The Insect Salivary Protein, Prolixin-S, Inhibits Factor IXa Generation and Xase Complex Formation in the Blood Coagulation Pathway*

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Prolixin-S is a salivary anticoagulant of the blood-sucking insect, Rhodnius prolixus, and known as an inhibitor of the intrinsic Xase. We report here its inhibitory mechanisms with additional important anticoagulation activities. We found prolixin-S specifically bound to factor IXa/I Xase in the presence of Ca2+ ions. Light scattering and surface plasmon resonance studies showed that prolixin-S interfered with factor IXa/I Xase binding to the phospholipid membrane, indicating that prolixin-S inhibit Xase activity of factor IXa by interference with its Xase complex formation. Furthermore, reconstitution experiments showed that prolixin-S binding to factor IX strongly inhibited factor IXa generation by factor XIa. We also found that prolixin-S inhibited factor IXa generation by factor VIIa-tissue factor complex and factor IXα generation by factor Xa. These results suggest that prolixin-S inhibits both intrinsic and extrinsic coagulations by sequential inhibition of all coagulation pathways in which factor IX participates. It was also suggested that prolixin-S may bind to factor IX/IXa by recognizing conformational change of the Gla domain induced by Ca2+ binding.

Rhodnius prolixus is a blood-sucking hemipteran insect, which is known as a vector of American trypanosomiasis. This insect has several physiologically active molecules in its saliva that assist its blood-sucking behavior (1, 2). Hellmann and Hawkins (3) first reported an anticoagulation activity in its saliva and named a possible anticoagulant prolixin-S. We have already reported purification, characterization, and the cDNA sequence of a salivary anticoagulant from this insect and pointed out that this inhibitor was the prolixin-S reported previously (4, 5). In addition, we reported that prolixin-S specifically prolonged activated partial thromboplastin time (APTT).1 This activity was explained by inhibition of the intrinsic Xase.

Prolixin-S is a hemoprotein with a molecular mass of approximately 20 kDa, structurally and immunologically related to other hemoproteins (nitrophorins) identified in the salivary gland of R. prolixus (4, 6–8). These hemoproteins, including prolixin-S, bind nitric oxide (NO) in their heme groups and are thought to transport NO to the host blood vessel, where NO acts as a vasodilator and a platelet aggregation inhibitor to assist blood feeding (9, 10). Our previous experiments demonstrated that prolixin-S was the sole anticoagulant among these hemoproteins (4). It was also demonstrated that its heme moiety, therefore also NO, was not involved in its anticoagulation activity (4).

Following initiation of the intrinsic coagulation pathway, generated factor Xa activates factor IX to factor IXa. On the other hand, in the extrinsic coagulation pathway, factor VIIa forms a complex with tissue factor on the phospholipid membrane surface and activates factor IX (11–13). Factor IXa activated by these different coagulation pathways forms an "intrinsic Xase complex" together with factor VIIIa and Ca2+ on the phospholipid membrane surface (11, 14, 15), which results in generation of thrombin and clot formation. In addition, generated thrombin further enhances the intrinsic blood coagulation pathway by activation of factor XI and subsequent generation factor IXa (16, 17). Therefore, factor IX plays a central role in both intrinsic and extrinsic coagulations.

In this paper, we show that prolixin-S directly binds to both factor IX and factor IXa. We suggest that prolixin-S binding to factor IXa may inhibit the intrinsic Xase complex formation itself. In addition, we demonstrated that prolixin-S binding to factor IX inhibits factor IX activation by factor VIIa-tissue factor complex and factor IX activation by factor Xa. Our results show that prolixin-S inhibits all coagulation pathways in which factor IX participates and that prolixin-S would act as an inhibitor of both intrinsic and extrinsic coagulations.

