
REVIEW

Vascular–Platelet and Plasma Hemostasis Regulators from Bloodsucking Animals

A. V. Basanova¹, I. P. Baskova^{1*}, and L. L. Zavalova²

¹*School of Biology, Lomonosov Moscow State University, Moscow, 119899 Russia; fax: (095) 939-1745*

²*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences,
ul. Miklukho-Maklaya 16/10, Moscow, 117997 Russia; fax: (095) 330-6538; E-mail: leech@humgen.siobc.ras.ru*

Received June 6, 2001

Revision received July 3, 2001

Abstract—Saliva of bloodsuckers (leeches, insects, ticks, vampire bats) contains various regulators of some hemostatic stages. This review summarizes information on their structural characteristics and mechanisms of action. Most bloodsuckers are shown to inhibit vascular–platelet hemostasis by blocking collagen-induced platelet adhesion/aggregation. Plasma hemostasis is inhibited by blocking activation of factor X or factor Xa directly.

Key words: bloodsuckers, leeches, insects, ticks, vascular–platelet hemostasis, platelet adhesion and aggregation, prothrombinase complex, factor Xa

Hematophages, i.e., animals adapted during evolution to bloodsucking as their exclusive feeding, secrete compounds blocking the hemostasis of the host. This is an absolute requirement for survival of the bloodsuckers [1]. Therefore, the appearance of antihemostatic and fibrinolytic compounds in secretions of hematophages has evolutionary sense. Hematophages belong to different classes and types of the animal world. Most of them are invertebrates; these include bloodsucking leeches (Annelides class) and insects (Arthropoda class). Some species of vampire bats are also bloodsuckers (vertebrate mammals). All hematophages are evolutionarily adapted to preferential feeding with blood of homoiothermic animals (including a man). Thus, mechanisms underlying optimization of the nutritional behavior of these animals provide the prevention of blood coagulation during bloodsucking and blood storage in the gut channel during digestion. These mechanisms are responsible for blockade of hemostasis and activation of the host's fibrinolytic system. They are regulated by various biologically active compounds elaborated by salivary glands of bloodsuckers, which are rather specific for each hematophage. In spite of the diversity of bloodsucking species, all compounds secreted by these animals to affect host hemostasis can be subdivided into the following groups (which correspond to certain stages of blood coagulation): 1) inhibitors of vascular–platelet hemostasis; 2) inhibitors of activation

of intrinsic mechanisms of blood coagulation and proteins of the prothrombinase complex; 3) regulators of fibrin formation; these include inhibitors of thrombin and factor XIIIa, fibrino(geno)lytic enzymes and activators of fibrinolysis [2].

In this review, we consider only the first two groups of inhibitors. The third group is considered in the accompanying paper [3].

INHIBITORS OF VASCULAR–PLATELET HEMOSTASIS

The functioning of platelets in the regulation of hemostasis is mainly determined by their reactivity to changes of the vascular wall in various physiological and pathological conditions. Platelets readily react to injury of the endothelial lining of the internal surface of blood vessels.

These properties of platelets are determined by cell surface receptors, which are integral proteins of the cell membrane. Most of these receptors are integrins, homodimer glycoproteins that consist of various α - and β -subunits with molecular masses of 130-200 and 90-130 kD, respectively. These receptors specifically react to changes in the platelet environment and immediately transduce this information inside the cells.

Integrin α IIb β 3 (glycoprotein IIb-IIIa, GP IIb-IIIa) plays the major role among platelet receptors. Each

* To whom correspondence should be addressed.

platelet contains about 50,000–80,000 copies of this receptor. In resting platelets, this surface receptor is inactive and does not react with ligands, plasma proteins, fibrinogen, and von Willebrand factor, which are responsible for binding to platelets during their aggregation. Thrombin, ADP, adrenalin, and thromboxane A_2 sharply increase the receptor affinity to its ligands. The exposure of integrin $\alpha IIb\beta 3$ to collagen and von Willebrand factor (the main adhesion proteins responsible for platelet binding to subendothelium) immobilized inside subendothelial structures also activates this receptor [4]. Besides integrin $\alpha IIb\beta 3$, there are some other specific receptors involved in functional transformation of human platelets: 1) proteinase activating receptors (PAR1 and PAR4); 2) subtype 2 purine-ergic ADP receptors (P2TAC, to inhibition of adenylate cyclase); 3) α_2 -adrenergic receptors (for adrenalin); 4) collagen GP VI, GP IV and integrin $\alpha_2\beta_1$ (GP Ia-IIa) receptors; 5) glycoprotein complex (GP Ib-V-IX) in which the receptor GP Ib α is specific for immobilized von Willebrand factor.

Receptors $\alpha IIb\beta 3$ and GP Ib-V-IX not only regulate aggregation and adhesion of platelets, causing vascular occlusion; they are also involved in control of growth of thrombi and their stability. Activated platelets secrete ADP and other agonists, stimulating neighboring platelets and provoking integrin $\alpha IIb\beta 3$ -mediated Ca^{2+} -dependent platelet aggregation. This integrin also mediates secondary adhesion and aggregation of platelets after GP Ib-V-IX-initiated primary contact between platelets and von Willebrand factor of the vascular wall [4]. Therefore, these receptors respond to pathology of the vascular wall at the place of platelet aggregation.

