and 80% dioleoyl phospatidylcholine in a calcium-dependent way. The phospholipid to VAC ratio at saturation was n=44 and n=29 moles/mol for 0.5 mM Ca^{+2} and 2 mM Ca^{+2}, respectively. Half maximal binding occurred at 0.4 mM Ca^{+2}.

P-VAC has a quenched intrinsic fluorescence signal, which is not affected neither by calcium nor by EDTA. With the use of serine proteases further evidence was obtained that calcium and EDTA do not induce a conformational transition of P-VAC.

In addition, the effects of the proteolytic enzymes on VAC demonstrate that it has a stabilized tertiary structure, with mainly glutamic acid and/or aspartic acid residues at the exterior side of the molecule.

The mechanism of the calcium dependent VAC binding to phospholipid is discussed.

INTRODUCTION

The calcium dependent binding of the vitamin K-dependent coagulation factors to negatively charged phospholipids is essential for blood clotting (1-3). Interference with this binding in one way or another will impair the process of blood coagulation, as is demonstrated by the following compounds.

1) Vitamin K-antagonists interfere with the posttranslational formation of γ-carboxyglutamic acid (GLA), which are essential for proper phospholipid binding. K-related coagulation factors, lacking GLA-residues are poor substrates in the coagulation process (4-6).

2) GLA-containing activation peptides of prothrombin (7) and factor X (8) have been reported to possess anticoagulant activity, due to their calcium dependent affinity for phospholipids.

3) Antibodies, directed against acidic phospholipids inhibit the phospholipid dependent procoagulant reactions (9).

Some basic phospholipases A_2 exhibit an anticoagulant activity (10,11), because of their binding to phsopholipids, which are subsequently hydrolyzed by these phospholipases.

In a previous paper we reported the existence of a vascular protein (VAC), which inhibit the factor X_a-catalyzed activation of prothrombin, only when phospholipids are present (12, chapter 2 of this thesis).

VAC can be isolated from human umbilical cord arteries, and bovine aortic intima (chapter 3 of this thesis). We have established that VAC, a non GLA-protein with no phospholipase activity, binds in a calcium dependent way to negatively charged phospholipids, and thereby it interferes with the assembly of prothrombinase on this surface. In this paper we show that VAC is also present in human placenta. We present a rapid and convenient purification procedure for VAC, as well as a further characterization of this vascular anticoagulant.

MATERIALS AND METHODS

Materials - The Fast Protein Liquid Chromatography - system (FPLC) was obtained from Pharmacia (Uppsala, Sweden). Polybuffer 74, DEAE-Sephacel, the Superose 12, Mono Q, and Mono P columns were also purchased from Pharmacia. The PhastGel electrophoresis system as well as the ready prepared polyacrylamide PhastGels were products of Pharmacia. The chromogenic substrate S2337 was purchased from AB Kabi Diagnostica (Stockholm, Sweden). Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were obtained from Sigma. Elastase (EC 3.4.21.36) and subtilisin (EC 3.4.21.14) were products of Boehringer Mannheim.

Proteins - Umbilical cord VAC (U-VAC) was purified from human umbilical cord arteries with the purification method described in (chapter 3 of this thesis). Placenta VAC (P-VAC) was isolated from human placenta, which was obtained immediately after delivery. The placenta was cooled at 4°C and rinsed free of adhered blood. Membraneous material was removed prior to homogenization of the placenta in Tris buffered saline (TBS, 50 mM Tris/HCl, 100 mM NaCl, pH 7.4), containing 1 mM EDTA, 1.6 g/l benzamidine and 1.57 mg/l soybean trypsin inhibitor. The 20% homogenate (w/v) was fractionated by the ammonium sulfate precipitation as described in (chapter 3 of this thesis). The VAC-containing fractions were further purified by batchwise adsorption to DEAE-Sephacel at 50 mM NaCl, pH 7.4. The bound VAC was released with a one step elution with 300 mM NaCl at pH 7.4. Column chromatography was subsequently performed at ambient temperature and increased pressure, using a FPLC-system. Gel filtration of the VAC-containing fractions was performed with a Superose 12 column, equilibrated with 20 mM bis-Tris/HCl, 100 mM NaCl, pH 6.3. Subsequently, the eluted VAC was submitted to ion exchange chromatography on Mono Q, equilibrated with the latter buffer. A linear gradient of NaCl in 20 mM bis-Tris/HCl, pH 6.3, was used to release VAC from the column. Finally, the VAC-containing fractions were rechromatographed on Superose 12. The

purified P-VAC was dialyzed against TBS, and stored at -70°C. During the purification procedure the fractions were evaluated for VAC-activty, protein concentration, and protein composition, with the MPT-test (12, chapter 2 of this thesis), the Sedmak procedure (13) and gel electrophoresis, respectively.
Bovine factors X_2, IX_a and thrombin were prepared as described by Van Dieijen et al. (14). Factor VIIIc was purified from bovine blood according to the method of Vehar & Davie (15) as modified by Van Dieijen et al. (16). Factor $VIII_a$ was prepared by activation of factor VIIIc (1.75 nM) with thrombin (4 nM) for 1 min. at 37°C (16). Concentrations of factors IX_a, and thrombin were determined by active-site titration with P-NGB-p-nitrophenyl-p'-guanidinobenzoate as described elsewhere (17,18). Factors VIIIc and X were quantified according to the methods of (15,16).

Assay of VAC-activity - The MPT-test, which is described in detail elsewhere (12, chapter 2 of this thesis), was used throughout the purification procedure in order to test for VAC-activity. Expression of this activity in arbitrarily defined units was also performed with the MPT-test, as described previously (chapter 3 of this thesis).

Electrophoresis - Polyacrylamide gel electrophoresis in the presence and absence of SDS was performed with ready prepared PhastGel gradient 10-15 gels in combination with PhastGel SDS and Native buffer strips. Staining with Coomassie Briliant Blue and destaining of the gels were carried following the recommendations of the manufacturer.

Amino acid analysis - U-VAC and P-VAC, dialyzed against distilled water, were hydrolyzed under vacuum in 6 N HCl, 01% phenol, at 110°C for 18, 48 and 72 hours. The hydrolysates were evaluated with a LKB 4400 amino acid analyser, operating with lithium buffers. The recovery of amino acids were plotted against the different times of hydrolysis. The extrapolated maximum recovery was estimated from these plots.

Intrinsic fluorescence - Fluorescence measurements were made with the SPF-500 spectrofluorometer of SLM-Aminco. Emission band pass was 2 nm and excitation band pass was 4 nm. Slits were closed in between the scans to avoid photodegradation. Temperature in the cell was maintained at 25°C. Samples were excitated at 285 nm. Emission intensity was monitored at 1 nm intervals from 300 to 400 nm. Back ground signals due to solvents were substracted, and dilution due to the addition of titrant was corrected.

Phospholipid vesicle preparation and phospholipid bilayer preparation on silicon slides - Phospholipid vesicles composed of 25% DOPS and 75% DOPC were prepared as described by (17). Phospholipid bilayers were stacked on silicon slides in a preparative Langmuir trough (Landa type FW-1) as described in detail earlier (19).

P-VAC binding to a phospholipid bilayer - The binding of P-VAC to a phospholipid bilayer (20% DOPS/80% DOPC) was examined with an automated ellipsometer. Details of this technique and the mathematical equations for calculation of the equilibrium binding parameters are described elsewhere (19, chapter 3 of this thesis).

Intrinsic factor X-activation - The determination of the factor IX_a- catalyzed factor X-activation in the presence of factor VIIIa and phospholipid vesicles was performed as described in detail by (20). The only difference being is the use of bovine factor IX_a instead of human factor IX_a.

RESULTS

Purification of placenta VAC - Application to a human placenta of the first purification steps, established for U-VAC and bovine VAC (chapter 2 and 3 of this thesis), revealed the presence of VAC in this tissue. A purification method for P-VAC was developped, involving the FPLC-system. This method is fast and can be performed at ambient temperature. Gel filtration on a Superose 12

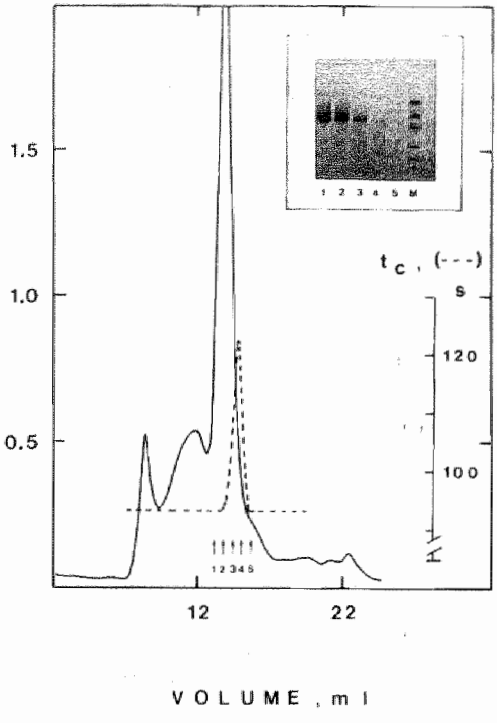


Fig. 1. Gel filtration on Superose 12.
This purification step is the first one in the sequence, which employes the FPLC-system. The column was equilibrated with 20 mM bis-Tris/HCl, 100 mM NaCl (pH 6.3). The effluent was monitored at 280 nm for protein contents. Samples were taken from the fractions and assayed in the MPT-test for VAC-activity. The inset shows a gel electrophoretic analysis of several fractions. The numbers of the lanes correspond with the numbers below the arrows, which in turn indicate the positions in the effluent of the analyzed samples. Lane M represents the low molecular weight markers, which are from top to bottom; 94 k, 67 k, 43 k, 30 k, 20 k, and 14 k Daltons.

column was completed within one hour. P-VAC elutes from this column in the molecular weight range of 32 to 35 kDA (fig. 1). The P-VAC fractions were pooled and subsequently applied to the anion exchanger Mono Q. P-VAC bound to this column at 100 mM NaCl, pH 6.3. A linear gradient of NaCl was established to release VAC from the Mono Q. Elution of VAC occurred at 190 mM NaCl (fig. 2).

The VAC-containing peak was reapplied to the Superose 12, and appeared to consist of about 85% VAC and some contaminants with higher molecular weights (fig. 3).

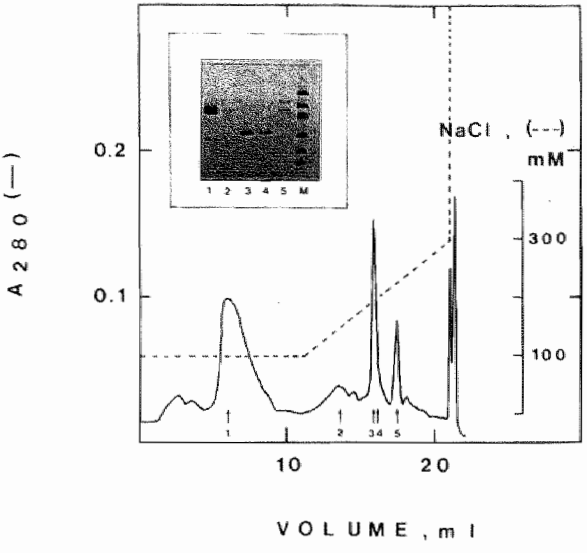


Fig. 2. Ion exchange chromatography on Mono Q.
The VAC-containing fractions from the Superose 12 (fig. 1) were applied to a Mono Q column, equilibrated with the elution buffer of the Superose 12. A linear gradient of NaCl was established for VAC-elution. The effluent was monitored at 280 nm, and assayed for VAC-activity (not shown), which was only associated with the protein peak, indicated by the arrows 3 and 4.
The inset presents the gel electrophoresis of several samples of the effluent. The numbers of the lanes correspond with the numbers below the arrows. Lane M give the markers (from top to bottom); 94 k, 67 k, 43 k, 30 k, 20 k, and 14 k Daltons.

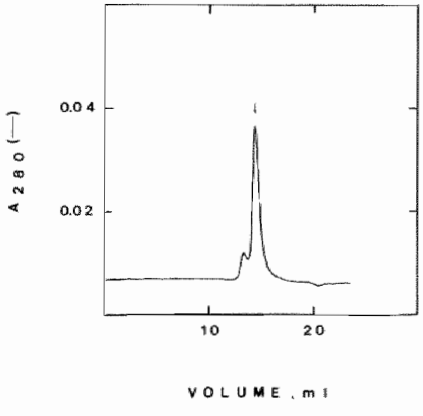


fig. 3. Gel filtration on Superose 12; The final purification step for P-VAC.
The protein peak, which eluted from the Mono Q (fig. 2) and which contained the VAC, was re-applied to the Superose column, equilibrated with 20 mM bis-Tris/HCl, 190 mM NaCl (pH 6.3). The effluent was monitored at 280 nm and assayed for VAC-activity (data not shown). Only the protein peak, indicated by the arrow, expressed VAC-activity. This peak was homogeneous for a 32 kDa protein, as judged with gel electrophoresis.

Table 1

Summary of the purification of P-VAC

	protein[a] (mg)	units[b]	specific activity (u/mg)	yield (%)	purification (fold)
starting material[c]	225	5550	24.7	100	1
Superose 12	44	4540	103	82	4
Mono Q	2.0	4700	2350	85	96
Superose 12	1.5	3900	2600	70	105

[a] Protein was determined according to Sedmak & Grossberg (13).
[b] Units of P-VAC activity were quantified in the MPT-test as described in the materials and methods.
[c] Starting material was derived from 100 g of placenta, processed upto and including the DEAE - Sephacel step.

Table 2

Amino acid composition of P-VAC and U-VAC

	P-VAC	U-VAC
amino acid[a]	mol% ± S.D.[b]	mol%[c]
aspartic acid/asparagine	7.9 ± 1.0	8.5
threonine	6.9 ± 0.8	6.6
serine	7.4 ± 1.8	7.2
glutamic acid/glutamine	13.4 ± 2.2	14.9
proline	2.3 ± 1.2	3.4
glycine	8.8 ± 0.6	8.4
alanine	7.9 ± 1.8	8.4
valine	4.6 ± 0.4	4.1
methionine	1.4 ± 0.2	0.7
isoleucine	6.5 ± 0.6	5.9
leucine	12.0 ± 2.4	10.7
tyrosine	3.7 ± 0.4	3.9
phenylalanine	3.7 ± 0.6	3.9
lysine	6.5 ± 1.2	7.6
histidine	1.4 ± 0.8	1.4
arginine	5.6 ± 2.0	4.9

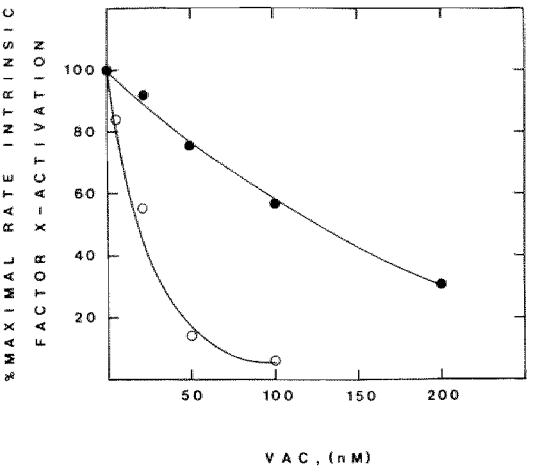
[a] Amino acids were determined after acid hydrolysis as described in the materials and methods. Tryptophan was not determined.
[b] The data are the mean of 4 separate purifications of P-VAC, and are given with the standard deviations.
[c] The data are from one purification of U-VAC.