EXPERIMENTAL PROCEDURES

Proteins and Phospholipids—Human factors IX, IXa, XIa, Xa, and VIIa were purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant human factor VIII was obtained from American Diagnostica Inc. (Greenwich, CT), and activated before use as described by Astermark et al. (18). Recombinant tissue factor was purchased from American Diagnostica Inc., and its relipidation was prepared as described by Zhong et al. (19). To determine protein concentrations, the following extinction coefficients (ε280 0.1%) and molecular weights were used: human factor IX, 1.33 and 57,000; factor IXa, 1.4 and 46,000; factor XIa, 1.34 and 143,000; factor X, 1.16 and 56,500; factor Xa, 1.16 and 46,500; factor VIIa, 1.16 and 56,500. The Gla-domainless factor IXa was prepared by chymotryptic digestion of factor IXa as described by Morita et al. (20, 21). Phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Sigma. Small unilamellar phospholipid vesicle suspension (PC and PS in a 7:3 (w/w) ratio) was prepared as described by Komiyama et al. (22) and quantified by standard method.

Recombinant Proteins—Recombinant prolixin-S (designated rProlixin-S hereafter) was produced using a baculovirus system, purified and reconstructed with hemin as described previously (10). rProlixin-S has been demonstrated to have the same activity as native protein (10).
obtained from injections of prolixin-S at different concentrations ranging from 3.125 to 100 nM at flow rate of 20 μl/min. Each of proteins was dialyzed in a buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 10% glycerol, 5 mM 2-mercaptoethanol, 0.01% BSA, 0.025% Triton X-100) as running buffer at 25 °C. Forty microliters of recombinant protein in concentrated PBS (pH 7.4) were incubated with glutathione-Sepharose beads bearing GST-prolixin-S (designated GST-prolixin-S hereafter) was produced in Escherichia coli (BL21 strain). In brief, cDNA fragments encoding prolixin-S without signal peptide were amplified by polymerase chain reaction and subcloned into an expression plasmid, pGEX6P-1 (Amersham Pharmacia Biotech). Produced GST-prolixin-S was purified by glutathione-Sepharose affinity chromatography. The specific activity (per weight) of GST-prolixin-S was estimated by injection of Xase activity in a reconstituted assay system (without VIIa, see below) and shown to be approximately 2% of rProlixin-S. Since the specific activity did not increase even after removal of GST from the fusion protein, reduction of the activity would be due to improper folding and degradation of the recombinant protein in E. coli.

Surface Plasmon Resonance (SPR) Analysis of Binding Kinetics between Prolixin-S and Factor IXa/IXa—Surface plasmon resonance studies were performed using a BIAcore X instrument (Bioacore AB, Sweden). Coupling factor was immobilized onto the surface of a sensor chip CM5 by amine coupling procedure according to the manufacturer’s instructions.

Binding analyses were performed using HEPES-buffered saline (HBS; 10 mM HEPES, pH 7.4, 150 mM NaCl containing 5 mM CaCl2 and 0.005% Tween 20) as running buffer at 25 °C. Forty microliters of varying concentrations of rProlixin-S (1.31–100 nM) was injected at a flow rate of 20 μl/min. Association was monitored during a 2-min injection of analyte. Dissociation was monitored during 2 min after return to buffer flow. Regeneration of the sensor chip surface was achieved by a pulse injection of 0.5 M EDTA. To determine the kinetic binding constants (k₂, k₋₁, and Kᵣ), the binding data were analyzed by non-linear regression (23) using the evaluation software (BIAevaluation 3.0). The experimental data fit well with a 1:1 binding model (Langmuir).

The Ca²⁺ dependence of rProlixin-S binding to factor IXa/IXa was also investigated by the same method. In this experiment, HBS buffer was passed through a column of Chelex 100 (Bio-Rad) to remove metal contaminants before adding CaCl₂. Each of proteins was dialyzed in a metal-chelating HBS buffer containing 0.1% Chelex 100. rProlixin-S (100 nM) was injected at different Ca²⁺ concentrations.

To monitor the factor IXa/IXa-phospholipid interaction, surface of a sensor chip HPA was coated with lipid monolayer (PC:PS 3.0). The experimental data fit well with a 1:1 binding model (Langmuir).