Glycoprotein Ib-V-IX, a specific integral membrane complex of platelet receptors, induces adhesion by binding to the adhesion glycoprotein, von Willebrand factor of the vascular wall or blood plasma [5].

Inhibitors of Platelet Adhesion

Leeches. *Hirudo medicinalis*. The first information on inhibition of platelet adhesion was obtained using salivary gland secretion of the medicinal leech [6, 7]. This secretion inhibited by 85–87% the total adhesion of human platelets to collagen surfaces coated by human collagens of genetic types I, II, and III; it completely inhibited the spreading of platelets on collagen surfaces. The inhibitory effect of the secretion on platelet adhesion was shown to be due to binding of secretion components to collagen [7]. In 1991, Munro *et al.* isolated and purified a platelet adhesion inhibitor from the leech secretion and named it calin [8]. Calin not only blocks platelet adhesion on collagen-coated surfaces but also prevents the formation platelet rich thrombi in hamsters [9]. It also inhibits binding of von Willebrand factor to

the collagen surfaces under conditions of high-shear stress; however, the former effect is more potent [10]. The molecular mass of calin is 65 kD [8], but its structure remains unknown. The inhibitor is suggested to possess two active sites; one site interacts with von Willebrand factor, which causes collagen binding to its platelet receptor, glycoprotein GP Ib (representing a fragment of the integral receptor complex GP Ib-IX-V), whereas the other site directly binds platelet $\alpha_2\beta_1$ -integrin (GP Ia-IIa) and provides attachment to collagen [11]. This suggestion is based on the fact that direct collagen binding to the platelet receptor, integrin $\alpha_2\beta_1$, and von Willebrand factor mediated collagen binding to platelets involves different sites of the collagen surface, and calin blocks both interactions [12].

Haementeria officinalis. LAPP (Leech Anti-Platelet Protein). This protein of molecular mass 16 kD was isolated from extracts of the Mexican leech *Haementeria officinalis* and identified as a highly specific inhibitor of collagen-stimulated platelet aggregation [13] (see below). The partial amino acid sequencing allowed cDNA and then the complete amino acid sequence of LAPP to be obtained. This protein consists of 126 amino acid residues. It does not have any homology with known proteins. The protein was expressed in yeast. The recombinant LAPP inhibited not only collagen-stimulated aggregation of platelets but also their adhesion to collagen; it also inhibited the release of the content of platelet dense granules [14].

Inhibitors of Platelet Aggregation

Leeches. *Hirudo medicinalis*. Hirudin, a highly specific thrombin inhibitor, was the first agent found to block thrombin binding to platelets, inhibiting thrombin-induced platelet aggregation of platelets and causing their disaggregation [15, 16]. In 1984 it was shown that salivary gland secretion of the medicinal leech that did not contain the antithrombin activity of hirudin inhibited ADP-stimulated platelet aggregation [6]. Thus, it was demonstrated that the leech secretion contains compounds other than hirudin that are responsible for blockade of platelet hemostasis. Later it was shown that the secretion inhibited platelet aggregation induced not only by ADP but also by the prostaglandin endoperoxide analog, U46619, Ca^{2+} -ionophore A23187, and arachidonic acid. The secretion possessing antithrombin activity was more effective in inhibition of thrombin-stimulated platelet aggregation than secretion lacking this activity [7]. Trypsin-resistant low-molecular-weight fraction of this inhibitor activates platelet membrane adenylate cyclase in a receptor-mediated manner and increases cAMP content. This activation is not mediated by adenosine receptors. It was suggested that platelet prostaglandin receptors are involved into this activation [7]. The secretion was

shown to inhibit platelet aggregation induced not only by ADP, but also collagen and adrenalin [17]. The secretion contained apyrase and collagenase activities, which might be involved in inhibition of platelet aggregation. Two isoforms of apyrase, low- and high-molecular-weight (45 and 400 kD, respectively) were recognized. This enzyme hydrolyzes ATP to ADP and P_i [17]. Later a fraction inhibiting PAF (Platelet Activating Factor)- and thrombin-induced platelet aggregation was isolated from the secretion [18]. Being released from an injured vessel, PAF is involved in hemostasis initiation and the development of inflammation [19]. The PAF inhibitor of the leech secretion is a phosphoglyceride because treatment with phospholipases C and D reduce its biological activity. The low-molecular-weight fraction isolated from the leech secretion inhibits endogenous PAF released from neutrophils stimulated by N-formyl-Met-Leu-Phe or Ca^{2+} -ionophore A23187 [18].

Destabilase is the endo- and exo- ϵ -(γ -Glu)-Lys isopeptidase. This protein of molecular mass 12.3 kD completely inhibits spontaneous aggregation of human platelets. Destabilase also blocks ADP-, PAF-, and collagen-induced platelet aggregation by 63, 50, and 65%, respectively. The mechanism of platelet inhibition by destabilase was suggested to involve the interaction of the enzyme with high affinity sites of the platelet membrane surface [20].

Calin, the platelet adhesion inhibitor from the leech secretion, blocking the interaction of collagen and von Willebrand factor with their platelet receptors, can also inhibit platelet aggregation stimulated by these inducers [10]. This effect involves the same mechanisms that are responsible for the inhibition of platelet adhesion to collagen and von Willebrand factor.