During the FPLC-procedure, VAC-activity was quantified and protein concentrations were determined. These results are summarized in table 1. 100 g placenta contains about 1.5 mg P-VAC, having a specific activity of about 2600 units/mg.

Comparison of P-VAC with U-VAC - Protein chemical characterization of P-VAC revealed that, like U-VAC, it is a single chain protein (Mr=32,000) with a non glycoprotein nature. Furthermore, the presence of GLA-residues in P-VAC could be excluded. The amino acid composition of P-VAC is comparable to that of U-VAC (table 2), as was determined after acid hydrolysis. This procedure features a conversion of asparagine and glutamine into aspartic acid and glutamic acid, respectively. Furthermore, cysteine, cystine, and tryptophan residues cannot be recovered. As to their anticoagulant activities both proteins are similar in all respects tested. The dose-responses of U-VAC and P-VAC in the MPT-test are identical. Moreover, prothrombinase activity is inhibited by P-VAC, only when the factor X_a-catalyzed activation of prothrombin takes place in the presence of negatively charged phospholipids. The amidolytic activities of factor X_a and prothrombin are not affected by P-VAC. Exposure of P-VAC to 56°C renders the molecule inactive. Hence, the mechanism through which P-VAC inhibits coagulation, is likely to be similar to that of U-VAC.

Effect of P-VAC on the intrinsic factor X-activation - Similar to U-VAC, P-VAC inhibits the phospholipid dependent activation of prothrombin. We have explored the effect of P-VAC on another phospholipid dependent procoagulant reaction, the intrinsic factor X-activation. As expected, the activation of factor X by factor IXa in the presence of phospholipids and factor $VIII_a$ is inhibited by P-VAC in a dose-dependent manner (fg. 4). Furthermore the extent of inhibition depends on the phospholipid concentration. At 2 μM phospholipids, 17.5 nM VAC inhibits with 50% the rate of factor X (1 μM) activation by factor IX_a (0.2 nM) and factor $VIII_a$ (0.35 nM) by 50%. At 10 μM phospholipids a 50% inhibition is accomplished with 125 nM VAC.

Fig. 4. Effect of P-VAC on the intrinsic factor X-activation.
0.2 nM factor IX_a, 0.35 nM factor $VIII_a$, varying amounts of P-VAC, 3 mM $CaCl_2$, and 2 μM 25% DOPS/ 75% DOPC vesicles (○) or 10 μM of these vesicles (●) were incubated at 37°C in TBS, containing 0.5 mg/ml human serum albumine. The activation of factor X was started with the addition of 1 μM factor X. The rates of factor X activation were determined as described in materials and methods and are expressed as percentage of the maximal rates, which were 1.8 nM X_a/min (●) and 1.1 nM X_a/min (○).



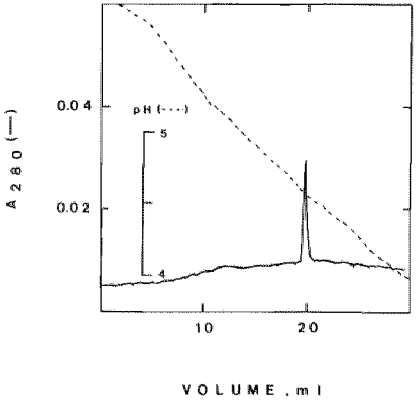


Fig. 5. Chromatofocussing of P-VAC on Mono P.
The Mono P column was preadjusted to pH 6.3 with 20 mM bis-Tris/ HCl (pH 6.3). 25 μg of purified P-VAC was applied to the column, and subsequently eluted by developing a pH gradient, which was established by elution with Polybuffer 74, adjusted to pH 6.3.

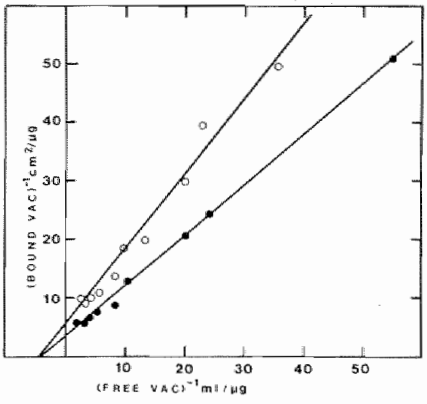


Fig. 6. Double reicprocal plots of adsorbed mass of P-VAC to a bilayer of 20% DOPS/80% DOPC as a function of the free P-VAC concentration.
Binding on a 20% DOPS/80% DOPC bilayer stacked on a silicon slide was measured in an automated ellipsometer. K_d and Γ_{max} were estimated from the double reciprocal plots at 0.5 mM (○) and 2.0 mM $CaCl_2$ (●) at 37°C.

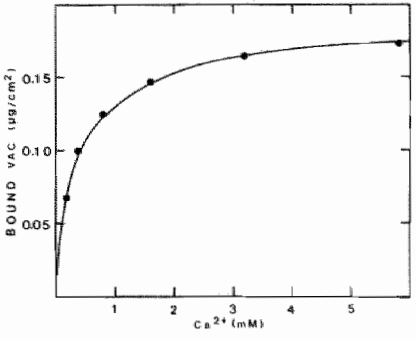


Fig. 7. Effect of the calcium concentration on the amount of P-VAC, adsorbed to a 20% DOPS/80% DOPC bilayer.
The calcium dependent binding of P-VAC to a bilayer of 20% DOPS/ 80% DOPC was measured on an automated ellipsometer. Equilibrium values are given as reached after stepwise addition of $CaCl_2$. The P-VAC concentration was 2 µg/ml.

Characterization of P-VAC - To investigate whether or not P-VAC excists in different formes with identical molecular weights, a sample of P-VAC was chromatofocussed on a Mono P column, from which it eluted as a single sharp peak at pH 4.6 (fig. 5).
P-VAC binding to a phospholipid bilayer (20% DOPS/80% DOPC) was quantified with an automated ellipsometer. Fig. 6 shows a double reciprocal plot obtained from the equilibrium values (amount of deposited VAC in µg/cm^2) as a function of the amount of P-VAC at two calcium concentrations. From these plots a K_d is calculated of 7.8×10^{-9} M with a Γ_{max}=0.18 µg VAC/cm^2 and Γ_{max}=0.27 µg VAC/cm^2 for 0.5 mM and 2 mM Ca^{+2}, respectively. The P-VAC binding depends on the Ca^{+2}-concentration (fig. 7). Half maximal binding, as measured with an excess of P-VAC (ten times the K_d) is obtained at 0.4 mM Ca^{2+}.

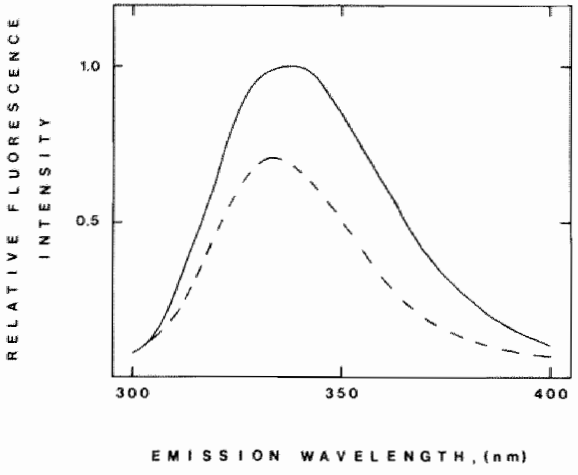


Fig. 8. Intrinsic fluorescence of P-VAC.
Emission spectra were made at 25°C in TBS. Excitation was at 285 nm. The emission spectrum of active P-VAC in the absence of EDTA and Ca^{2+} was the same as it was in the presence of 1.5 mM Ca^{2+} or 1.5 mM EDTA (-----).
Emission spectra were also monitored of P-VAC, which was inactivated by a 5 min exposure to 56°C (——). No differences were observed between the emission spectra of inactivated P-VAC, in the absence of Ca^{2+} and EDTA, and in the presence of 1.5 mM Ca^{2+} or 1.5 mM EDTA.
Emission spectra were monitored for several P-VAC concentrations. The concentrations of the active P-VAC, as well as those of the inactivated P-VAC, were linear with the integrated surfaces of the corresponding emission spectra.

Since P-VAC shows a calcium-dependent binding to negatively charged phospholipids, we investigated whether a conformational change of P-VAC is required for binding by studying the effects of calcium and EDTA on the intrinsic fluorescence signal of P-VAC. At all concentrations tested (upto 5 mM), both titrants did not alter the intrinsic fluorescence signal. Together with the observation that trypsin is unable to cleave P-VAC either in the presence or in the absence of calcium, this indicates that P-VAC does not undergo a conformational transition as a conse-

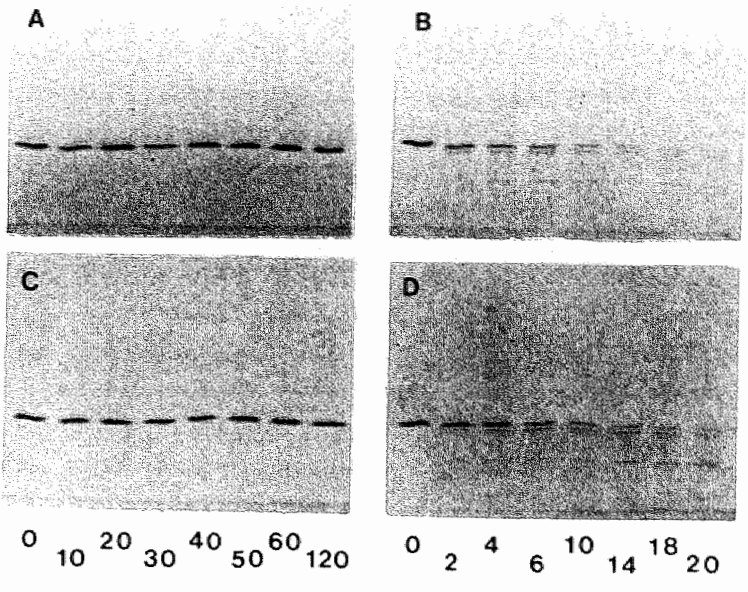


Fig. 9. Effects of serine proteases on active P-VAC and heat-inactivated P-VAC.
25 μg of active P-VAC was incubated with 0.1 μg of trypsin at 37°C. At the indicated time points, samples were withdrawn, immediately boiled in SDS and subsequently submitted to gel electrophoresis, either under non-reducing (panel A) or reducing conditions (panel C). The same procedure was carried out for P-VAC, which was inactivated by 5 min. exposure to 56°C (B and D). Panel B presents gel electrophoresis under non-reducing conditions and panel D shows the reduced gel. Identical incubations were performed with chymotrypsin and elastase. Both proteases cannot cleave the active P-VAC, but readily hydrolyze the inactivated P-VAC (gels not shown).
The incubations of P-VAC with the proteases were repeated with either 0.5 mM EDTA or 0.5 mM Ca^{2+} being present. The electrophoretic patterns obtained from the incubations in the presence of one of these agents did not differ from the patterns obtained in their absence (not shown).

quence of calcium ions. The intrinsic fluorescence signal of P-VAC, however, could be increased upon exposure to 56°C (fig.8). This heat exposure also results in an inactivation of P-VAC. Together with the fact that trypsin, chymotrypsin and elastase could not cleave the active P-VAC, but readily cleaved the inactivated P-VAC (fig. 9), this suggests that the inactivation

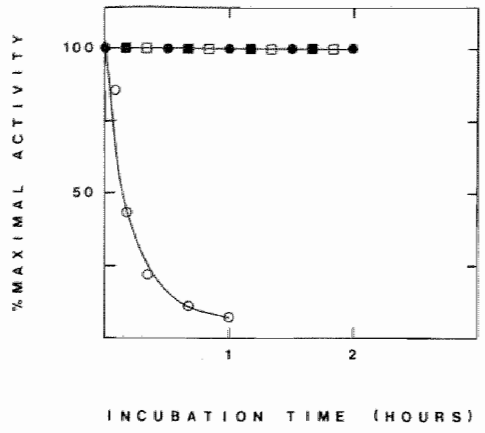


Fig. 10. Effect of serine proteases on the activity of P-VAC. 25 μg of P-VAC was incubated with either 0.5 μg trypsin (●), 0.5 μg chymotrypsin (■), 0.5 μg elastase (□) or 0.5 μg subtilisin (○). At the indicated time points a sample was taken, that contained theoretically 2 units of VAC-activity, and was assayed in the MPT-test. The residual activity is expressed as percentage of the maximal activity. The amount of protease carried over from the incubation to the MPT-test, had no effect on its blank clotting time.

of P-VAC by heat exposure is associated with a conformational transition.

In contrast to trypsin, chymotrypsin and elastase, subtilisin, a serine protease from procaryotes, can inactivate native P-VAC (fig. 10).

DISCUSSION

Vascular tissue of human umbilical cords contains an anticoagulant protein (VAC), that inhibits the phospholipid dependent factor X_a-catalyzed prothrombin activation (12, chapter 2 of this thesis). Bovine aortic intima appears to contain a similar anticoagulant, a purification procedure of which has been developed (chapter 3 of this thesis). Part of its anticoagulant

mechanism has already been unraveled: through a high affinity binding of VAC to phospholipids it interferes with the assembly of prothrombinase complex on a negatively charged phospholipid surface. We have, furthermore, demonstrated the presence of VAC in endothelial cells, and have suggested that VAC is an intracellular extrinsic membrane protein, with still unknown physiological functions (chapter 5 of this thesis).

In this paper we demonstrate the presence of a similar anticoagulant in human placenta. Comparison of the placenta VAC (P-VAC) with the umbilical cord VAC (U-VAC) reveals that both proteins are likely to be identical. P-VAC and U-VAC have, apart from their similar molecular weights, comparable amino acid compositions. Furthermore, antibodies raised against U-VAC bind to P-VAC.