GST Pull-down Assays—Three micrograms of factor IX or factor IXa were incubated with glutathione-Sepharose beads bearing GST-prolixin-S (approximately 1 μg of protein/1 μl of bed volume) in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 10% glycerol, 5 mM 2-mercaptoethanol, 0.01% BSA, 0.025% Triton X-100) containing 5 mM CaCl₂ or 5 mM EDTA. After incubation for 1 h at 4 °C, the beads were washed three times, boiled in SDS-PAGE loading buffer, and directly subjected to 12.5% SDS-PAGE. Separated proteins were stained with Coomassie Brilliant Blue or transferred to a nitrocellosulfur membrane for detection with antibody.

Solid-phase Binding Assay—Wells of a microtiter plate (Immulon 2, Dynatech Laboratories, Inc.) were incubated with different coagulation factors in buffer A overnight at 4 °C, blocked with 5% BSA, washed twice, and incubated with GST or GST-prolixin-S in buffer A containing 1% BSA for 1 h. Wells were then washed three times with buffer A containing 0.05% Tween 20, incubated with anti-GST polyclonal antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc) for 1 h, and washed five times. Peroxidase activity was detected with 0.05% (w/v) 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic acid) and 0.05%/v/v H₂O₂, and absorbance was read at 405 nm.

The effect of Ca²⁺ concentration on the binding of prolixin-S to factor IXa/IXa was investigated by the same procedure with modifications as follows. The buffer used in this assay was passed through a column of Chelex 100. Protein solution (GST-prolixin-S, factor IX, and factor IXa) were dialyzed against buffer containing 0.1% Chelex 100 resin. The wells coated with factor IX or factor IXa (10 nM) were incubated with GST-prolixin-S (4.7 μg/ml) and then washed in buffer containing various concentrations of CaCl₂.

Light Scattering Analysis—Factor IXa (9 μg, 200 pmol) was incubated with various concentrations of rProlixin-S in buffer A for 10 min and then added to 600 μl of the same buffer containing 9 μg of phospholipid vesicles. The interaction of factor IXa with phospholipid membrane surface was evaluated by the relative 90° light-scattering technique using excitation and emission wavelengths of 320 nm as described by Nelsestuen and Lim (24). For the assay in the presence of factor VIIIa, factor IXa (500 pm, in final concentration) was preincubated at 37 °C for 10 min with rProlixin-S (0, 5, 10, 20, 50, and 100 nM) in buffer A containing 1% BSA and added to reaction mixture (the same buffer containing 1.5 units/ml factor VIIIa, 40 μg phospholipid suspension and 400 μl factor X). For the assay in the absence of factor VIIIa, factor IXa (50 nM, in final concentration) was preincubated at 37 °C for 10 min with rProlixin-S (0, 25, 50, 100, 150, and 250 nM) and added to the reaction mixture without factor VIIIa. After 10 min of incubation, the activity of generated factor Xa was determined using chromogenic substrate S-2222 (Chromogenix AB).

Assay for the Effect of Prolixin-S on Factor IXa Activation by Factor IXa— For the assay in the presence of factor VIIIa, factor IXa (500 pm, in final concentration) was preincubated at 37 °C for 10 min and then activated by addition of factor VIIa. Factor VIIIa (100 pmol) in the mixture without factor VIIIa. After 10 min of incubation, the activity of generated factor Xa was determined using chromogenic substrate S-2222 (Chromogenix AB).

Assay for the Effect of Prolixin-S on Factor IXa Generation by Factor VIIIa—Tissue Factor Complex—To prepare the reaction mixture containing factor VIIa/tissue factor/phospholipid-enzyme complex, lipidated tissue factor (35 nM) was incubated with factor VIIa (25 nM) and 1 μg phospholipid in buffer B at 37 °C for 30 min. Factor IX (600 pm) was preincubated with rProlixin-S (0, 600, or 1800 nM) in buffer B at 37 °C for 10 min and added to the reaction mixture. Aliquots were removed from the reaction at designated time points, and the progress of the activation was evaluated as described above.