Thus, the inhibition of platelet aggregation by salivary gland secretion of the medicinal leech involves various mechanisms and is realized by hirudin, apyrase, PAF inhibitor, adenylate cyclase activator, calin, and destabilase.

Potent inhibitors of platelet aggregation called decorsin and ornatin were isolated from the North American leech *Macrobdella decora* and the tortoise leech *Placobdella ornata*, respectively. These potent platelet aggregation inhibitors act as GP IIb-IIIa antagonists. Decorsin is a polypeptide of molecular mass 4379 daltons; it consists of 39 amino acid residues including six cysteines and six prolines. There are three isoforms of decorsin (1, 2, and 3), two of which (2 and 3) are shorter than the first by two and three N-terminal amino acid residues, respectively. The C-terminal region of decorsin contains the specific sequence Arg-Gly-Asp [21], which is typical for desintegrins; it is the recognition site typical of many adhesion proteins exhibiting affinity for GP IIb-IIIa [22, 23]. Decorsin isoforms 1 and 3 effectively inhibited GP IIb-IIIa binding to immobilized fibrinogen ($IC_{50} \sim 1.5$ nM), whereas peptide Gly-Arg-Gly-Asp-Val, containing the sequence Arg-Gly-Asp was less active ($IC_{50} \sim$

40 nM). The anti-aggregant property of decorsin is due to its anti-thrombotic effect. Decorsin administration (1.5–3 μ g/mouse) effectively protected mice against death from pulmonary thromboembolism [24]. The molecular mass of ornatin is about 6 kD. Six isoforms of ornatin vary in the length of polypeptide chain (from 41 to 52 residues). Comparison of their amino acid sequences revealed that in addition to six cysteines, they all contain the sequence Arg-Gly-Asp. The isoforms of ornatin inhibited GP IIb-IIIa binding to immobilized fibrinogen with IC_{50} values of 2.9–5.3 nM. The amino acid sequence of ornatin shares 40% identity with decorsin and the location of Arg-Gly-Asp and five (of six) cysteines are the same in both inhibitors. The existence of these isoforms may be explained by proteolytic degradation of precursor proteins by leech exopeptidases [25]. It is possible that decorsin and ornatin molecules are formed during proteolytic processing of larger proteins. There is no reliable homology of ornatin and decorsin with desintegrins, GP IIb-IIIa antagonists from snake venom [26].

The spatial structure of decorsin has some resemblance with fragments of the spatial structure of hirudin, ornatin, and antistasin [27].

Haementeria officinalis. As already mentioned above, LAPP is a protein isolated from the soluble fraction of the whole extract of the Mexican leech *Haementeria officinalis*. It selectively inhibits collagen-induced platelet aggregation (IC_{50} for aggregation index is about 60 nM) without any influence on platelet aggregation induced by ADP, arachidonic acid, U46619, thrombin, and ionophore A23187 even at concentrations producing total inhibition of the collagen-induced platelet aggregation. However, it should be noted that the initial extract of the salivary glands of *Haementeria officinalis* exhibited inhibitory activity with respect to all these agonists except thrombin (1 U/ml) and 2 mM A23187 [13].

Arthropods. Many hemostatic agents from arthropods are of the lipocaine protein family [28]. These include platelet aggregation inhibitors from the saliva of bloodsucking bugs *Triatoma pallidipensis* (palladipin) and *Rhodnius prolixus* (*Rhodnius prolixus* Aggregation Inhibitor; RPAI) and ticks *Ornithodoros moubata* (moubatin). Palladipin is a protein of molecular mass 19 kD that specifically inhibits collagen-induced platelet aggregation [27]. It is an equipotent inhibitor of platelet aggregation studied using washed platelets and platelet-rich plasma; this effect is due to palladipin inhibition of the collagen receptor GP IV. Palladipin is inactive with respect to platelet aggregation induced by ADP, thrombin, the thromboxane A_2 analog U46619, and phorbol ester. It does not influence platelet adhesion on collagen, but it inhibits ATP release from platelets. The recombinant protein obtained by expressing the palladipin gene in cultured hamster kidney cells and in *E. coli* cells inhibited platelet aggregation, and this effect was similar to that obtained with the wild type protein [29–31].

In platelet-rich plasma, RPAI-1 inhibits platelet aggregation induced by low concentrations of ADP, collagen, arachidonic acid, U46619, and very low doses of thrombin. The N-terminal sequence (49 amino acid residues) of one isoform of this protein was used for primer synthesis and screening of cDNA library of salivary gland cells of *Rhodnius prolixus*. Expression of the RPAI-1 gene in *E. coli* yielded active protein. RPAI-1 inhibits ADP-induced platelet aggregation by binding to ADP. In contrast to platelet aggregation inhibitors blocking adhesion and/or aggregation of collagen-stimulated platelets, RPAI-1 does not inhibit collagen receptors: $\alpha_2\beta_1$ and GP IV. Blockade of collagen-induced platelet aggregation is due to inhibitor binding to ADP, which is released from platelets by low concentrations of collagen, arachidonic acid, and the thromboxane mimetic U46619 [32].