As to their anticoagulant activities, both vascular anticoagulants inhibit the factor X_a-catalyzed activation of prothrombin only when phopholipids are present. Moreover, both P-VAC, as well as U-VAC, lose their activities upon exposure to 56 ^{o}C.

With the discovery of VAC in placenta a source of human VAC has been found that enables its purification in amounts sufficient for characterization of the protein, in spite of the fact that it is a minor component of the tissue.

We have developed a convenient and a rapid purification procedure for VAC, using a FPLC-system. Special columms and the increased working pressure shorten the purification time considerably. Because of the stability of VAC at room temperature, this quick procedure permits the purification to be carried out at ambient temperature.

P-VAC and bovine VAC (chapter 3 of this thesis) show comparable affinities for a phospholipid surface, containing negatively charged phospholipids. Consequently their mechanisms of anticoagulant action are likely to be identical. This mechanism predicts an interference of VAC with the pholpholipid dependent intrinsic factor X-activation, since the organisation of the latter complex strongly resembles the structure of the prothrombinase (14,17). In both complexes the phospholipids function as a membrane

surface to which the vitamin K-dependent coagulation factors involved bind in a calcium dependent way (21-24).
P-VAC, indeed, inhibits the activation of factor X by factor IX_a, in the presence of phospholipids and factor $VIII_a$. Because the intrinsic factor X-activation preceeds prothrombinase in the coagulation cascade (6), VAC is, thus, more effective as an anticoagulant.
To gain insight in the mechanism of the binding of VAC to negatively charged phospholipids, we have investigated whether this binding requires a conformational change, as has been suggested for prothrombin (26,27). To this end the effect of calcium and EDTA on intrinsic fluorescence was measured. Both compounds were without any effect. Since, moreover, the proteases, trypsin, chymotrypsin and elastase appeared to be unable to cleave VAC (both in the absence and presence of calcium), this strongly indicates that VAC does not show a calcium ion dependent conformational transition. Consequently, such a process is unlikely to be involved in the binding of VAC to negatively charged phospholipids.
The native tertiairy structure of VAC, however, is required for the phospholipid binding and for the anticoagulant activity, since inactivation of VAC by its exposure to 56°C was associated by a change in its tertiairy structure, as reflected by an increase of the intrinsic fluorescence. Moreover, the heat exposed VAC became susceptible to proteolytic attack by the serine proteases. Finally, the heat inactivated form of VAC does not bind to negatively charged phospholipids anymore (unpublished experiments).
Active VAC is not proteolytically cleaved by trypsin, chymotrypsin, and elastase. Regarding the preferential cleaving sites of these proteases, the conformation of the active VAC molecule is likely to have only few or no accessible arginine and lysine residues. Electrostatic forces between the latter and the negative charges in the phospholipid membrane are therefore unlikely to occur. Since VAC does not bind to pure phosphatidylcholine it can be excluded that predominant hydrophobic interac-

tions with the membrane, as described for factor IX (27), are unlikely to occur between VAC and the phospholipid membrane.
Active VAC can be cleaved by subtilisin, therefore, the VAC molecule probably contains quite some accessible glutamic acid and aspartic acid residues. Whether these are essential for the binding of VAC to the negatively charged phospholipids remains to be seen.
In conclusion we can say that VAC has a stabilized conformation with quenched intrinsic fluoresence. This conformation shows a calcium dependent binding to negatively charged phospholipids. A possible mechanism of this binding is the bridging of the calcium ions between the glutamic acid and/or aspartic acid residues of VAC with the negatively charged surface.
The real physiological function(s) of VAC is/are, as yet, unknown. However, the location of the protein in endothelial cells, can strategically implicate a function in anticoagulation. The importance of VAC as a physiological anticoagulant strongly depends on whether VAC is secreted by endothelial cells or whether exposure of VAC to plasma is only a result of cell injury. This is now under investigation.

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CHAPTER 5

IMMUNOLOGICAL AND CHEMICAL DETECTION OF VASCULAR ANTICOAGULANT (VAC) IN CULTURED HUMAN VASCULAR ENDOTHELIAL CELLS

IMMUNOLOGICAL AND CHEMICAL DETECTION OF VASCULAR ANTICOAGULANT (VAC) IN CULTURED HUMAN VASCULAR ENDOTHELIAL CELLS

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SUMMARY

Recently we have isolated from vessel wall homogenates a protein (VAC) with anticoagulant properties completely different from the known physiological anticoagulants like antithrombin III and activated protein C (Eur. J. Biochem. 1985. 151:626-629).
By means of immunological and chemical techniques we now demonstrate qualitatively the presence of VAC in cultured human vascular endothelial cells. Endothelial VAC appears to be an intracellular protein, which is not secreted in detectable amounts by quiescent endothelial cells. Whether or not VAC is synthesized de novo by the endothelial cell is not known at the moment. In the presence of 1 mM calcium, endothelial VAC binds to subcellular structures, thereby diminishing the procoagulant activities of these structures. Since the intracellular calcium content can become as high as 1 mM in the case of cell injury, we hypothesise that VAC provides an anticoagulant mechanism, which locally competes with the surface bound enzyme complexes of blood coagulation and thus controls thrombin formation at sites of damaged endothelial cells.

INTRODUCTION

Endothelial cells in culture, originating from various vascular tissues, play a dual role in hemostatic processes. The endothelial cell surface supplies the antithrombin III anticoagulant pathway with heparin-like structures (1). Moreover, the protein C anticoagulant pathway is supplied with thrombomodulin (2), protein S (3), and cofactor activity for the assembly of the activated protein C - protein S complex (4).

Apparently in contradiction, the endothelial cell surface also can propagate a sequence of procoagulant reactions leading to fibrin formation (5-7).

The perception of this duality has led to a concept of a balance of procoagulant and anticoagulant reactions occurring at the endothelial cell surface. Non-perturbed endothelial cells favour the anticoagulant reactions, this can be reversed upon exposing the cells to perturbing agents, like tissue necrosis factor (8) and interleukin 1 (9).

A disturbance is also likely to occur in case of endothelial cell damage. Procoagulant activities, associated with intracellular structures (10-12), will then be exposed to the plasma.

Up till now, no anticoagulant mechanisms are known that become active after cell injury and that contribute to a normalization of the disturbed procoagulant - anticoagulant balance.

This study describes the presence in endothelial cells of a recently discovered (13) vascular anticoagulant (VAC), which binds in the presence of calcium to negatively charged phospholipids (14). As a consequence of this binding, the phospholipid dependent procoagulant reactions (intrinsic factor X- and the prothrombin activation) are inhibited by VAC. In this article we demonstrate that the endothelial VAC binds in the presence of calcium to intracellular structures and, thereby, inhibits the procoagulant activities of these structures. Based on these features, we propose that VAC provides an anticoagulant mechanism, which can control thrombin formation at sites of damaged endothelial cells.

MATERIALS AND METHODS

Proteins - A standard pool of human platelet free plasma (PFP) was prepared (13). Human brain thromboplastin was isolated following the method described by van Dam-Mieras et al. (15). VAC was purified from carefully dissected human umbilical cord arteries in a way identical to the purification procedure of bovine VAC (14). The purified preparation was homogenous for the Mr=32,000 band as judged with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The modified prothrombin time test (MPT-test) employed for quantifying of VAC-activity, has been described in detail in (13).

Antibodies - Normal swine serum and horse radish peroxidase (HRP)- labeled swine anti-rabbit IgG were purchased from DAKO (Denmark). Rabbit antibodies to human VAC were produced with standard techniques: Rabbits were injected subcutaneously with 500 μg purified human VAC in 1 ml Freund's complete adjuvant. After four weeks, rabbits were boostered with 100 μg of purified VAC in Freund's incomplete adjuvant. Boosters were repeated four times with two week intervals. Finally, the rabbit serum was collected and fractionated with 35 % ammoniumsulfate precipitation. The precipitated proteins were dialyzed into Tris-buffered saline (TBS, 50 mM Tris/HCl, pH 7.9, 100 mM NaCl) and subsequently applied to a DEAE-Sephadex column (Pharmacia, Uppsala Sweden), which was equilibrated with TBS. The unbound protein fraction, containing the anti-VAC antibodies, is referred to as purified a-VAC Ab.

Cell culture - Endothelial cells derived from human umbilical cord veins were prepared according to the method of Thornton (16) as modified in our laboratory (17). In brief, after harvesting endothelial cells from the vein of the umbilical cord by collagenase digestion, the cells were cultured in flasks pretreated with fibronectin in RPMI 1640 tissue culture medium supplemented with 20 % human serum and growth factors. Cultures were characterized

as endothelial cells based on morphology, presence of angiotensin converting enzyme and presence of factor VIII related antigen/von Willebrand antigen.

Fractionation of endothelial cells - Confluent cell cultures (passage 2 and 3) were washed three times with ice cold TBS. Following the last wash, cold homogenization buffer (0.25 M sucrose, 10 mM Tris/ HCl. pH 8.1, 1 mM leupeptin (Boehringer Mannheim, W.Germany), 1 mM EDTA or 1 mM $CaCl_2$) was added and the cells were removed from the surface of the flask with a rubber policeman. The suspended cells were homogenized using a tight fitting teflon pestle tissue grinder (Braun-Melsungen, W. Germany). The homogenates were submitted to differential centrifugation, following the scheme depicted in fig. 1, in a Beckman TL-100 table top ultracentrifuge. Pellets were resuspended in a volume of TBS, which equals the starting volume of the homogenate. Homogenization and fractionation were carried out at 0^{o} C. Protein contents of the different fractions were determined following the method as described by Sedmak (18). Lactate dehydrogenase activity was assessed by the procedure of Wroblewski (19).

Electrophoresis and immunoblotting - SDS-PAGE was carried out as described by Laemmli (20) on slab gels containing 10 % acrylamide, 0.27 % N,N^3-methylenebisacrylamide and 0.1 % SDS. The gels were either stained with Coomassie Brilliant Blue (13) or used for immunoblotting as described by Towbin et al. (21). Proteins were electrophoretically transferred from the gel into a nitrocellulose paper (Bio-Rad Richmond, California). The part of the paper into which the marker proteins were blotted, was stained with Amido Black. The rest of the paper was incubated for one hour at 37^{o}C with phosphate buffered saline (PBS, 10 mM KH_2PO_4/K_2HPO_4, pH 7.4, 150 mM NaCl, 0.02 % (v/v) Tween-20, and 1 mg/ml bovine serum albumine) containing 0.2 % normal swine serum. Subsequently the paper was incubated for another hour at 37^{o}C with PBS, containing a-VAC Ab (25 μg/ml). After this period, the

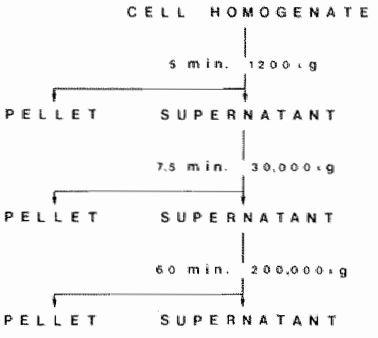


Fig. 1. Differential centrifugation scheme.
Homogenates, which were prepared from endothelial cells as described in materials and methods, were submitted to differential centrifugation following the above depicted routine. Centrifugation was carried out at 0°C in a Beckman TL-100 table top ultracentrifuge. Pellets were resuspended in TBS with a volume, which equals the starting volume of the homogenate.

paper was rinsed with PBS, and further incubated for one hour at 37°C with HRP-labeled swine anti-rabbit IgG, diluted 1:500 in PBS. The paper was rinsed free of unbound HRP-labeled IgG, and the bound HRP-labeled Ab was visualized by incubating the paper with 0.5 mg/ml DAB (3,3-diamino-benzidinetetra-chloride, Fluka AG Buchs, Switzerland) in 50 mM Tris/HCl, pH 7.5, 0.025 % H_2O_2. In each immunoblot experiment a) omission of a-VAC Ab, b) replacement of a-VAC Ab by preimmune rabbit serum, and c) employment of a-VAC Ab, which was preincubated with purified VAC, were included as controls. In one experiment (fig. 2a) electrophoresis was carried out with ready-prepared polyacrylamide gradient gels (10-15%) from Pharmacia in the Pharmacia Phast System. Following electrophoresis, the proteins in the gel were transferred by diffusion into nitrocellulose paper at 37°C. The paper was further processed as described above.