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lixin-S (100 nM) at flow rate of 20 μl/min. This mixture was added to the reaction mixture (buffer B containing 400 μg phospholipid suspension and 50 nM factor X). The progress of the factor IX conversion to factor Xa was monitored and analyzed as described above.

RESULTS

Prolinx-S Binds to Factors IX and IXa in the Presence of Ca^{2+} Ions—In the previous paper (4), we demonstrated that prolixin-S inhibited intrinsic Xase activity in the absence of factor VIIIa. This result suggests that prolixin-S may inhibit intrinsic Xase activity by direct interaction with factor IXa. Therefore, we performed SPR analysis in order to examine direct interactions between prolixin-S and factor IXa/IX. As shown in Fig. 1, the injections of prolixin-S onto immobilized factor IX/IXa gave significant responses, indicating that prolixin-S binds both zymogen and enzyme form of factor IX. At a standard condition (pH 7.4 and [Ca^{2+}] = 5 mM), $k_0$, $k_{a1}$, and $K_d$ for prolixin-S binding to factor IXa were $3.11 \pm 0.35 \times 10^5$ M$^{-1}$s$^{-1}$, $4.06 \pm 0.61 \times 10^{-3}$ s$^{-1}$, and $13.2 \pm 0.24$ nM, respectively. On the other hand, those values to factor IX zymogen were $5.03 \pm 0.46 \times 10^5$ M$^{-1}$s$^{-1}$, $6.47 \pm 0.62 \times 10^{-3}$ s$^{-1}$, and $13.0 \pm 0.14$ nM, respectively. In contrast, Prolixin-S showed no interactions with other coagulation factors tested, even when excess of ligand was immobilized (Fig. 2). In addition, prolixin-S failed to interact with Gla-domainless factor IXa. This indicates that the binding site would be located in the Gla domain of factor IXa (see “Discussion”).

Since it had been reported that factor IXa changes its conformation depending on Ca^{2+} binding (see “Discussion”), we monitored the rProlixin-S binding to factor IXa/IX at different Ca^{2+} concentrations. Responses were not observed at 0.2 mM or less, rapidly increased depending on Ca^{2+} concentrations over 0.2 mM, and nearly reached plateau at 1 mM.

The results presented above were also supported by assays performed with GST-prolixin-S. GST-pull down assay showed that factors IX and factor IXa bound to GST-prolixin-S only in the presence of Ca^{2+} ions (data not shown). Solid phase binding assay showed that GST-prolixin-S bound to factor IX/IXa but not to other factors tested (factor XIa, factor X, and factor VIIIa). Solid phase binding assay also showed that binding potency of GST-prolixin-S to factor IXa/IXa rapidly increased dependent on the Ca^{2+} concentrations especially between 0.3 and 1 mM (data not shown).

Prolinx-S Inhibits Factor IXa-Phospholipid Interaction—In order to investigate whether prolixin-S binding to factor IXa affects its interaction with phospholipids, we monitored the interactions (association phases) in the presence of rProlixin-S by SPR analysis. As shown in Fig. 3, the response decreased with increasing rProlixin-S content, showing that rProlixin-S inhibits the factor IXa binding to phospholipids.

Light-scattering experiment also supported this result. The light scattering intensities due to factor IXa-phospholipid vesicle complex formation dramatically decreased concomitant with increasing rProlixin-S content (data not shown). The scattering intensity was reduced to 28% after incubation with equimolar rProlixin-S.