Moubatin is a protein of molecular mass 17 kD that consists of 156 amino acid residues [33]. Moubatin inhibited aggregation of washed collagen-induced human platelets ($IC_{50} = 50$ nM) and did not influence aggregation induced by arachidonic acid, thrombin, ristocetin, and the Ca^{2+} ionophore A23187. Moubatin was a weak inhibitor of platelet adhesion to collagen [33, 34]. At micromolar concentrations (>1 μ M), moubatin inhibited aggregation induced by low concentrations of ADP or U46619 [32]. It is suggested that mechanisms of the moubatin effect is similar to that of RPAI-1 [28, 32]. A polymerase chain reaction-generated hybridization probe (produced using primers based on moubatin protein sequence) identified the entire cDNA sequence of tick moubatin. cDNA cloning in the yeast produced a biologically active protein [34].

Two groups of platelet aggregation inhibitors were recognized in saliva of the tsetse fly (*Glossina morsitans*). The first group consists of protein fraction with molecular mass exceeding 30 kD. It was active in inhibiting the first and the second stages of ADP-induced platelet aggregation, the second stage of adrenaline-induced platelet aggregation, and collagen-induced platelet aggregation; this fraction also caused disaggregation of platelets stimulated by ADP and adrenaline. These effects are attributed to the ADP-hydrolyzing activity found in this fraction. The second fraction (previously described as the antithrombin fraction) of molecular mass 11–13 kD inhibited thrombin-induced aggregation and did not influence aggregation induced by ADP, adrenaline, or collagen [35].

These results emphasize the importance of platelet interaction with collagen in the mechanism of activation of vascular–platelet hemostasis in man and animals. Invertebrate bloodsuckers block this component of hemostasis as the most sensitive regulatory target.

INHIBITORS OF PLASMA HEMOSTASIS

The injuries of skin, surrounding tissues, and blood vessels caused by insects and leeches result in the activa-

tion of the plasma system of host hemostasis. Tissue factor (TF) release initiates the extrinsic mechanism of blood coagulation. In the presence of calcium ions the TF–factor VII complex activates factors IX and X, and TF inhibitor plays an important regulatory role in this process [36]. Activation of the intrinsic mechanism of blood coagulation is mediated by components of kallikrein–kinin system [37]. Kallikrein activates factor XII, which converts factor XI to factor XIa. The latter in turn activates factor IX, and activation of the intrinsic mechanism leads to the formation of the tenase complex, which consists of factors IXa, VIIIa, and phospholipids. In the presence of Ca^{2+} , this complex activates factor X (into Xa). Thus activation of the extrinsic and intrinsic mechanisms of blood coagulation results in formation of factor Xa, the ultimate component of the prothrombinase complex (factor Va, negatively charged surfaces of membrane phospholipids, and calcium ions), responsible for conversion of prothrombin into thrombin.

Hirudo medicinalis. Studies of the effects of salivary gland secretion from the medicinal leech on the activation of extrinsic and intrinsic mechanisms of blood coagulation revealed that the secretion inhibited only intrinsic blood coagulation of experimental animals. Leech secretion lacking antithrombin activity of hirudin did not increase the prothrombin time of rat blood plasma, but it sharply increased recalcification time of plasma dextran sulfate-activated plasma [38]. Intravenous administration of the leech secretion to rats (even at a dose 0.3 ml) did not protect animals against lethal thrombosis initiated by intravenous administration of sub-lethal doses of brain thromboplastin. However, the secretion blocked thrombus formation stimulated by implantation of negatively charged glass surfaces into jugular vein of rats [39]. Munro obtained similar results studying extracts from leech *Haementeria granulosa* [40]. We demonstrated data suggesting preferential inhibition of the contact stage of intrinsic blood coagulation [38]. According to these results, minor injuries comparable with bites of the medicinal leech are accompanied by activation of only the intrinsic mechanism of blood coagulation. Identification of plasma kallikrein inhibitor in the salivary gland secretion of *Hirudo medicinalis* [41] should be attributed to the manifestation of one of the adaptation mechanisms for feeding with blood of homoiothermic animals. The presence of factor Xa inhibitor in the salivary gland secretion of the bloodsuckers is also a manifestation of such adaptation.

PROTHROMBINASE INHIBITORS

Most members of this group inhibit factor Xa, which plays the central role in the blood coagulation system because it is formed during activation of both the intrinsic and extrinsic mechanisms of blood coagulation.

Leeches. *Hirudo medicinalis*. Factor Xa inhibitor was isolated from diluted saliva of the medicinal leech in 1995 [42]. It is a protein of molecular mass 13–14 kD; it consists of 85 amino residues including 14 cysteines, which form seven disulfide bonds. The inhibitor forms tight equimolar complex with factor Xa. It effectively inhibits (with $IC_{50} = 1$ pM) amidolytic activity of factor Xa characterized by the following constants: $k_{cat} = 130 \text{ sec}^{-1}$, $K_m = 15 \text{ } \mu\text{M}$. The complete amino acid sequence was deduced from the cDNA sequence [43]. The recombinant factor Xa inhibitor (obtained by gene expression of one isoform of the inhibitor) contains 133 amino acid residues including 22 cysteines that form 11 disulfide bonds. Its molecular mass is 14.4 kD. The homology between recombinant and wild type factor Xa inhibitor is 42% [44].