Immunofluorescence and immunoperoxidase staining - Immunofluorescence was used for localisation of VAC on the cell-surface and in

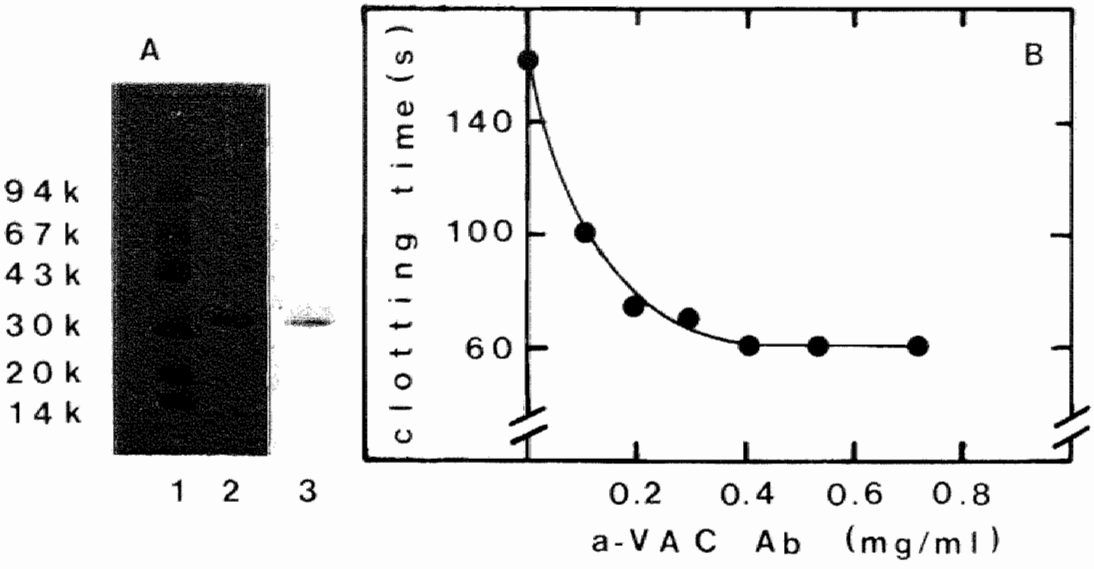


Fig. 2. Evaluation of the specificity of the a-VAC Ab.
A: 0.4 μg purified human VAC was electrophoreted on a SDS polyacrylamide gel, employing the Pharmacia Phast system. The gel was either stained with Coomassie Brilliant Blue (CBB) (lane 2) or used for immunoblotting (lane 3) as described in materials and methods. In the blot experiment, 3 μg/ml a-VAC Ab was used. Lane 1 gives the low molecular weight markers, stained with CBB.
B: 2 μg purified VAC was incubated with varying amounts of a-VAC Ab in TBS, containing 1 mg BSA/ml, at 37° for 30 minutes. Subsequently the activity of VAC was tested in the MPT-test. The control clotting time in the absence of VAC was about 60 seconds.

the cytoplasma. For detection of cell-surface bound VAC, endothelial cells were detached from culture flasks with EDTA and subsequently transferred into PBS, containing 0.1% BSA and 0.01% NaN_3. Intracellular fluorescence for detection of VAC within the cells was assessed by making the cells permeable for the reagents by fixation with 70% ethanol in ice for 30 minutes under constant stirring. Subsequently, the intact and the fixed cells were incubated with a-VAC Ab, washed 3 times, and incubated with swine anti-rabbit IgG, labeled with FITC. Following this incubation, the cells were washed 3 times and the fluorescence of the cells as read on a FACS IV (Beckton and Dickenson, Sunny Vale, Los

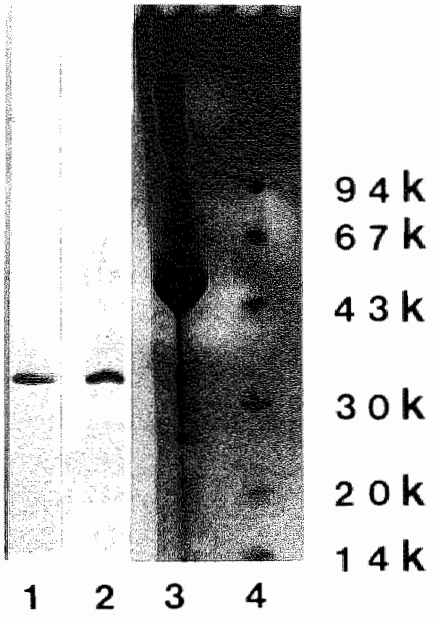
Angelos). Controls consisted of cells treated with rabbit anti-dog IgG (as nonspecific control) and subsequent incubation with fluorescent conjugate.
Immunoperoxidase staining of ethanol fixed endothelial cells was performed on endothelial cells, cultured on slides and fixed as described above. The cells were first incubated with a-VAC Ab, and after 3 washes, with swine anti-rabbit IgG, labelled with peroxidase. The visualization occurred with DAB. The cells were counterstained with hematoxiline and mounted as usual.

RESULTS

Characterization of the a-VAC Ab - The purified a-VAC Ab, obtained from the serum of rabbits as described in the materials and methods, was tested for its reactivity with purified VAC in an immunoblot experiment and in the MPT-test. Both tests revealed that the a-VAC Ab reacts with the purified VAC molecule: the a-VAC Ab not only binds to purified VAC (fig. 2A) but also inhibits in a dose-dependent manner the anticoagulant activity of VAC (fig. 2B). The specificity of the a-VAC Ab was examined by immunoblotting the protein contents of a vessel wall homogenate, prepared from human umbilical cord arteries. The immunoblot (fig. 3) shows that the a-VAC Ab reacts with a 32k daltons band, which comigrates with purified VAC.
Control immunoblots were all negative. Minor cross reactions can be observed with some other proteins present in the homogenate.
Immunoblot analysis of vessel wall homogenates, prepared from aortas of other species than man, reveals that horse and rat aorta contain a 32k daltons protein, which is recognized by the a-VAC Ab, and bovine aorta contains a 32k daltons and a 34k daltons band, which react with the a-VAC Ab.

Detection of VAC in cultured human endothelial cells - Using the immunoblot technique, it was demonstrated that homogenates of human umbilical cord venous endothelial cells (HUVE) contain a 32k daltons antigen which is recognized by the a-VAC Ab and which

fig. 3: Evaluation of the specificity of the a-VAC Ab.
The human umbilical cord arteries were homogenized in cold TBS, containing 1 mM EDTA. The homogenate was centrifuged for 6,000 x gmin. at 0°C. The resulting supernatant was submitted to SDS-PAGE. The separated proteins were either stained with CBB (lane 3) or transferred into nitrocellulose paper, which was thereafter treated with a-VAC Ab (3 μg/ml) as described in materials and methods (lane 2). Lane 1 represents 1 μg of purified VAC, and lane 4 gives the CBB stained low molecular weight markers.



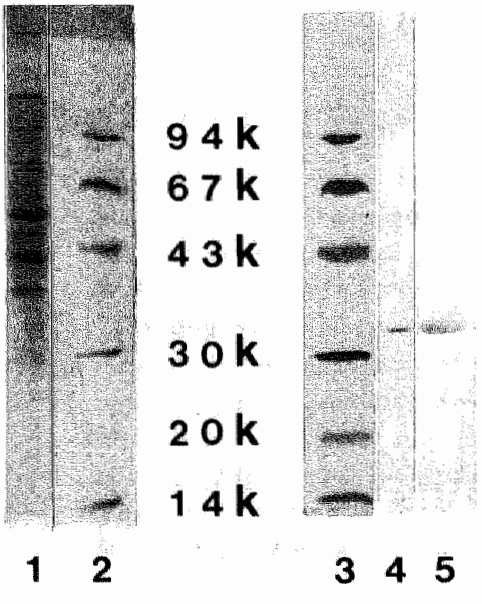


Fig. 4. Immunochemical detection of VAC in cultured endothelial cells.
10^6 Confluent endothelial cells were homogenized in homogenization buffer, containing 1 mM EDTA. The proteins in the homogenate were separated with SDS-PAGE and subsequently stained with CBB (lane 1) or immunoblotted with 3 μg a-VAC Ab/ml (lane 4). Lane 5 contains 1 μg of immunoblotted purified VAC. Lane 2 and 3 give the low molecular weight markers. Lane 2 was stained with CBB, and lane 3 was first blotted into nitrocellulose paper and subsequently stained with Amido black.

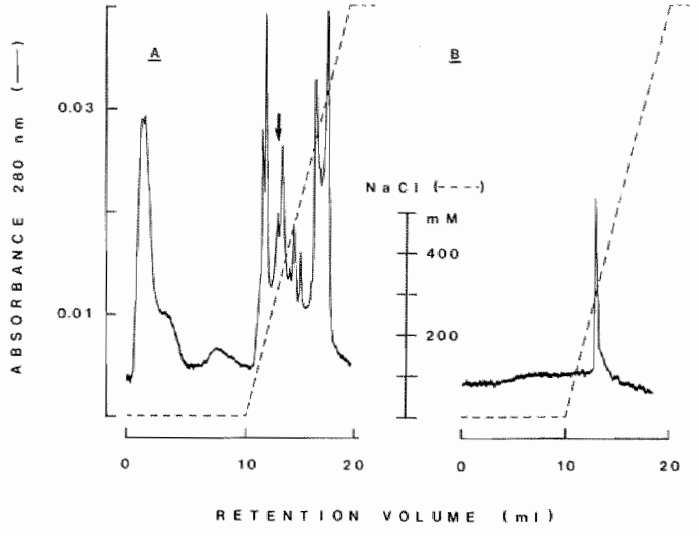


Fig. 5: Chromatography of lyzed endothelial cells and purified VAC on the anion exchanger Mono.

A) 10^6 confluent endothelial cells were resuspended in 20 mM bis-Tris/HCl, pH 6.3, and lyzed by 3 cycles of freezing and thawing. The lysate was submitted to chromatography on Mono Q. The linear NaCl gradient was constructed in 20 mM bis-Tris/HCl, pH 6.3. The fractions (160 μl each) were analyzed with immunoblotting using a-VAC Ab. The VAC-antigen eluted at 300 mM NaCl with a retention volume, which is indicated by the arrow.

B) 8.5 μg purified VAC was submitted to chromatography on Mono Q with conditions identical as under A. The fractions were tested in the MPT-test for VAC-activity, which co-elutes with the protein peak.

comigrates with purified VAC (fig. 4). Control immunoblots were negative. Since it is most likely that this antigen represents the VAC-molecule, the antigen was further characterized by chromatography on Mono Q. A homogenate of HUVE was applied to the Mono Q column at a 100 mM NaCl concentration. A linear gradient of NaCl was employed for elution of the bound proteins. Immunoblot analysis of the eluted fractions revealed that the 32k daltons antigen elutes from the Mono Q column with a 300 mM NaCl concentration (fig. 5A), which is the concentration required for

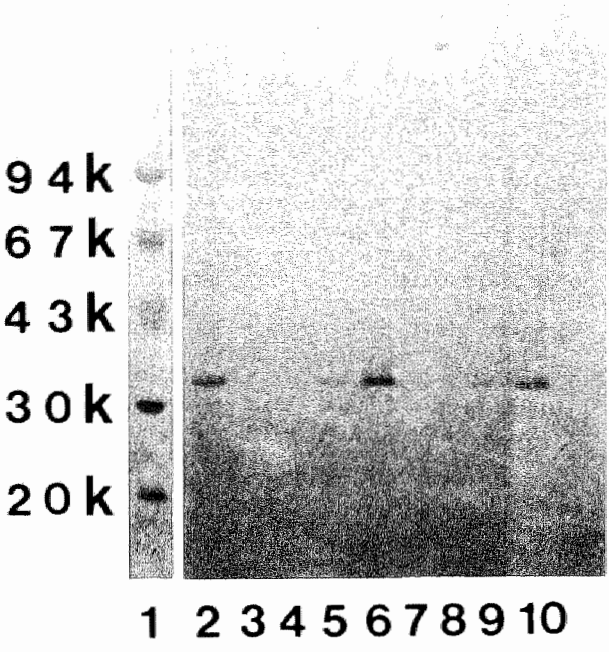


Fig. 6: Distribution of VAC over the subcellular fractions of an endothelial cell homogenate. Effect of EDTA and calcium.
10^6 Confluent endothelial cells were homogenized in 400 μl homogenization buffer as described in materials and methods, either in the presence of 1 mM EDTA (lane 2-5) or 1 mM calcium (lane 7-10). The homogenates were fractionated following the scheme of fig. 1. Pellets were resuspended in 400 μl TBS, containing 1 mM EDTA. Equal volumes of each fraction were submitted to immunoblotting using a-VAC Ab (3 μg/ml). The lanes are as follows: Lane 1; low molecular weight markers, which are stained with Amido black. Lanes 2 and 7; vesicle free supernatants. Lanes 3 and 8; 12.10^6 x gmin. pellets. Lanes 4 and 9; 227,000 x gmin. pellets. Lanes 5 and 10; 6,000x gmin. pellets. Lane 6; 1 μg purified VAC.

elution of the purified VAC from the Mono Q under identical conditions (fig. 5B). Therefore the physical properties of the 32k daltons antigen and the VAC molecule are identical in this regard. Another similarity between the antigen and the VAC-molecule is the reversible binding to intracellular structures in the presence of calcium. Fractionation of an endothelial cell homogenate in the presence of 1 mM calcium results in an attachment of the antigen to the intracellular endothelial structures. The antigen is mainly present in the 6,000 x gmin pellet. EDTA induces a release of the antigen into the vesicle free supernatant (fig. 6). However, EDTA induced depletion of the 6,000 x gmin pellet from the antigen is not complete.

This feature of the VAC-antigen enabled us to assess its possible anticoagulant activity. Homogenates of HUVE were prepared and fractionated as described above. The fractions were analyzed for

Table 1

Effect of subcellular fractions of endothelial cell homogenates on the recalcification time of citrated platelet free plasma: influence of EDTA and calcium.

EDTA-fractions[a]	protein[b] (μg)	LDH[c] (% of total)	clotting time[d] (s)
6,000 x gmin. pellet	6.0	3	228
227,000 x gmin. pellet	5.0	1	252
12.10^6 x gmin. pellet	6.0	2	684
vesicle free supernatant	20.0	94	1900[e]
blank	-	-	1100
Calcium-fractions[f]			
6,000 x gmin. pellet	9.0	4	432
227,000 x gmin. pellet	7.5	1	456
12.10^6 x gmin. pellet	8.0	1	786
vesicle free supernatant	11.5	94	1020
blank	-	-	1100

10^6 endothelial cells were homogenized in 400 μl homogenization buffer as described in material and methods, either in the presence of 1 mM EDTA ([a]) or in the presence of 1 mM calcium ([f]). The homogenates were fractionated as described in fig. 1. The pellets were resuspended in 400 μl TBS. [b] Protein contents were determined according to the method of Sedmak (18). [c] Lactate dehydrogenase activity was assessed following the method described by Wroblewski (19). [d] 75 μl of a fraction was added to 50 μl citrated platelet free plasma. The mixtures were stirred at 37^0 C. At time point zero prewarmed calcium-containing buffer was added. Final volume of the reaction mixture was 500 μl and final calcium concentration was 10 mM. Clotting is monitored optically (13). Blank clotting time is about 1100 s. [e] Preincubation of an aliqout of this fraction with a-VAC Ab results in a reduction of the clotting time to its blank value. The data are the mean of two separate experiments.

protein concentration and lactate dehydrogenase activity (table 1). Furthermore aliquots of the fractions were added to citrated normal human platelet free plasma, which was thereafter recalcified. Resulting clotting times are given in table 1.

The intracellular structures significantly shorten the clotting time of the recalcified plasma. Intracellular structures prepared in the presence of calcium, however, possess less procoagulant

activity in comparison with the structures prepared in the presence of EDTA. This diminished procoagulant activity coincides with the presence of the VAC antigen in these fractions (fig. 6). On the other hand the vesicle free fraction, prepared in the presence of calcium, lacks an anticoagulant activity (table 1) as well as the VAC antigen (fig. 6). In contrast to this, the vesicle free EDTA fraction prolongs the clotting time and contains the VAC antigen. This prolongation of the clotting time is prevented by incubating the vesicle free EDTA fraction with a-VAC Ab prior to addition to the PFP. These results demonstrate that the VAC antigen and the purified VAC are identical, moreover, the endothelial VAC lowers the procoagulant activities of intracellular structures.