Prolinx-S Inhibits Factor IXa-catalyzed Activation of Factor X—Our previous study showed that prolixin-S inhibited factor IXa-catalyzed activation of factor X (4). However, the inhibition of this reaction by prolixin-S did not appear so potent because complete inhibition was not observed, even when an excess of prolixin-S was used. In the previous experiments, prolixin-S was preincubated together with factor IXa, factor VIIIa, and phospholipids and the reaction was started with addition of factor X. We thought the inhibitory activity of prolixin-S would have been underestimated in this procedure, if the inhibitory effect by prolixin-S resulted from interference with Xase complex formation itself. Therefore, in these experiments, we altered the order of rProlixin-S addition as follows. rProlixin-S was preincubated with factor IXa in Ca^{2+}-containing buffer and reaction was started by adding it to a reaction mixture containing phospholipid in the presence and absence of factor VIIIa. As shown in Fig. 4A, in the presence of factor VIIIa, rProlixin-S inhibited factor X activation by factor IXa in a dose-dependent manner. Addition of excess rProlixin-S re-
resulted in almost complete inhibition. Dose-dependent inhibition by rProlixin-S was also observed in the absence of factor VIIIa (Fig. 4B).

Prolixin-S Inhibits Factor XIa-catalyzed Activation of Factor IX—Our previous studies using a synthetic substrate showed that prolixin-S could not inhibit factor Xa amidolytic activity (4, 5). However, this result does not rule out a possibility that prolixin-S could inhibit activation of a physiological substrate, factor IX. Furthermore, it was shown above that prolixin-S binds to factor IX in the presence of Ca\(^{2+}\) ions. Based on this reason, we investigated the inhibitory effect of prolixin-S on factor XIa-catalyzed activation of factor IX in a reconstitution system. In these experiments, factor IX was preincubated with rProlixin-S before addition of factor XIa, and activation of factor IX was checked by SDS-PAGE after time intervals following addition of factor XIa. Fig. 5 shows the results of the time-course study. Factor IXa generation by factor XIa was inhibited by the addition of rProlixin-S in a dose-dependent manner. The rate of factor IX activation was reduced by 91% after incubation with equimolar rProlixin-S and almost completely inhibited after incubation with a 3-fold molar excess of rProlixin-S. These results suggest that prolixin-S binds to factor IX stoichiometrically 1:1, resulting in almost complete inhibition of factor IX activation by factor XIa.

On the other hand, also shown in Fig. 5, when molar ratios between rProlixin-S and factor IX were 1:2, factor IX was completely activated by factor XIa within 60 min. This make clear contrast to strong inhibitory effect of rProlixin-S observed when molar ratios between rProlixin-S and factor IX were 3:1 and 1:1. We suppose that the result would be explained by the fact that rProlixin-S binds to the reaction product (factor IXa) as well as factor IX with almost the same kinetics, i.e. in the progress of the reaction, a ratio of prolixin-S to bound factor IXa gradually increased and then excess factor IX was sequentially activated, resulting in the complete activation. In order to clearly demonstrate this assumption, we compared the activation of factor IX between the following conditions, that is, with molar ratios between prolixin-S and factor IX 2:1 and 2:4 and the concentrations of factor IXa (1.35 nM) and prolixin-S (1500 nM) kept constant. As shown in Fig. 6, factor IXa was almost fully activated within 30 min when an inhibitor/factor IXa ratio was 2:4. In contrast, a considerable amount remained unactivated when the inhibitor/factor IXa ratio was 2:1. This result means that a larger amount of substrate was digested although other conditions were identical. These results would be explained only by the assumption presented above.