Like heparin, the recombinant factor Xa inhibitor protected experimental animals against vein thrombus formation. However, it was a more effective anti-thrombotic agent than heparin, although its effect on bleeding time in experimental animals did not differ from that of heparin [44].

Two structurally related inhibitors of factor Xa were isolated from *Haementeria* leeches: antistasin (*Haementeria officinalis*) and ghilanten (*Haementeria ghilianii*). These highly homologous proteins of molecular mass 15 kD consist of 119 amino acid residues including 20 cysteines. Antistasin and ghilanten are highly specific reversible inhibitors of factor Xa forming a stoichiometric complex with it with dissociation constants in the nanomolar range [45–47]. The amino acid sequence of domain I (1–55 residues) shares 56% homology with domain II (56–110 residues). Five isoforms of ghilanten differing by amino acid sequence and designated as P1–P5 were isolated. These proteins can bind heparin. Binding involves residues 32–47 and 87–101 of antistasin and the C-terminus of ghilanten P5. In both proteins the reactive site for factor Xa includes Arg34 [47–49]. Besides anticoagulant activity, antistasin also exhibits metastasizing activity of tumor cells. Recombinant forms of these proteins [50, 51] are used in preclinical and clinical trials. Antistasin was the first member of the family of serine proteinase inhibitors that were then denominated as the antistasin-like inhibitors [52].

Lefaxin is a factor Xa inhibitor isolated from salivary gland of *Haementeria depressa*. This protein of molecular mass 30 kD lacks homology with antistasin and ghilanten. However, it shares some homology with NO heme protein carriers: miohemeritrin from the annelid *Nereis diversicolor* and prolixin S from *Rhodnius prolixus*. It can block amidolytic activity of factor Xa with respect to its chromogenic substrate S-2765 ($K_i \sim 4$ nM) and thrombin formation in the prothrombinase complex [53].

Therostasin, a cysteine-rich protein of molecular mass 8991 daltons, was isolated from salivary glands of the leech *Theromyzon tessulatum*. It consists of 82 amino

acid including 16 cysteines. Therostasin inhibits mammalian factor Xa with $K_i = 34$ pM. Its amino acid sequence does not share any homology with other factor Xa inhibitors. Nevertheless, this protein contains a sequence typical for vasoconstrictor proteins of the endothelin family isolated from mammals and snake venom [54].

Insects. Factor Xa inhibitor of molecular mass 35.5 kD [55] was isolated from salivary glands of the female yellow-fever mosquito *Aedes aegypti*. Factor Xa inhibitory activity was determined in blood coagulation tests and by inhibition of cleavage of the chromogenic substrate chomazin X. This inhibitor exhibited kinetics of reversible noncompetitive inhibition. The anticoagulant activity depended on the presence of Ca^{2+} (with optimum at 4 mM) but not magnesium ions. The optimum pH was 8.0. The anticoagulant activity disappeared after boiling for 10 min or moderate heating at 56°C for 30 min.

Salivary gland extracts of Simuliidae buffalo gnats contain factor Xa inhibitory activity. This was found in *Simulium vittatum* Zetterstedt, *Simulium argus* Williston, *Simulium metallicum* Bellardi, and *Simulium ochraceum* Walker. The highest and lowest activities were determined in *Simulium ochraceum* and *Simulium vittatum*, respectively [56].

A factor Xa inhibitor of molecular mass 18 kD forming a stoichiometric complex with the factor was isolated from salivary glands of the buffalo gnat *Simulium vittatum* [57].

Anticoagulant activity of *Simulium vittatum* Zetterstedt salivary glands was not related to inhibition of factor Xa or thrombin. Purified fractions exhibiting anticoagulant activity were investigated using the test of partial thromboplastin time of normal and factor V-, VIII-, IX-, XI-, and XII-deficient plasmas. It was shown that only factor V deficit influenced coagulation time of the analyzed samples. Thus, in addition to known factors Xa inhibitors of salivary gland secretion of *Simulium vittatum* contains factor V inhibitory activity [58].

Salivary glands of *Rhodnius prolixus* contain an NO carrier known as nitrophorin. These ferric heme proteins play some role in the dysfunction of the host coagulation system by the transport of NO into host blood circulation, where NO causes vasodilatation and inhibition of platelet aggregation. Prolixin S is one of the members of this group. At nanomolar concentrations and in the presence of Ca^{2+} this protein binds only to factors IX and IXa. (Under these conditions it does not bind to other blood coagulation factors, including factor IXa lacking the Gla-domain.) Prolixin S also inhibits factor XIa-dependent activation of factor IX, activation of factor IX catalyzed by the factor VIIa–TF complex, and also proteolysis of factor IX by membrane bound factor Xa. Thus, prolixin S can inhibit both the extrinsic and the intrinsic mechanisms of blood coagulation. The

activity of prolixin S was studied using wild and recombinant forms of this protein [59]. The use of sequential chromatographic procedures allowed the purification of another anticoagulant protein from the salivary glands of these bugs. This protein of molecular mass ~19.7 kD is called nitrophorin 2. It inhibits factor VIII-dependent activation of factor Xa, and therefore its effect is additional to anticoagulant activity of other salivary proteins of *Rhodnius prolixus* [60].