Preliminary studies on the localization of VAC in the endothelial cell - In the previous section we have described the presence of VAC in HUVE and its attachment to intracellular structures in the presence of 1 mM calcium. This condition does not represent the physiological situation of the intact cell, which maintains a free calcium concentration of about 0.1 μM. To get an insight in the localization of VAC within HUVE, we have conducted immunofluorescence studies on HUVE using a-VAC Ab. The FACS data show, that VAC is not present in detectable amounts on the surface of the HUVE. However, fixed cells, permeable for the a-VAC Ab, show significant increase in fluorescence intensity (fig.7). Immunoperoxidase staining of HUVE with a-VAC Ab reveals a perinuclear staining, which is granular and diffuse over the cytoplasm (fig. 8). Immunoperoxidase staining on intact cells is negative using a-VAC Ab, (data not shown).

The presence of VAC in other vascular cells - Cell homogenates prepared from cultured human omentum capillary endothelial cells and human aortic smooth muscle cells were submitted to immunoblotting as described for HUVE homogenates. Also these cells appear to be positive for the 32k daltons antigen. These results indicate that the presence of VAC is not restricted to the endothelial cell, which is derived from the human umilical cord vein.

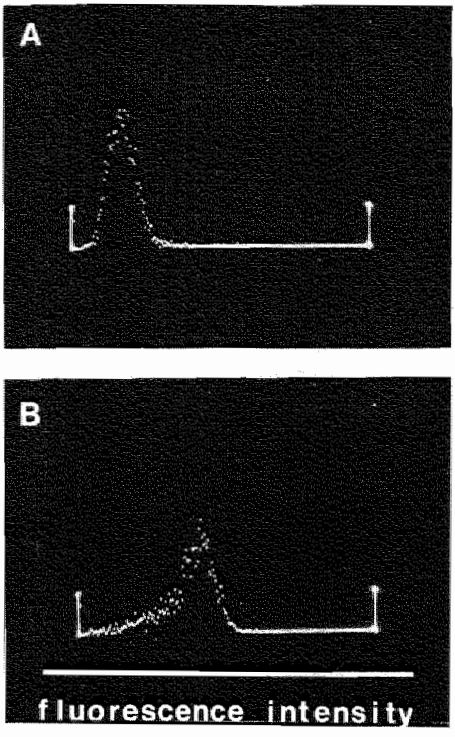


Fig. 7. Immunofluorescence staining of intact and permeabilized endothelial cells, using a-VAC Ab.
A) Intact endothelial cells, detached from the culture flask, were incubated with a-VAC Ab, and swine anti-rabbit IgG, labeled with FITC. The cells were evaluated on the FACS.
B) Prior to the incubation with a-VAC Ab and FITC-labeled anti-rabbit IgG, the intact endothelial cells were permeabilized by fixation with 70% ethanol on ice. The cells were analyzed with the FACS. Control immunofluorescence images of intact and permeabilized endothelial cells, cannot be distinguished from the image in panel A.

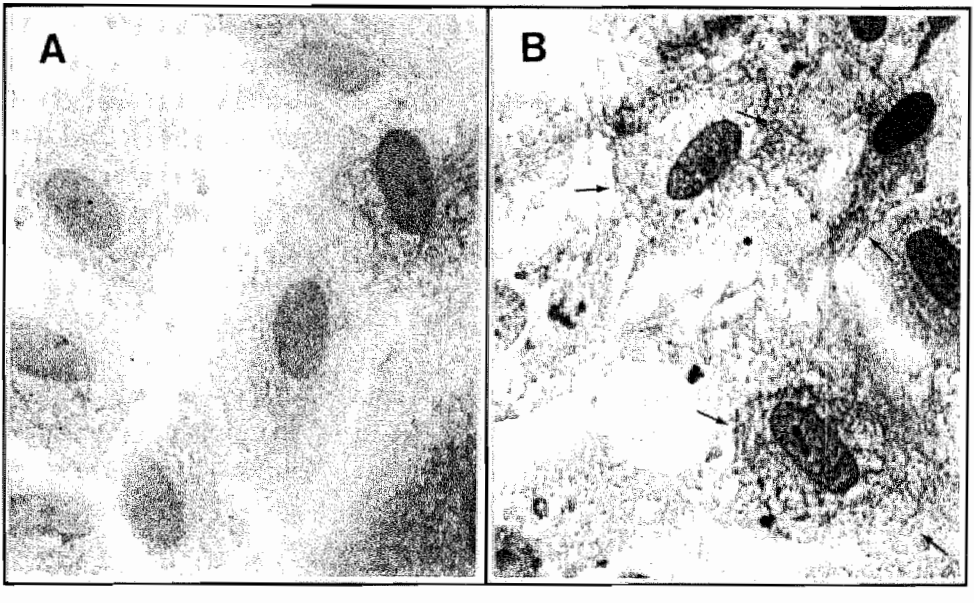


Fig. 8: Immunoperoxidase staining of spread permeabilized endothelial cells.
Endothelial cells, spread on gelatin coated glass cover slips, were made permeable for antibodies with ethanol fixation, and subsequently incubated with preimmune serum (A) or with a-VAC Ab (B), and swine anti-rabbit IgG, conjugated with horse radish peroxidase. The arrows indicate some of the granulated brown precipitates.

DISCUSSION

The discovery in vessel wall homogenates of a unique anticoagulant activity and the elucidation of its assosiation with a single chain protein (VAC) with Mr=32,000 (13), have prompted us to investigate the localization of VAC in the vessel wall. This paper reports on this study, which is conducted with immunological and chemical techniques, applied to cultured vascular endothelial cells.

Recently, a complete purification procedure (14) for VAC was established using bovine aorta, which contains an anticoagulant activity similar to that found in human umbilical cord arteries (13). Application of this procedure using human umbilical cord arteries as a source, results in a purified VAC preparation, which is homogenous for a 32k daltons protein. Rabbits immunized with this purified VAC gave rise to IgG antibodies, which bind to the purified VAC and which inhibit the anticoagulant activity of purified VAC, as was judged from immunoblotting studies and functional tests, respectivily. The antibodies bind to a 32k daltons antigen, which is present in the homogenate prepared from human umbilical cord arteries and which comigrates with the purified VAC. Minor cross reactions of the antibodies with other proteins, present in the homogenate, are observed. These findings indicate that we have obtained a polyclonal IgG preparation (a-VAC Ab), which recognizes specifically the VAC molecule.

In order to obtain an insight in the localization of VAC in the vessel wall, we have employed the a-VAC Ab in immunoblotting studies, in which lysates of cultured cells were used. We have focussed this study on endothelial cells. Endothelial cells, derived from human umbilical cord veins (HUVE), appeared to contain a 32k daltons antigen, which comigrates with purified VAC. Besides the similarity in molecular weight, this antigen and VAC share various other features:

1) Both the antigen and VAC bind reversibly in the presence of calcium to intracellular structures.
2) The interaction of both substances with the anionic resin

Mono Q is identical, indicating similarities in physical properties.

3) The antigen cofractionates with an anticoagulant activity, which can be inhibited with a-VAC Ab, suggesting that the anticoagulant activity is coupled to the antigen.

These findings clearly demonstrate the presence of VAC in cultured endothelial cells. Whether or not the endothelial VAC originates from de novo synthesis is unknown. Studies to elucidate this topic, are under way. The presence of VAC in the cell may implicate (a) physiological function(s) of VAC, which is/are not clear at the moment. From the localization of VAC as well as on the basis of its phospholipid-binding characteristics, VAC can be classified as an extrinsic membrane protein, and thus, VAC might play a role in membrane dependent processes. At present we know that, due to its high affinity for negatively charged phospholipids (14), VAC inhibits the phospholipid dependent activation of prothrombin by factor X_a and calcium (13,14), and the phospholipid dependent activation of factor X by factor IX_a and calcium (chapter 4 of this thesis).

The distribution of VAC in the cell differs strikingly from that of the secretable proteins, von Willebrand factor (22) and protein S (3). Together with our failure to detect VAC in the cell-conditioned medium and the absence of VAC at the outside of the plasma membrane, the intracellular localization of VAC apparently precludes a function for VAC as an physiological active extracellular anticoagulant. However, the employed endothelial cells in the secretion study were quiescent, indicating, that VAC is not secreted constitutively, but not excluding the secretion of an intracellular pool of VAC upon cell perturbation. Furthermore, factor V, which is demonstrated to be secreted by endothelial cells (23), shows an intracellular distribution, which resembles the distribution of VAC. Therefore, further studies have to be undertaken to elucidate whether under certain conditions VAC can be secreted by endothelial cells.

The data obtained so far indicate that intracellular VAC may play a role in hemostatic processes following cell damage. Intracel-

lular structures, which then become accessable to plasma and which probably consist of intracellular membranes, a Hageman factor activating compound (12) and tissue factor (10,11), excert a procoagulant activity. It is likely that the negatively charged phospholipids of these structures play an accelerating role in the procoagulant reactions. Our study, in which we measured the procoagulant activity of the intracellular structures, prepared in the presence of calcium or EDTA, demonstrates that endothelial VAC, which is bound to intracellular structures in the presence of calcium, diminishes the procoagulant activity of these structures. However, this procoagulant activity is not completely blocked by endogenous VAC, indicating that there is not sufficient VAC present for complete inhibition and/or the intracellular structures exhibit a procoagulant activity, which cannot be inhibited by VAC.

The demonstration in endothelial cells of the presence of VAC with an anticoagulant activity, adds a new possibility to the list of anticoagulant mechanisms through which endothelial cells can modulate thrombin formation. VAC provides protection against a surplus of procoagulant phospholipids at the site of a wound.

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CHAPTER 6

A NEW HOMEOSTATIC MECHANISM TO CONTROL THROMBIN FORMATION AT THE VESSEL WALL LEVEL.

A NEW HOMEOSTATIC MECHANISM TO CONTROL THROMBIN FORMATION AT THE VESSEL WALL LEVEL.

Reutelingsperger, C.P.M., Hornstra, G., Hemker, H.C.

SUMMARY

Vessel wall procoagulant activity was studied by exposing under non-flow conditions rat aortic tissue to citrated autologous plasma, which was recalcified. By fixing the tissue in a specially designed tissue template a standardized area of a pre-determined part of the tissue could be investigated.
The vascular procoagulant activity is enhanced by platelets. Platelet adhesion to and platelet thrombi formation on the subendothelium parallels vessel wall induced thrombin formation, suggesting that these platelet responses and the coagulation process are interrelated.
The procoagulant activity of a standardized area of several vascular structures was measured. Among the vascular structures investigated, the subendothelium exhibits the lowest procoagulant activity. This activity could be abolished by phospholipase C from Bacillus cereus and is therefore likely due to tissue factor activity. The balloon catheter treated luminal side and the abluminal side of the aorta posses higher procoagulant activity.
VAC, a single chain vascular protein, which inhibits the phospholipid dependent procoagulant reactions through a calcium dependent high affinity binding to the phospholipids, completely inhibits the procoagulant activity of the subendothelium, under the standardized conditions. The activity of the more severly damaged aorta, however cannot be fully blocked by VAC, under those conditions. Since VAC is present in endothelial cells we conclude that VAC provides a novel mechanism to control thrombin formation at the superficially damaged vessel wall. Moreover, VAC may be a useful anticoagulant without the risk of bleeding.

INTRODUCTION

The endothelium of a vessel wall provides in its quiescent state a nonthrombogenic surface, to which platelets do not adhere (1), and on which anticoagulant activities dominate (2,3).
In experimental studies of the response of blood to vessel injury under flow conditions, it is observed that desquamation of the endothelium leads to a coagulation-independent adherence of platelets to subendothelial components, such as collagen (4) and Von Willebrand factor (5). The adhered platelets spread to cover the exposed subendothelial surface and to form a continuous carpet, on which via coagulation-independent processes, transient thrombi can be formed (6). The spread platelets release some of their granular contents, which penetrate the vessel wall (7). This sequence of apparently coagulation-independent events is believed to contribute to the process of smooth muscle cell proliferation and the development of atherosclerotic lesions (8). Following the removal of endothelium in vivo, a neointima of smooth muscle cells is formed (9). Injury to this neointima leads to a platelet accumulation, in which the coagulation cascade seems to be involved (10,11). Tissue thromboplastin activity, reported to be present in the vessel wall (12-14), could be responsible for the generation of thrombin, which then could induce thrombus formation onto the injured neointima.
Experiments carried out under conditions in which blood flow is impaired or absent, showed that the injured vessel wall which has not yet developed a neointima, also can induce the generation of thrombin (15).
In this paper we present further evidence that injured vessel wall induces the formation of thrombin, which in turn can activate platelets. These vessel wall induced processes can be regulated by the novel anticoagulant protein VAC, which has been discovered and characterized in our laboratory (16, chapter 2 and 3 of this thesis). By using a specially designed tissue template, we were able to discriminate between the procoagulant activities

of the different structures of the vessel wall. Structures very close to the vascular lumen possess low procoagulant activity which can be inhibited completely by VAC. Structures more remote from the vascular lumen appear to possess a higher procoagulant activity, which cannot be fully inhibited by VAC within the concentration range tested. Based on these results, we suggest that VAC is involved in the homeostatic regulation of coagulation at the vessel wall lining. Upon superficial vascular injury VAC efficiently inhibits the procoagulant activity of the vessel wall and thereby prevents thrombin-mediated platelet activiation and thrombus formation. When deeper injuries occur, and especially at vessel transsection, the stronger procoagulant potential of the deeper vascular layers overrules VAC-activity, as a result of which thrombin can be formed to promote platelet plug formation, fibrin generation and ultimately haemostasis. Thus, VAC may also be a usefull anticoagulant without increasing bleeding tendency. Part of this work has been presented in abstract-form before (17).

MATERIALS AND METHODS

Materials - Indomethacin, hirudin grade IV, and phospholipase C (EC 3.1.4.3) from Bacillus Cereus were products of Sigma (St. Louis, USA). Bovine vascular anticoagulant was purified from bovine aortic intima following the procedure described in chapter 3 of this thesis. The purified VAC had a specific activity of 1500 units/mg, as assessed in the MPT-test (16).

For the purpose of studying vessel wall-induced platelet aggregation and clot formation, a template-like device was designed, in which a piece of vascular tissue can be fixed. This tissue template made of arsenite, enables the exposure of a choosen part of the vascular tissue to a medium. The dimensions of the device (fig. 1) allow it to be put into a glass cuvette, which, in turn, fits into a Payton Dual Channel aggregometer module. Hence, when platelet rich plasma forms the medium exposed to the tissue, platelet aggregation and clot formation can be monitored optically.