Prolixin-S Inhibits Factor VIIa-Tissue Factor-catalyzed Activation of Factor IX—The factor VIIa-tissue factor complex proteolytically activates factor IX on phospholipid membrane in the presence of Ca\(^{2+}\) ions. Factor IX should bind to the phospholipid membrane surface to be catalyzed as a substrate of this reaction. Thus, it was supposed that prolixin-S would inhibit factor IX activation by interference with its binding to the phospholipid membrane. We examined this possibility by the time-course study in a reconstitution system. As shown in Fig. 7A, factor IXa generation by factor VIIa was inhibited by the addition of rProlixin-S in a dose-dependent manner. Addition of a 3-fold molar excess rProlixin-S resulted in almost complete inhibition (99.7%) of factor IXa generation.
In this study, we attempted to clarify the mechanism by which prolixin-S inhibits blood coagulation. We found that prolixin-S had the specific ability to bind to factor IX and factor Xa. Prompted by this finding, we found that prolixin-S binding to factor IXa interfered with its association with phospholipids. This interference would lead factor IXa to failure to form intrinsic Xase on the phospholipid membrane surface and results in reduction of its Xase activity. We also demonstrated that prolixin-S binding to factor IX inhibited activation of factor IX by factor XIa. This finding shows that prolixin-S could act on the earlier phase of the intrinsic coagulation than it has been believed. Since the activation of factor IX by factor XIa does not require a phospholipid membrane, the inhibition of factor IX activation cannot be explained by interference with factor IX binding to phospholipids. As discussed later, we supposed that Gla domain binding of prolixin-S would directly interfere with interaction between factor IX and factor XIa. We think these distinct inhibitory mechanisms may contribute to the intrinsic pathway inhibition and severe prolongation of APTT by prolixin-S.

In addition to the intrinsic pathway inhibition mentioned above, we found that prolixin-S also acts as an inhibitor of the extrinsic coagulation pathway. Reconstitution experiments showed that prolixin-S inhibited factor IX activation by the factor VIIa-tissue factor complex. In addition, prolixin-S inhibited the conversion of factor IX to IXa by factor Xa (12, 13, 19). These inhibitory activities may be due to its interference with factor IX binding to phospholipids because both proteolysis of factor IX should proceed on the phospholipid membrane surface (13). We previously reported that prolixin-S hardly elongated prothrombin time. It made a clear contrast with its potent prolongation of APTT. This result would be due to the fact that, in prothrombin time assays, a high concentration of tissue factor and phospholipid was used to initiate the reaction. Direct factor X activation by excess amount of factor VIIa-tissue factor complex would have masked the inhibitory effect of prolixin-S on factor IX activation by the factor VIIa-tissue factor complex (13). On the other hand, prolixin-S could act as an extrinsic pathway inhibitor in physiological hemostasis because, in vivo, only a limited amount of tissue factor contacts the blood and forms a complex with factor VIIa. Since extrinsic coagulation is known to be crucial for initiation of the physiological hemostasis following vesicular injury, the extrinsic pathway inhibition would be important for the blood sucking insect to arrest blood coagulation in the initial phase.

We think that prolixin-S shows these activities by binding to the Gla domain of factor IX (IXa). Gla domain of factor IX is known to be a phospholipid binding domain of factor IXa and dramatically change its conformation with Ca²⁺ binding, especially in Ca²⁺ concentrations between 0.1 and 1 mM. This conformation change enables this domain to interact with phospholipid membranes (25–27). Our results of SPR analysis and solid phase binding assays showed that binding property of prolixin-S to factor IX and factor Xa dramatically changed between 0.3 and 1 mM. This suggests that prolixin-S binds to factor IXa by recognizing a Ca²⁺-dependent conformation of the Gla domain and inhibits its interactions with phospholipid vesicles. It was strongly supported by the findings that prolixin-S could not interact with factor IXa without the Gla domain (Fig. 2). Interestingly, inhibitory properties of prolixin-S are similar to those of the Ca²⁺-induced conformation specific antibodies (27). These antibodies bind to the Gla domain of factor IXa by recognizing its Ca²⁺-dependent conformation change and inhibit the binding to phospholipid vesicles. Furthermore, these antibodies inhibit the activation of factor IX by factor Xa (27, 28). These coincidences suggest that prolixin-S and some of these antibodies recognize the same or a related
site on the Gla domain formed by Ca$^{2+}$ binding. It is also suggested that the Gla domain plays an important role in the activation of factor IX by factor XIa as well as in the binding to phospholipids. In fact, Wojcik et al. (29) have provided evidence that the conformational change of Gla domain is important for the factor IX activation by factor XIa.

In conclusion, we identified factor IX and factor IXa as target molecules of prolixin-S. We show that prolixin-S affects all known proteolytic reactions in which factor IX participates and that the vital point of this factor may be present in the Gla domain.

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