Ticks. A low-molecular-weight factor Xa-specific serine proteinase inhibitor TAP (Tick Anticoagulant Peptide) was isolated and purified from *Ornithodoros moubata*. It has some homology with Kunitz type inhibitors. However, in contrast to other inhibitors of this group TAP inhibited only factor Xa. Even at 300-fold excess of TAP did not influence factor VIIa, kallikrein, trypsin, chymotrypsin, thrombin, urokinase, plasmin, plasminogen tissue activator, and elastase [61]. TAP is a protein consisting of 60 amino acid residues (molecular mass 6977 daltons); its structure is stabilized by three disulfide bonds. Structural and kinetic studies were carried out using wild and recombinant protein (the latter was obtained during expression of the TAP gene in *Saccharomyces cerevisiae*). They demonstrated that TAP is a highly specific, reversible competitive inhibitor of factor Xa; it stoichiometrically binds to the enzyme with a dissociation constant of 180 ± 2 pM [62, 63].

The other inhibitor was purified from salivary glands of the tick *Rhipicephalus appendiculatus*. The purification procedure included gel-filtration, ion-exchange, and affinity chromatography. The purified anticoagulant represents a thermolabile protein of molecular mass 65 kD. It is stable over a wide pH range, but is readily inactivated by trypsin. This anticoagulant inhibited factor Xa-induced coagulation of bovine plasma, but it did not influence factor Xa amidase activity with respect to the synthetic substrate. It is likely that this anticoagulant interacts with factor Xa at sites distinct from the active center; it is also possible that this anticoagulant influences other components of the prothrombinase complex [64].

Vampire bat (*Desmodus rotundus*). Draculin is an anticoagulant isolated from saliva of the vampire bat *Desmodus rotundus*. It consists of a single polypeptide chain glycoprotein of molecular mass 80–83 kD and *pI* 4.1–4.2. Draculin inhibits factors IXa and Xa without any influence on thrombin, trypsin, and chymotrypsin. It lacks fibrinolytic activity. The presence of one coagulation factor (IXa or Xa) does not affect its binding with the other. It is suggested that there are two structurally distinct forms of draculin, each of which can bind both these factors, but one isoform selectively inhibits factor IXa, whereas the other is more active with respect to factor Xa [65]. The anticoagulant activity of draculin depends on its posttranslational glycosy-

lation. Study of highly purified vampire bat saliva draculin preparations with high and low anticoagulant activity revealed different composition and localization of the carbohydrate components in these proteins. Controlled chemical deglycosylation of draculin resulted in sequential loss of its biological activity [66]. Draculin forms equimolar complexes with factor Xa (IXa) within seconds. The formation of draculin–factor Xa is a two-stage process. The first reversible stage is characterized by the following constants: $k_1 = 1.117 \cdot 10^6 \text{ M}^{-1} \cdot \text{sec}^{-1}$, $k_{-1} = 15.388 \cdot 10^{-1} \text{ sec}^{-1}$. The second irreversible (concentration-independent) stage is characterized by the forward reaction rate constant $k_2 = 0.072 \text{ sec}^{-1}$. The dissociation constant determined as the ratio $k_{-1}/k_1 = 13.76 \text{ nM}$. Draculin acts as a non-competitive inhibitor [67].

This review shows that salivary gland secretions of all species of bloodsuckers contain not only inhibitors of adhesion and platelet aggregation, but also highly specific inhibitors of factor Xa. The latter is the ultimate component of the prothrombinase complex completing the cascade activation of blood coagulation factors by conversion of prothrombin to thrombin. The high affinity of wild and recombinant forms of these proteins for factor Xa determine their potential pharmacological importance. Although there is no generally accepted viewpoint on the role of factor Xa inhibitors in blockade of tumor metastasizing, antistasin exhibits such activity, which clearly requires further investigation in factor Xa inhibitors of bloodsuckers. Little is known about inhibitors influencing activation of the intrinsic mechanism of blood coagulation, which directly affect activation of vascular–platelet hemostasis. Inhibitor of plasma kallikrein was found in secretion of the leech *Hirudo medicinalis* and in extract of the leech *Haementeria granulosa*. However, there is no evidence for lack of such inhibitors in most bloodsuckers.

REFERENCES

1. Markwardt, F. (1996) *Clin. Appl. Thrombosis/Hemostasis*, **2**, 75–82.
2. Arocha-Pinango, C. L., Marchi, R., Carvajal, Z., and Guerrero, B. (1999) *Blood Coagul. Fibrinol.*, **10**, 43–68.
3. Zavalova, L. L., Basanova, A. V., and Baskova, I. P. (2002) *Biochemistry (Moscow)*, **67**, 135–142.
4. Stättl, S. J. (1999) *Thromb. Haemost.*, **82**, 318–325.
5. Andrews, R. K., Shen, Y., Gardiner, E. E., Dong, J., Lopez, J. A., and Bendt, M. C. (1999) *Thromb. Haemost.*, **82**, 357–364.
6. Baskova, I. P., Misselvit, F., and Nikonov, G. I. (1984) *Byul. Eksp. Biol. Med.*, **97**, 696–698.
7. Baskova, I. P., Nikonov, G. I., Mazurov, A. V., Misselvit, F., Leitin, V. L., Repin, V. S., Avdonin, P. V., and Svitina-Ulitina, I. V. (1987) *Biokhimiya*, **52**, 1461–1468.
8. Monro, R., Jones, C., and Sawyer, R. (1991) *Blood Coagul. Fibrinolysis*, **2**, 179–184.