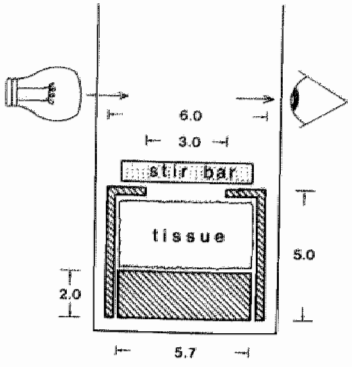

Fig. 1. Schematic presentation of the template, designed to study vessel wall procoagulant activity. The template is constructed of arsenite and its dimensions (expressed in mm) allows it to be placed into a glass cuvette, which fits into a Payton Dual Aggregometer. Hence turbidimetric measurements in the medium contacting the tissue is possible.

Preparation of platelet rich plasma - After puncturing the abdominal aorta of ether-anaesthetized adult male Wistar rats blood was collected and anticoagulated with trisodium citrate (final concentration, f.c., 0.38% (w/v)). Platelet rich plasma (PRP) was prepared through centrifugation of the anticoagulated blood for 10 min. at 800 x g. The residue was recentrifuged for 10 min. at 2000 x g to obtain platelet poor plasma (PPP). Subsequently, the PPP was centrifuged for 20 min. at 10,000 x g to give platelet free plasma (PFP).

Isolation of the aorta - Following blood collection, the thoracic and abdominal aorta was dissected, and rinsed with ice-cold Krebs-Henseleit buffer (K-H,120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 11.6 mM glucose, 25 mM $NaHCO_3$, pH 7.4). The aorta was prepared free of adhering tissue and opened longitudinally. Before further use the isolated aorta was kept 1 hour in K-H buffer, containing 10 μg/ml indomethacin, in order to block prostacyclin formation (18).

Vessel wall induced platelet aggregation and clot formation - A piece of aorta (5.0 mm in diameter) was punched out mounted in the tissue template and placed into a siliconized aggregation cuvette. Prior to incubation with PRP, PPP, or PFP, the vascular tissue was washed twice with 0.5 ml buffered saline (BS, 4 mM

KCL, 20 mM $NaHCO_3$, 14 mM glucose, 4 mg/ml bovine serum albumine and 110 mM NaCl, pH 7.4). After the second wash, 450 μl BS buffer, containing varying amounts of $CaCl_2$, was added to the aggregation cuvette, which was then placed into the Payton Dual Channel Agregometer and stirred at 450 rpm at 37°C. After 3 min. (= time point zero), 50 μl of PRP, PPP, or PFP, was added and platelet aggregation and clot formation, as reflected by change in optical density, were monitored continuously with a flat bed recorder (BD 41, Salm and Kipp). The clotting time t_c, which is the time (seconds) between addition of the plasma and the occurrence of clot formation, was used to calculate the clotting index, S, in the following way:

$$S = t_c^{-1} \times 1000,$$

Coagulation is directly proportional to this clotting index.

Electron microscopy - For electron microscopy, the tissue, mounted in the template, was washed twice with 0.5 ml of BS buffer, and subsequently fixed by the addition of glutaardialdehyde (f.c. is 2.5% in 0.1 M phosphate buffer, pH 7.3).

For transmission electron microscopy, the tissue was further processed, according to the epoxy embedding method described by Luft (20). In brief, the glutaardialdehyde-fixed tissue was washed with phosphate buffer (0.1 M, pH 7.3) and fast-fixed with osmium tetroxide (1% in phosphate buffer). Subsequently, the tissue was dehydrated in a graded series of ethanol concentrations and finally embedded in Epon 812. Ultra-thin sections, obtained with a diamond knife, using a Reichert Ultracut Microtome, were stained with uranyl acetate and lead citrate. Micrographs were taken with a Philips CM 12 microscope.

For scanning electron microscopy, the tissue was postfixed in osmium tetraoxide, dehydrated in graded ethanol, critical point dried from CO_2, mounted on scanning electron microscope stub, and gold-coated before they were viewed on a Philipis CM 12 microscope with a S.E. detector.

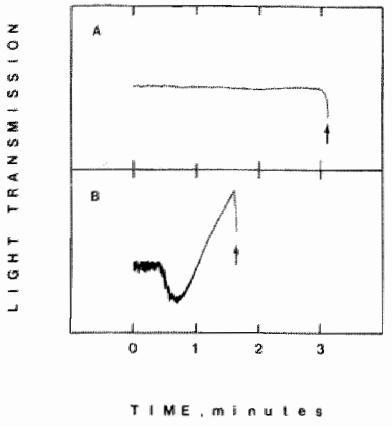

Fig. 2. Optical tracing during the contact of the aortic subendothelium with platelet free plasma (A) and platelet rich plasma (B).
A piece of indomethacin-treated aorta was fixed in the template, exposing the luminal side. Following two rapid washes with BS, the tissue was preincubated with BS, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After 3 min. 50 μl PFP (A) or PRP (B) was added. Light transmission through the medium was recorded with a Payton Dual Aggregometer. The arrows indicate the moments of clot formation.

RESULTS

Effects of the aortic tissue on recalcified plasma - Aortas, isolated from rats according to the procedure described in materials and methods, appear to lack an intact endothelium, as judged from electron microscopy (fig. 5B). The lamina elastica interna is covered with amorphous material, which is likely to be composed of the basal membrane and residues of the endothelium. When a piece of this aorta is punched out, mounted in the holder (fig. 1) and exposed to a medium, containing Coomassie Brilliant Blue, it is demonstrated that the part of the tissue, ment to be exposed, is indeed the only part being in contact with the medium. Typical tracings reflecting changes in optical density during the contact between aortic tissue and recalcified PFP and PRP are shown in fig. 2 A and B, respectively. Exposure to plasma of the structures, covering the lamina elastica interna

reveals a procoagulant activity resulting in fibrin formation, which, in the presence of platelets, is preceded by platelet shape change and platelet aggregation. Extensive washing did neither reduce the procoagulant activity of the exposed structures nor did it result in the appearance of procoagulant activities in the washing buffer. Therefore this activity is strongly associated with the tissue and is not released into the medium, under the prevailing experimental conditions.

The procoagulant activity, expressed as the clotting index S, depends on the calcium and magnesium concentration of the medium (fig. 3).

The presence of platelets significantly enhance the expression of the procoagulant activity of the aortic tissue (fig. 4). In PRP the platelets in the medium clearly change their shape and start to aggregate as a consequence of the exposure to the tissue. These platelets responses are mediated by thrombin, since heparin (1 U/ml) and hirudin (1 U/ml) completely abolish these platelet effects.

Electron microscopy of the luminal surface of the aortic tissue following the exposure to PRP revealed that when the platelets in suspension start to change shape, the tissue becomes covered with adhered and spread platelets (fig. 5C). When the platelets in suspension start to aggregate, large platelet aggregates have already been formed on the luminal surface of the vascular tissue; these platelet thrombi persist during later aggregation (fig. 5D). The platelets in the aggregates, attached to the tissue, still contain secretion granules. During aggregation no fibrin was observed in or nearby aggregates attached to the tissue. When clot formation has occurred in the plasma, large platelet aggregates are still attached to the vascular surface (5E). This surface and these platelet aggregates are, furthermore, covered with fibrin strands.

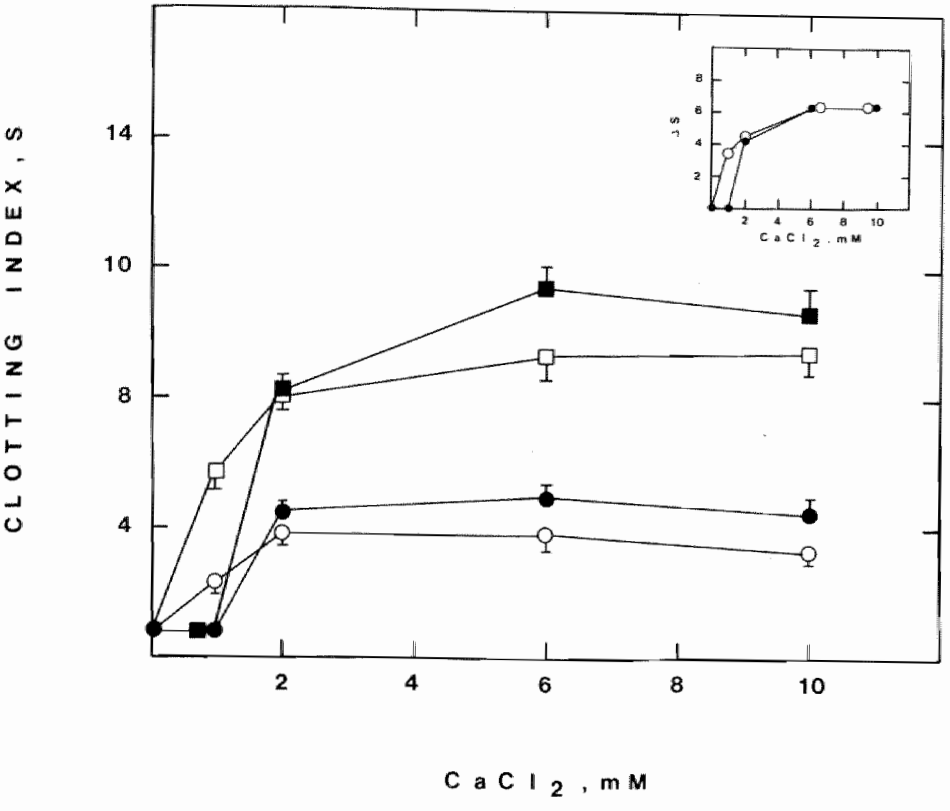

Fig. 3. The effects of calcium and magnesium on the vessel wall procoagulant activity as measured in platelet free plasma.
Indomethacin-treated aorta, fixed in the template, was exposed with its luminal side to 50 μl with 450 μl BS, containing varying amounts of $CaCl_2$ with 1 mM $MgCl_2$ (open symbols) or without $MgCl_2$ (closed symbols). The resulting coagulation times are expressed as clotting indices (■ , □). In control series, coagulation times were recorded under identical conditions, the only difference being the absence of tissue in the template (● , ○). The data are the mean + SEM (n=4).
The insert shows the difference between the clotting indices obtained in the presence of tissue and the ones obtained in the absence of tissue.

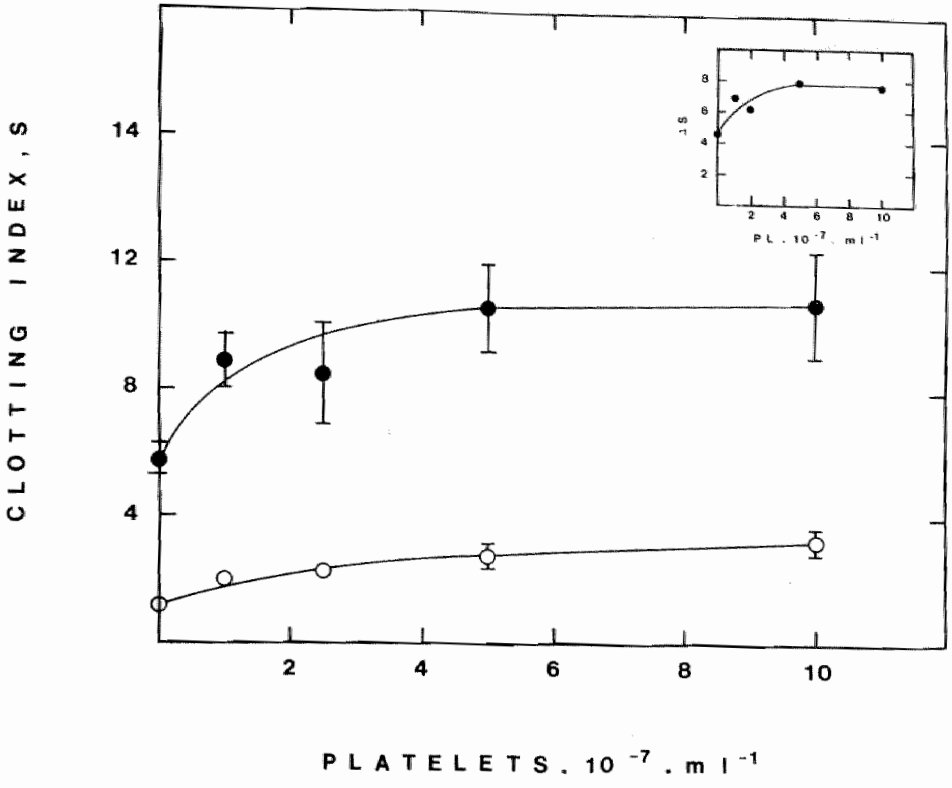

Fig. 4. Effect of the platelet count on the vessel wall procoagulant activity.
Indomethacin treated aorta, fixed in the template, was exposed with its luminal side to 50 μl PRP with varying platelet counts, and 450 μl BS, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The resulting coagulation times are expressed as clotting indices (●).
In control series, coagulation times were recorded under identical conditions but in the absence of tissue (○). The data are the mean + SEM of 6 separate experiments. The insert shows the difference in clotting indices between the ones obtained in the presence of tissue and the ones obtained in the absence of tissue.