9. Deckmyn, H., Stassen, J. M., Vreys, L., van Houtte, E., Sawyer, R. T., and Vermeylen, J. (1995) *Blood*, **85**, 712-719.
10. Harsfalvi, J., Stassen, J., Hoylaerts, M., van Houtte, E., and Sawyer, R. (1995) *Blood*, **85**, 705-711.
11. Saelman, E. V., Nieuwenhus, H. K., Hese, K. M., de Groot, P. G., Heijnen, H. F., Sage, E. H., Williams, S., McKeown, L., Gralnick, H. R., and Sixma, J. J. (1994) *Blood*, **83**, 1244-1250.
12. Depraetere, H., Karekes, A., and Deckmyn, H. (1999) *Thromb. Haemost.*, **82**, 1160-1163.
13. Connolly, T. M., Jacobs, J. W., and Condra, C. (1992) *J. Biol. Chem.*, **267**, 6893-6898.
14. Keller, P. M., Schultz, L. D., Condras, C., Karczewski, J., and Connolly, T. M. (1992) *J. Biol. Chem.*, **267**, 6899-6904.
15. Kato, I., Kohr, W. J., and Laskowski, M. (1978) *Proc. FEBS Meet.*, **47**, 197-206.
16. Tschesche, H., and Kolkenbrock, H. (1984) in *Chemistry of Peptides and Proteins* (Voelter, W., Bayer, E., Ovchinnikov, Y. A., and Wunsch, E., eds.) Vol. 2, Walter de Gruyter, Berlin, pp. 349-355.
17. Rigbi, M., Levy, H., Eldor, A., Iraqi, F., Tetelbaum, M., Orevi, M., Horovitz, A., and Galun, R. (1987) *Comp. Biochem. Physiol.*, **88C**, 95-98.
18. Orevu, M., Rigbi, M., Hy-Am, E., Matzner, Y., and Eldor, A. (1992) *Prostaglandins*, **43**, 483-485.
19. Braquet, P., Tiuqui, L., and Shen, T. S. (1987) *Pharmacol. Rev.*, **39**, 97-145.
20. Baskova, I., Zavalova, L. L., Berezhnoy, S., Avdonin, P., Afanasjeva, G., Popov, E., and Gabbasov, Z. (2000) *Platelets*, **11**, 83-86.
21. Seymour-Ulmer, J., Henzel, W. J., Nevins, B., Stults, J. T., and Lazarus, R. A. (1990) *J. Biol. Chem.*, **265**, 10143-10147.
22. Ruoslahti, E., and Pierschbacher, M. D. (1987) *Science*, **238**, 491-493.
23. Hynes, O. (1992) *Cell*, **69**, 11-15.
24. McLane, M. A., Gabbeta, J., Rao, A. K., Bevilgia, L., Lazarus, R. A., and Niewiarowski, S. (1995) *Thromb. Haemost.*, **74**, 1316-1322.
25. Sawyer, R. T. (1986) in *Leech Biology and Behavior*, Vol. 2, Clarendon Press, Oxford, pp. 518-519.
26. Mazur, P., Henzel, W. J., Seymour, J. L., and Lazarus, R. A. (1991) *Eur. J. Biochem.*, **202**, 1073-1082.
27. Kresel, A. M., Wagner, G., Seymour-Ulmer, J., and Lazarus, R. A. (1994) *Science*, **264**, 1944-1947.
28. Monfort, W. R., Weichel, A., and Andersen, J. F. (2000) *Biochim. Biophys. Acta*, **1482**, 110-118.
29. Noeske-Jungblut, C., Kratzchmar, J., Haendler, B., Alagon, A., Possani, L., Verha, L., Donner, P., and Shleuning, W. D. (1994) *J. Biol. Chem.*, **269**, 5050-5053.
30. Haendler, B., Becker, A., Noeske-Jungblut, C., Kratzschmar, J., Donner, P., and Schleuning, W. D. (1995) *Biochem. J.*, **307** (Pt. 2), 465-470.
31. Haendler, B., Becker, A., Noeske-Jungblut, C., Kratzschmar, J., Donner, P., and Schleuning, W. D. (1996) *Blood Coagul. Fibrinolysis*, **7**, 183-186.
32. Francischetti, I. M., Ribeiro, J. M., Champagne, D., and Andersen, J. (2000) *J. Biol. Chem.*, **275**, 12639-12650.
33. Waxman, L., and Connolly, T. M. (1993) *J. Biol. Chem.*, **268**, 5445-5449.
34. Keller, P. M., Waxman, L., Arnold, B. A., Schultz, L. D., Condra, C., and Connolly, T. M. (1993) *J. Biol. Chem.*, **268**, 5450-5456.
35. Mant, M. J., and Parker, K. R. (1981) *Br. J. Haematol.*, **48**, 601-608.
36. Broze, G. Y. (1995) *Blood Coagul. Fibrinolysis*, **6**, S7-S13.
37. Rojckaer, R., and Schmaier, A. H. (1999) *Immunopharmacology*, **43**, 109-114.
38. Baskova, I. P., Khalil, S., and Nikonov, G. I. (1984) *Byul. Eksp. Biol. Med.*, **97**, 142-143.
39. Khalil, S. (1987) *Mechanisms of thrombolysis and inhibition of blood coagulation by preparations from the medicinal leech *Hirudo medicinalis**: Candidate's dissertation [in Russian], Moscow State University, Moscow.
40. Munro, R. (1986) *Leech News Lett.*, **1**, 5-7.
41. Baskova, I. P., Khalil, S., Nikonov, G. I., Rabinovich, E. E., Nartikova, V. F., and Pashkina, T. S. (1988) *Biokhimiya*, **53**, 1467-1473.
42. Rigbi, M., Jackson, C. M., Atamna, H., Giguzin, I., Goldlust, A., Zeelon, E., Guy, R., Kook, M., Levanon, A., Werber, M., and Panet, A. (1995) *Thromb. Haemost.*, **73**, 1306.
43. Zeelon, E. P., Werber, M. M., and Levanon, A. (1995) Patent USA No. 469,219.
44. Zeelon, E. P., Werber, M. M., and Levanon, A. (1997) Patent USA No. 5,858,970.
45. Tuszynski, G. P., Gasic, T. B., and Gasic, G. J. (1987) *J. Biol. Chem.*, **262**, 9718-9723.
46. Dunwiddie, C., Thornberry, N. A., Bull, H. G., Sardana, M., Friedman, P. A., Jacobs, J. W., and Simpson, E. (1989) *J. Biol. Chem.*, **264**, 16694-16699.
47. Theunissen, H. J., Dijkema, R., Swinkels, J. C., de Poorter, T. L., Vink, P. M., and van Dinter, T. G. (1994) *Thromb. Res.*, **75**, 41-50.
48. Blankenship, D. T., Brankamp, R. G., Manley, G. D., and Cardin, A. D. (1990) *Biochem. Biophys. Res. Commun.*, **166**, 1384-1389.
49. Brancamp, R. G., Manley, G. G., Blankenship, D. T., Bowlin, T. L., and Cardin, A. D. (1991) *Blood Coagul. Fibrinolysis*, **2**, 161-166.
50. Han, J. H., Law, S. W., Kellr, P. M., Kniskern, P. J., Silberklang, M., Tung, J.-S., Gasic, T. B., Gasic, G. J., Friedman, P. A., and Ellis, R. W. (1989) *Gene*, **75**, 47-57.
51. Brankamp, R. G., Sreekrishna, K., Smith, P. L., Blankenship, D. T., and Cardin, A. D. (1995) *Protein Exp. Purif.*, **6**, 813-820.
52. Baskova, I. P., and Zavalova, L. L. (2001) *Biochemistry (Moscow)*, **66**, 703-714.
53. Faria, F., Kelen, E. M., Sampaio, C. A., Bon, C., Duval, N., and Chudzinski-Tavassi, A. M. (1999) *Thromb. Haemost.*, **82**, 1469-1473.
54. Chopin, V., Salzert, M., Baert, J., Vandenbulcke, F., Sautiere, P. E., Kerckaert, J. P., and Malecha, J. (2000) *J. Biol. Chem.*, **275**, 32701-32707.
55. Stark, K. R., and James, A. A. (1995) *Exp. Parasitol.*, **81**, 321-331.
56. Abebe, M., Cupp, M. S., Ramberg, F. B., and Cupp, E. W. (1994) *J. Med. Entomol.*, **31**, 908-911.
57. Jacobs, J. W., Cupp, E. W., Sardana, M., and Friedman, P. A. (1990) *Thromb. Haemost.*, **64**, 235-238.
58. Abebe, M., Ribeiro, J. M., Cupp, M. S., and Cupp, E. W. (1996) *J. Med. Entomol.*, **33**, 173-176.
59. Isawa, H., Yuda, M., Yoneda, K., and Chinzey, Y. (2000) *J. Biol. Chem.*, **275**, 6636-6641.

60. Ribeiro, J. M., Schneider, M., and Guimaraes, J. A. (1995) *Biochem. J.*, **308** (Pt. 1), 243-249.
61. Waxman, L., Smith, D. E., Arcuri, K. E., and Vlasuk, G. P. (1990) *Science*, **248**, 593-596.
62. Jordan, S. P., Waxman, L., Smith, D. E., and Vlasuk, G. P. (1990) *Biochemistry*, **29**, 11095-11100.
63. Vlasuk, G. P. (1993) *Thromb. Haemost.*, **70**, 212-216.
64. Limo, M. K., Voigt, W. P., Tumbo-Oeri, A. G., Njogu, R. M., and ole-MoiYoi, O. K. (1991) *Exp. Parasitol.*, **72**, 418-429.
65. Apitz-Castro, R., Beguin, S., Tablante, A., Bartoli, F., Holt, J. C., and Hemker, H. C. (1995) *Thromb. Haemost.*, **73**, 94-100.
66. Fernandez, A. Z., Tablante, A., Bartoli, F., Beguin, S., Hemker, H. C., and Apitz-Castro, R. (1998) *Biochim. Biophys. Acta*, **1425**, 291-299.
67. Fernandez, A. Z., Tablante, A., Beguin, S., Hemker, H. C., and Apitz-Castro, R. (1999) *Biochim. Biophys. Acta*, **1434**, 135-142.