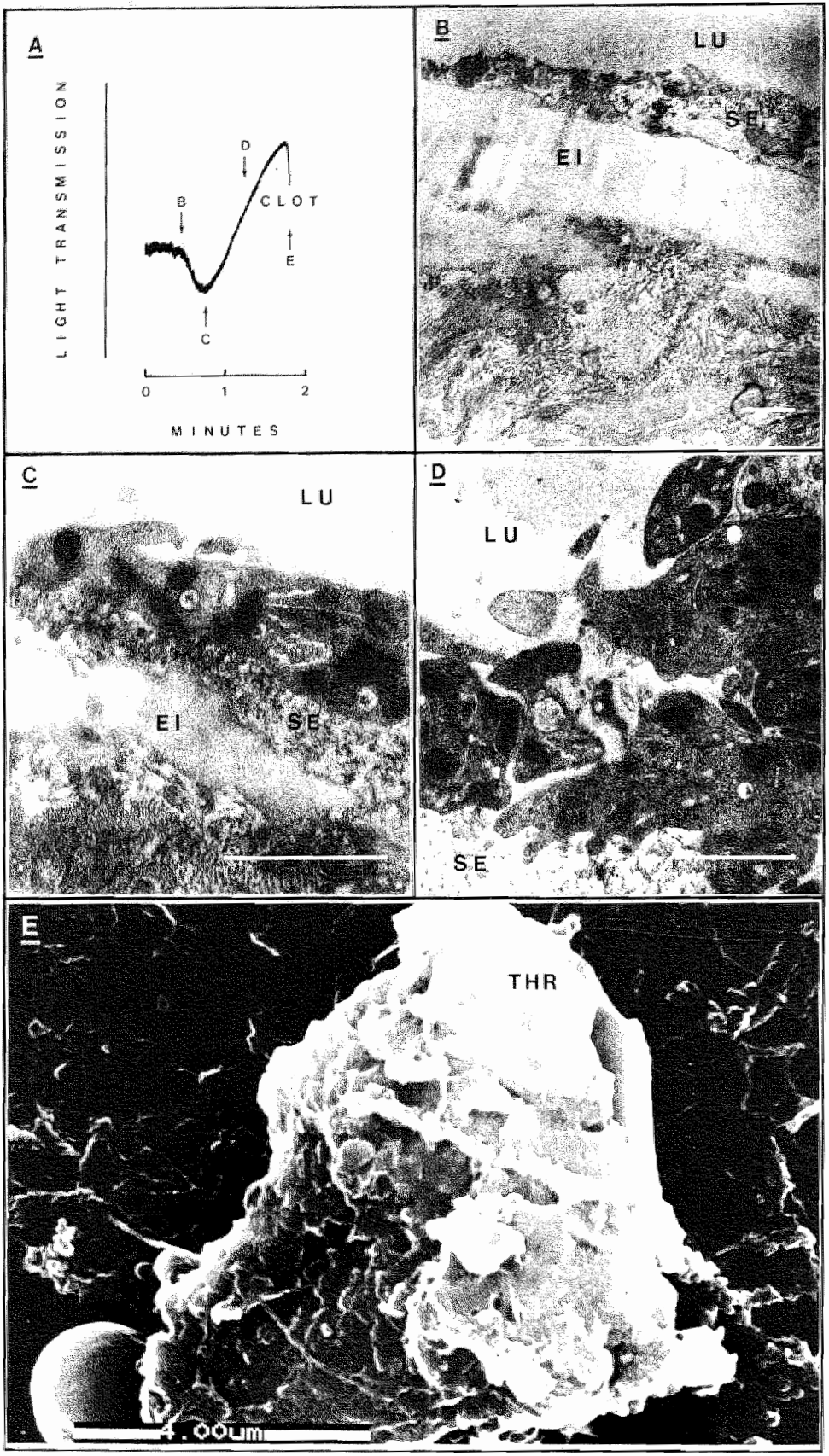
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Fig. 5. Electron microscopy of the platelet-vessel wall interaction during determination of vessel wall procoagulant activity.
Indomethacin-treated aorta was fixed in the template and exposed with its luminal side to 50 μl PRP and 450 μl BS, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. A typical optical tracing is shown in panel A. The arrows indicate the time points at which the incubation was interrupted, the vascular tissue was rapidly washed, fixed with glutaardialdehyde, and prepared for electron microscopic analysis as described in materials and methods. The letters at the arrows correspond with those of the subsequent panels, which show the electron micrographs, obtained at those time points. B-D are transmission micrographs. E is a scanning micrograph. LU: lumen. SE: subendothelial structures,probably composed of basal lamina and endothelial residues. EI: lamina elastica interna. THR: platelet thrombus. The white bars of panels B-D represent the length of 1 μm.

Effect of the degree of vessel wall damage on the exposed procoagulant activity - In the previous section, it is shown that the aorta, isolated from a rat, already has a superficially damaged luminal side, which exhibits a procoagulant activity. When, prior to its isolation, the aorta is additionally damaged by insertion of a balloon catheter followed by its withdrawal when still inflated, the luminal side of the aorta has a higher procoagulant activity (fig. 6). A severly damaged aorta, which can be mimicked by exposure of the abluminal side of the tissue to the medium, exhibits a very high procoagulant activity (fig. 6).

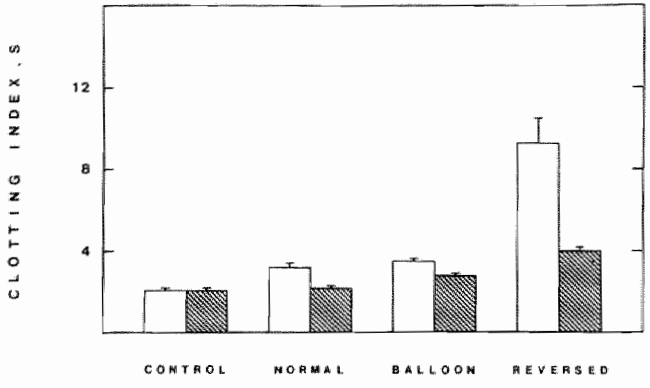

Fig. 6. Effect of the degree of vascular damage on the vessel wall procoagulant activity, and the effect of phospholipase C on the procoagulant activities of the variously damaged vessel walls.
Indomethacin-treated aorta, which was not additionally damaged (normal) or damaged by an indwelling balloon catheter (balloon), was fixed in the template and exposed with its luminal side to 50 μl PFP and 450 μl BS, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Normal aorta was also exposed with its abluminal side to this medium (reversed). Coagulation times are expressed as clotting indices (open bars).
Prior to exposure to PFP, the aortic tissues, fixed in the template, were incubated for 30 min. at 37°C with 6 I.U. phospholipase C. Subsequently, the tissue was washed and exposed to the PFP containing medium. Resulting clotting indices are given by the hatched bars. Control represents the clotting indices, obtained in the absence of tissue. The presented data are mean $\pm$ SEM (n=4).

Effects of phospholipase C and VAC on the procoagulant activities of the vessel wall - In order to investigate whether the vascular procoagulant activity is expressed by tissue factor, phospholipase C was employed. Since, antibodies against rat tissue factor were not available, phospholipase C was an acceptable alternative (23). Treatment of the damaged aortas with phospholipase C, prior to the exposure to PFP greatly reduces the procoagulant activities of the aortic tissues (fig. 6). The procoagulant activity of the superficially damaged aorta could be completely inhibited by

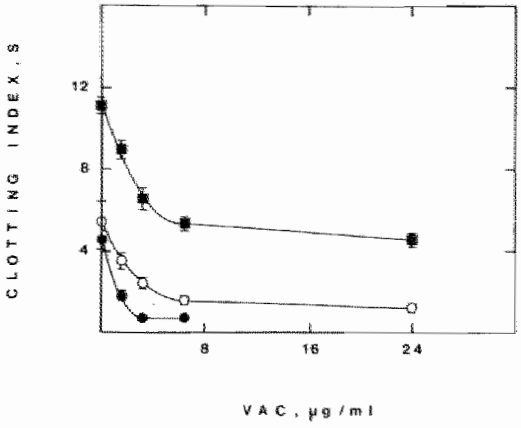

Fig. 7. Effect of VAC on the procoagulant activities of the variously damaged vessel walls.
Indomethacin-treated aorta, not additionally damaged (●) or damaged by an indwelling balloon catheter (○), was fixed in the template and exposed with its luminal side to 50 µl PFP and 450 µl BS, containing varying amounts of VAC, 1 mM $CaCl_2$ and 1 mM $MgCl_2$.
Untreated aorta was also exposed with its abluminal side to the medium (□). Registrated coagulation times are expressed as clotting indices (mean ± SEM, n=6).

phospholipase C. Furthermore, aortic tissue treated in this way did not induce platelet shape change and aggregation when brought into contact with PRP. The more severly damaged aortas, however, retain a certain procoagulant activity after phospholipase C treatment, under the given conditions. They are also able to induce platelet aggregation prior to clot formation. Comparable results were obtained when the procoagulant activity of the aortic tissue was measured in the presence of VAC (fig. 7). VAC, dose-dependently, inhibits the procoagulant activity of the superficially damaged aorta, which, in turn, fails to activate the platelets in suspension. Control experiments revealed that VAC has no effect on the ADP, collagen and thrombin induced platelet activation (data not shown). A complete inhibition by VAC, within the concentration range tested, can apparently not be reached when the aorta is more severly damaged.

The reduced procoagulant activity of the abluminal side, which was treated with phospholipase C, could, however, be completed inhibited by VAC within the concentration range of VAC, given in fig. 7 (data not shown). To examine the possible binding of VAC to the damaged aorta and to investigate as to whether the reduced procoagulant activity is a consequence of such a binding, aortas were incubated with an excess of VAC in the presence of $CaCl_2$, thoroughly washed with BS buffer containing 1 mM $CaCl_2$, and subsequently exposed to PFP in a medium containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The subendothelial structures preincubated with VAC in this way, did not exhibit any procoagulant activity. The presence of $CaCl_2$ during incubation of the tissue with VAC is a prerequisite for this effect, since the omission of $CaCl_2$ prevented the reduction of the vessel wall procoagulant activity, following the incubation with VAC and subsequent washing.

DISCUSSION

This paper presents a new method to investigate the procoagulant activity of vascular tissue and its effects on platelets and plasma. By means of a tissue template a standardized part of the vascular tissue can be exposed to a medium, in which turbidimetric measurements of clotting and platelet aggregation can then be performed.

In our study we have investigated the procoagulant activity of rat aorta. Inherent to the isolation procedure applied, the aorta lacks an intact endothelial layer. Instead, the lamina elastica interna is covered with subendothelial structures, which appear to exhibit a procoagulant activity. During the experiments performed, this activity remains associated with the subendothelial tissue.

Platelets significantly enhance the tissue procoagulant activity, as is in agreement with earlier observations (15). Platelets, once activated, trigger the intrinsic coagulation system (20) and, moreover, expose procoagulant sites on their membrane surface (21), as a result of which thrombin formation is accele-

rated.
The described experimental set-up to study vessel wall procoagulant activity shows that platelets, when present, first become adherant to the subendothelial structures, after which fibrin free platelet aggregates are formed. The platelets forming these aggregates still contain most of their secretion granules.
The absence of flow in our experiments has the advantage that any thrombin formed during the experiments remains in the investigated medium, and is thereafter easily detectable in the presence of platelets. Hence, it is observed that the vessel wall-induced formation of minor amounts of thrombin, which cause platelets to change shape but which do not induce fibrin formation, parallels the platelet adhesion to the subendothelium. The generation of larger amounts of thrombin, which induce platelet aggregation and ultimately fibrin formation, occurs simultaneously with the formation of platelet thrombi on the subendothelial surface. These results suggest that platelet adherence, thrombi formation on the subendothelial surface, and vessel wall induced thrombin formation are interrelated.
Baumgartner and Muggli (6) observed a similar sequence of platelet events, when perfusing subendothelial structures with citrated blood. The presence of the anticoagulant suggests these platelet responses to be coagulation-independent. Ardlie and Han (22), however, reported that platelets have a microenvironment (the "platelet atmosphere"), containing coagulation factors. Within this environment formation of small amounts of thrombin is possible even in the presence of citrate. In agreement to this, Hornstra and Hemker (15) observed vessel wall induced thrombin formation in the presence of platelets at very low calcium concentrations.
EDTA, on the other hand, disturbs the microenvironment of the platelet in such a way that thrombin formation is impossible (22). In the perfusion chamber of Baumgartner (6) EDTA prevents the spreading of platelets on subendothelial structures, and, moreover, the formation of thrombi. Together with the "platelet atmosphere" theory, these observations suggest that thrombin can

be formed in the vicinity of the vessel wall and platelets, even in the presence of the anticoagulant citrate. This thrombin may be required for platelet spreading and thrombus formation. Hence, the role of thrombin during the early events (platelet spreading, platelet thrombi formation, etc.) following vessel wall injury, requires reevaluation.

The vascular procoagulant activity appears not to be uniformly distributed over the vessel wall. Structures in the vessel wall more remote from the vascular lumen exhibit the highest procoagulant activity, whereas the subendothelial structures apparently posses the lowest activity. This latter activity can be completely inhibited by phospholipase C from bacillus cereus, which inactivates tissue factor activity (23), indicating that tissue factor constitutes the procoagulant activity of the subendothelium. The presence of tissue factor activity in vascular tissue and the synthesis of tissue factor by vascular cells, has already been described (12,13,15,24,25). Whether tissue factor is secreted by the vascular cells and forms a part of the extracellular matrix is unknown at the moment.

Structures more remote from the vascular lumen apparently exhibit a procoagulant activity, which could not be completely inhibited by phospholipase C, under the given conditions. The reason for this effect remains to be resolved. The procoagulant activity of the superficially damaged aorta can be fully inhibited by VAC. Since VAC inhibits the phospholipid dependent procoagulant reactions through its binding to the phospholipids (chapter 3 and 4 of this thesis), we conclude that phospholipids play an essential role in the vascular procoagulant process. Whether VAC binds to the phospholipid moiety of the tissue factor is not known but this is theoretically very well possible.

The amount of VAC, required for complete inhibition of the procoagulant activity of the superficially damaged aorta, is far less than the amount needed for a complete inhibition of the activity of the more severly damaged aorta. The procoagulant activity of the aortic abluminal side persisted even at the highest concentration of VAC tested (750 nM).

Together with the presence of VAC in endothelial cells as an anticoagulant compound (chapter 5 of this thesis), we conclude from these results that VAC supplies an anticoagulant mechanism, which can control thrombin formation efficiently at the superficially damaged vessel wall. A more severe damage to the vessel wall, like transsection of the vessel, results in an exposure of a surplus of procoagulant activity which is not efficiently inhibited by VAC. VAC may, therefore, provide an anticoagulant compound, which can be used in the treatment of thrombotic diseases without the risk of bleeding.

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CONCLUSIONS AND SUMMARY

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The discovery of an anticoagulant, that interferes with the interaction between coagulation factors and a phospholipid surface was the result of a study on the vascular procoagulant activity. During this investigation, this activity appeared to be inhibited by a vascular protein, which anticoagulant mechanism differed from the ones existing in plasma. Preliminary characterization revealed the essential role of phospholipids in this anticoagulant mechanism. The subsequent extensive study on this vascular anticoagulant was not only made possible by the infrastructure of the biochemical department of the University of Limburg, but was also stimulated by it. The present thesis describes the study on this vascular anticoagulant in extenso.
During our attempts to purify this vascular anticoagulant (VAC), we also developed a method to assay VAC-activity and to express this activity in arbitrarily defined units. The resulting modified prothrombin time test (MPT-test) suits these purposes very well, and was employed during the isolation and partial purification of VAC from human umbilical cord arteries. The VAC-activity, present in this tissue, appeared to be associated with a single chain protein with M_r=32,000. From experiments with the MPT-test it was already obvious that VAC interferes with thrombin formation and not with thrombin action. Studying VAC-activity in a system with purified coagulation factors and phospholipids, revealed that the factor X_a-catalyzed prothrombin activation is inhibited by VAC, only in the presence of phospholipids. The first indication that phospholipids play an essential role in the anticoagulant action of VAC was, thereby, confirmed.
The limited source of human umbilical cord vessels urged a new one to be found, in order to achieve further characterization of VAC. This new source was found with the demonstration of the presence of VAC-activity in bovine aortic intima. The nearly unlimited supply of this tissue guaranteed the establishment of a purification procedure of bovine VAC (B-VAC). B-VAC activity appeared to be associated with two single chain water-soluble

proteins (M_r=32,000 and M_r=34,000). The similarities in chemical properties of both proteins prevented their separation sofar. Both proteins were shown to bind to large volume phospholipid vesicles. The binding of B-VAC to a phospholipid bilayer, consisting of 20% DOPS/80% DOPC was quantified with the use of an ellipsometer. This binding requires both calcium and the presence of DOPS in the phospholipid surface. Both proteins have the same affinity (K_d=6x10^{-9} M) for this surface, which contains, moreover , a single class of binding sites for these proteins. Therefore, we assume that both proteins exhibit the same anticoagulant activity.

As a consequence of the high affinity binding of B-VAC to a phospholipid surface, it interferes with the binding of prothrombin and factor X_a binding to this surface. This interference is reflected by the effects of B-VAC on the kinetic parameters of the enzymatic prothrombin activation by factor X_a in the presence of phospholipids. B-VAC increases the K_m and decreases the V_{max} of this reaction. The increase in K_m is readily explained by the interference of B-VAC with the prothrombin binding, whereas the decrease in V_{max} likely originates from an interference with the binding of factor X_a. Based on these findings we have postulated the following anticoagulant mechanism for B-VAC: Negatively charged phospholipids function as catalyzing components in the procoagulant reactions, involving vitamin K-related coagulation factors. These factors have to bind to the phospholipid surface, for this catalyzing effect. In the presence of B-VAC, the binding of these factors to the phospholipids is impaired as a result of the occupation of the phospholipid surface by B-VAC. Hence, the phospholipids are shielded by B-VAC for the binding of K-related coagulation factors. The nature of the binding between B-VAC and the phospholipid surface is not known as yet. Unlike the vitamin K-related coagulation factors, which require GLA-residues for their phospholipid binding, B-VAC has no GLA-residues.

Having resolved the purification procedure of B-VAC and part of its anticoagulant mechanism, we further investigated human VAC. Using a FPLC-system, which allow a rapid and convenient purifi-

cation of VAC, we demonstrated that also human placenta contains VAC. This P-VAC appeared identical to the human umbilical cord VAC (U-VAC), as was concluded from a comparative study on the protein chemistry and the anticoagulant action.

P-VAC and B-VAC have similar affinities for a phospholipid surface, containing negatively charged phospholipids. Hence, their anticoagulant mechanism is likely to be identical. This mechanism predicts a P-VAC induced inhibition of the phospholipid dependent intrinsic factor X-activation. This, indeed, appeared to be the case.

An insight into the mechanism of the binding of P-VAC to negatively charged phospholipids was obtained by studying the intrinsic fluorescence of P-VAC and the effects of several serine proteases on P-VAC. Both studies indicated that P-VAC does not show a conformational transition prior to phospholipid binding. The tertiairy structure of the P-VAC molecule, that binds to the phospholipid surface in a calcium dependent way, has few or no accessible arginine and lysine residues, but quite some accessible glutamic acid and aspartic acid residues. From these results it can be concluded that P-VAC binds with a stabilized conformation to the phospholipid surface, and calcium ions may bridge between some of VAC's glutamic acid and/or aspartic acid residues, and the negatively charged moieties in the phospholipid surface.

The study with the serine proteases further indicate, that the serine proteases of the coagulation system are not able to cleave P-VAC.

Sofar VAC has been isolated and purified from homogenates of vascular tissue. In order to gain insight in the localization of VAC in the intact vessel wall, polyclonal antibodies raised against purified U-VAC, were employed to demonstrate the presence of VAC in cultured vascular cells. Both immunological and chemical studies demonstrated the presence of active VAC in cultured endothelial cells. VAC appeared to be localized intracellularly, associated with granular structures. This endogenous VAC was shown to reduce the procoagulant activity of the intracellular

structures, which are likely to contain negatively charged phospholipids, which accelerate the procoagulant reactions. By binding to these structures VAC impairs the clot promoting effects of these phospholipids. The presence of a compound with anticoagulant activity within endothelial cells, suggests a strategic function of VAC in the control of thrombin formation at the vessel wall lining. Whether endothelial VAC becomes active as an anticoagulant after endothelial cell damage only, depends on whether or not endothelial VAC can be secreted by the intact endothelial cell. This remains to be resolved.

Using a tissue template specially designed to study the effects of damaged vessel wall on platelets and coagulation, it was shown that the subendothelium of the vessel wall has a low procoagulant activity. This activity, nonetheless, induces the formation of thrombin, which in turn activates platelets and converts fibrinogen into fibrin. This procoagulant activity can be blocked with relatively small amounts of VAC. Structures of the vessel wall more remote from the vascular lumen exhibit a higher procoagulant activity, which requires relatively large amounts of VAC for complete inhibition. This difference is likely to originate from the difference in amounts of procoagulant phospholipids exposed after damage. The superficially damaged vessel wall may expose smaller amounts of phospholipids, than the more severly damaged blood vessel, for example after transsection. It is expected that the small amounts of VAC, which enable full inhibition of the procoagulant activity of the superficially damaged vessel is insufficient to block the procoagulant activity of the transsected vessel. In this latter case, coagulation will proceed and normal haemostasis occurs.

Based on the elucidated features of VAC, we conclude that we have found with VAC a new physiological mechanism of anticoagulation. Moreover, this anticoagulant mechanism may provide a useful antithrombotic concept without the risk of bleeding, associated with the currently used anticoagulants.

CONCLUSIES EN SAMENVATTING

De ontdekking van een anticoagulans, dat de interactie tussen een stolfactor en een fosfolipid oppervlak belemmerd, was het resultaat van een studie van de vasculaire procoagulant activiteit. Tijdens deze studie bleek deze activiteit geremd te worden door een vasculair eiwit via een mechanisme, dat fundamenteel verschilde van de in het plasma werkzame anticoagulante mechanismen. Uit een eerste onderzoek werd de belangrijke rol van fosfolipiden in dit mechanisme duidelijk. De hierop volgende intensieve studie van dit vasculaire anticoagulans werd niet alleen mogelijk gemaakt door de infrastructuur van de vakgroep Biochemie van de Rijksuniversiteit Limburg, maar werd ook gestimuleerd door deze vakgroep. Dit proefschrift beschrijft deze studie.
Tijdens de zuivering van het vasculaire anticoagulans (VAC) werd een test ontwikkeld, waarmee de VAC-activiteit zowel kwalitatief als kwantitatief bepaald kon worden. De gemodificeerde protrombin tijd test (MPT-test) voldeed aan deze voorwaarden en werd toegepast tijdens de isolering en gedeeltelijke zuivering van VAC uit humane navelstreng arterien. De in dit weefsel aanwezige VAC-activiteit bleek gekoppeld te zijn aan een eiwit met een molecuulgewicht van 32.000 daltons. Experimenten met VAC en de MPT-test maakten al snel duidelijk, dat VAC wel de vorming van trombine remde maar niet de activiteit van trombine. Verder onderzoek in een modelsysteem met gezuiverde stolfactoren en fosfolipiden leerde dat VAC de omzetting van protrombine in trombine door factor X_a remde, alleen wanneer deze omzetting plaatsvond in aanwezigheid van fosfolipiden. Hiermee werd de belangrijke rol van fosfolipiden in het anticoagulant mechanisme van VAC bevestigd. Verdere karakterisering van VAC werd bemoeilijkt door de beperkte hoeveelheid beschikbaar navelstreng weefsel. Daarom werd naar een nieuwe bron van VAC gezocht. Deze werd gevonden door het aantonen van VAC-activiteit in de intima van runder aorta, dat in vrijwel onbeperkte hoeveelheden beschikbaar was. Een zuiverings procedure van runder VAC (B-VAC) kon ontwikkeld worden. B-VAC bleek te bestaan uit twee eiwitten met molecuul

gewichten van 32.000 en 34.000 daltons. Door hun overeenkomsten in chemische eigenschappen was het onmogelijk deze eiwitten van elkaar te scheiden.
Beide eiwitten bonden met een hoge affiniteit (K_d=6x10^{-9} M) aan negatief geladen fosfolipiden. De bindingskinetiek liet zien dat beide eiwitten dezelfde bindingsplaatsen op het fosfolipid oppervlak hadden. Als gevolg van de hoge affiniteit van B-VAC voor negatief geladen fosfolipiden werd de binding van protrombine en factor X_a aan fosfolipiden belemmert in aanwezigheid van B-VAC. Dit kwam tot uitdrukking in de effecten van B-VAC op de kinetische parameters van de omzetting van protrombine in trombine door factor X_a in aanwezigheid van fosfolipiden. B-VAC verhoogde de K_m en verlaagde de V_{max} van deze reactie. De verhoging van de K_m kon verklaard worden door de invloed van B-VAC op de binding van protrombine aan de fosfolipiden. De verlaging van de V_{max} was het gevolg van de belemmering van de binding van factor X_a aan het oppervlak. Op grond van deze gegevens postuleerden wij het volgende: Negatief geladen fosfolipiden functioneren als katalysators voor de procoagulant reacties waarin vitamin K afhankelijke stolfactoren betrokken zijn. Deze stolfactoren moeten aan het fosfolipid oppervlak binden voor het katalyserende effect. In de aanwezigheid van B-VAC wordt deze binding belemmerd doordat B-VAC het fosfolipid oppervlak bezet en afschermt voor de stolfactoren. De aard van de binding van B-VAC is nog niet bekend. Echter in tegenstelling tot de vitamine K afhankelijke stolfactoren, die GLA-residuen nodig hebben voor deze binding, heeft B-VAC geen GLA-residuen.
Nadat, door de studie in het runder systeem de zuiverings procedure en het anticoagulante mechanisme van VAC, bekend waren, werd het humane VAC verder bestudeerd. Met behulp van een FPLC-systeem was het VAC op een snelle wijze uit humaan placenta weefsel te zuiveren. Het in dit weefsel gevonden VAC (P-VAC) bleek na een vergelijkende studie identiek te zijn met het VAC, dat in humane navelstreng arterien voorkwam.
Het P-VAC vertoonde een affiniteit voor een negatief geladen fosfolipid oppervlak, dat vergelijkbaar was met die van B-VAC.

Het anticoagulante mechanisme van P-VAC, dat hierdoor waarschijnlijk identiek aan dat van B-VAC was, voorspelde een remming door P-VAC van de fosfolipid afhankelijke intrinsieke factor X-activering. P-VAC bleek inderdaad deze activering te remmen.
Een eerste inzicht in de aard van de binding van P-VAC aan negatief geladen fosfolipiden werd verkregen met behulp van intrinsieke fluorescentie en serine proteasen. Zowel de intrinsieke fluorescentie als de serine proteasen toonden aan dat P-VAC onder invloed van calcium of EDTA geen conformatie verandering onderging. De tertiaire structuur van het P-VAC molecuul, dat aan fosfolipiden bond, had weinig of geen arginine en lysine residuen aan de buitenkant georienteerd maar daarentegen wel een aantal glutamine zuur en/of asparagine zuur residuen. Voor de binding aan fosfolipiden vormden de calcium ionen mogelijk een overbruggings lading tussen de glutamine zuur en/of asparagine zuur residuen en de negatief geladen groepen in het fosfolipid oppervlak.
De studie van de effecten van de serine proteasen op VAC toonden tevens aan dat de serine proteasen van het stolsysteem VAC niet konden knippen.
Tot dusver hadden wij VAC gezuiverd uit homogenaten van vaatweefsel. Hierdoor bleef de lokalisatie van VAC in de intacte vaatwand onbekend. Ter opheldering van deze lokalisatie werden immunologische studies uitgevoerd met antilichamen, die gericht waren tegen VAC. Deze studies lieten zien dat VAC in gekweekte vasculaire endotheelcellen voorkwam als een intracellulair eiwit, dat verbonden was met granulaire structuren. Chemische studies toonden aan dat dit endotheliale VAC een anticoagulante activiteit bezat, dat de procoagulante activiteit van de intracellulaire structuren van de endotheelcel kon remmen. Deze structuren bevatten waarschijnlijk fosfolipiden, waaraan het VAC via calcium bond en waardoor de stolbevorderende capaciteit van deze fosfolipiden verloren ging. De aanwezigheid van VAC in endotheelcellen suggereerde een strategische functie van VAC in de controle van de trombine vorming, die op het vaatweefsel plaatsvond. Of VAC voor deze functie beschikbaar kwam alleen na endotheelcel beschadiging ofwel doordat intacte endotheelcellen VAC konden uit-

scheiden, was nog niet bekend.
Met behulp van een zogenaamde "template", die ontwikkeld was voor de bestudering van de vasculaire procoagulant activiteit, werd aangetoond dat verschillende structuren in de vaatwand een verschillende hoeveelheid procoagulante activiteit bezaten. Het subendotheel van de vaatwand bezat relatief de laagste activiteit, terwijl de structuren, die verder van het vasculaire lumen verwijderd waren, een relatief hogere activiteit bezaten. Dit verschil was mogelijk het gevolg van het verschil in de hoeveelheid beschikbaar gekomen fosfolipiden. Het subendotheel (de oppervlakkig beschadigde vaatwand) exposeerde waarschijnlijk minder fosfolipiden dan de dieper gelegen structuren van de vaatwand. De procoagulant activiteit van de vaatwand structuren werd door VAC geremd. De hoeveelheid VAC, die in staat was de activiteit van het subendotheel volledig te remmen, kon de activiteit van de dieper liggende structuren in de vaatwand niet remmen. In dit laatste geval kon stolling gewoon plaats vinden.
Tot besluit kunnen we concluderen dat we met VAC een nieuw fysiologisch anticoagulant mechanisme hebben gevonden. Bovendien levert dit mechanisme wellicht een nieuw antitrombotisch concept, dat geen risico's tot bloedingen met zich meedraagt, zoals dat bekend is van de huidige toegepaste anticoagulantia.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren te Schaesberg op 4 oktober 1955. Hij behaalde in 1974 het diploma Atheneum-B aan het Eyckhagencollege te Schaesberg. Vanaf 1974 studeerde hij scheikunde aan de Rijksuniversiteit Utrecht. Na het kandidaatsexamen in 1978 werd met het keuzevak biochemie en de bijvakken klinische chemie en immunologie in 1981 het doctoraal diploma behaald.
Vanaf december 1981 is hij werkzaam als wetenschappelijk ambtenaar bij de vakgroep Biochemie van de Rijksuniversiteit van Limburg, alwaar hij onder leiding van dr. G. Hornstra het onderzoek verrichtte dat heeft geleid tot dit proefschrift.
In februari 1987 is hij benoemd door de Koninklijke Nederlandse Academie der Wetenschappen als Academie-onderzoeker.