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THE ACTION OF ECHIS CARINATUS VENOM ON THE BLOOD COAGULATION SYSTEM. DEMONSTRATION OF AN ACTIVATOR OF FACTOR X.

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ABSTRACT

It is shown that Echis carinatus venom activates both coagulation factor II and coagulation factor X. The activation of both proenzymes by the venom is Ca^{++}-dependent; phospholipids are not necessary.
The activation of factor II by the venom is a slow process and, in the absence of factor X, the clotting activity towards fibrinogen is generated only very slowly. Because Echis carinatus venom clots plasma readily, we postulate that under conditions where the prothrombinase complex can be formed from the factor X activated by the venom it is this complex, rather than the venom itself, that is responsible for the major part of the thrombin formation.

INTRODUCTION

The proteolytic action of the prothrombin activating fraction from the venom of Echis carinatus (ECV-prothrombin-activator) is well documented (1-8). Incubation of prothrombin with ECV-prothrombin-activator results in the splitting of the prothrombin molecule at the site that links the A-chain and B-chain region of thrombin, the resulting molecule is called meizothrombin. Prolonged incubation (20 hours) in the presence of inhibitors of thrombin activity results in the splitting of meizothrombin to meizothrombin-(des-F1) and fragment-1 (6,7). The resulting molecules, meizothrombin and meizothrombin-(des-F1) possess an active centre comparable to that of normal thrombin. Secondary binding sites may differ considerably, however, as may be concluded from the fact that the capability of meizothrombin to split fibrinogen is strongly reduced (2,3,8). By autocatalytic digestion of meizothrombin a thrombinlike molecule arises that does have the capability to clot fibrinogen (2,3,6,7). Echis carinatus venom (ECV) acts on both normal pro-

Key words: Echis carinatus venom, factor II, factor X, coagulation assay, chromogenic substrate assay.

thrombin and decarboxyprothrombin (PIVKA-II). It therefore has been used as a means to assess the total concentration of these two proteins in plasma (9). It will be shown here that the Echis carinatus venom is also able to activate coagulation factor X and that, in the presence of factor X and phospholipid, the venom exerts its procoagulant action via factor X rather than via a direct action on prothrombin.

MATERIALS AND METHODS

Chemicals: All chemicals used were analytical grade; all solutions were prepared with distilled water. Buffers: 0.05 M Tris/HCl, 0.100 M NaCl, pH 7.4 containing albumin (0.5 mg/ml); Michaelis buffer: 0.15 M Na-veronal, 0.109 M, 0.15 M NaCl, pH 7.35.

Plasma: Platelet poor plasma was obtained from fresh blood collected in plastic tubes containing Na-citrate (9 volumes of blood + 1 volume of Na-citrate) by centrifugation (15 minutes at 13000 g). Platelet free plasma was obtained by centrifugation of platelet poor plasma (20 minutes at 20000 g at 4°C). Pooled plasma was obtained by pooling equal amounts of platelet free plasma from at least 30 healthy individuals (15 males and 15 females, average age 30 years) and was stored in 1 ml portions at -20°C until use. $BaSO_4$-adsorbed plasma was obtained by adsorbing oxalated normal plasma (final conc. 0.01 M Na-oxalate) with 1% (w/v) of $BaSO_4$.

Procoagulants: Purified prothrombin and purified factor X were prepared as described before (10). The purified proteins were stored in 1 ml portions at -80°C; prothrombin in a concentraton of 100 nM and factor X in a concentration of 15 nM.

Thromboplastin was prepared from human brain according to a modification of the method of Owren and Aas (10,11). Inositin was obtained from Associated Concentrates Inc. (N.J. U.S.A. Lot no. 4170)

Echis carinatus venom was obtained from Sigma laboratories (St. Louis USA Lot 31F-0490)

Activation experiments: Incubations of Echis carinatus venom with plasma or with solutions of purified coagulation factors were carried out at room temperature in plastic tubes. The incubation mixtures were prepared in Tris/HCl buffer, pH 7.4. The exact compostions of the incubation-mixtures is given in the description of the individual experiments. From the mixture, samples were drawn for clotting and chromogenic assays.

Clotting assays: 0.2 ml $BaSO_4$-absorbed plasma, diluted 1:5 with Michaelis buffer was prewarmed to 37°C. At the incubation time indicated a 100 µl sample of the incubation mixture was added and the clotting time, which is the time interval between the addition of the sample and the appearance of the fibrin clot, was registrated. The thrombin concentrations were read from a reference curve obtained with a standard solution of thrombin diluted to known concentrations.

Chromogenic substrate assays: For thrombin: 2.3 ml Tris/HCl buffer (pH 7.4 prewarmed at 37°C) and 100 µl S2238 (5.33 mM in distilled water) were pipetted into a plastic cuvette contained in the thermostated (37°C) cuvette holder of a spectrophotometer (Beckman Model 25). At the incubation time indicated a 100 µl sample of the incubation mixture is added and the increase in adsorption, due to the p-nitroaniline production, is registrated at 405 nm. If the increase in absorbance is >0.2 absorbancy units/minute the sample volume is reduced.

Factor Xa was assayed in the same way but 250 µl of a 1.9 mM solution of S2337 in distilled water was used as a substrate.

Column chromatography: 200 mg crude Echis carinatus venom dissolved in 5 ml of Tris/HCl buffer (0.05 M Tris/HCl, pH 8) was applied to a 2.5 x 100 cm column of preswollen and degassed Sephadex G 150. At a flow rate of 20 ml/hour 4 ml samples were collected. The procoagulant activity was found in

the first peak. The middle part of this peak was applied to a DEAE-Sephadex A50 column (2.5 x 50 cm) equilibrated with Tris/HCl buffer (0.05 M Tris, pH 8) and eluted with a linear gradient of 250 ml of 20% glycerol in the same buffer and 250 ml of 0.6 M NaCl in the same buffer. The flow rate was 15-20 ml per hour and 4 ml fractions were collected. The tubes 65-to 90 contained factor II and factor X activators that were incompletely separated. When these fractions were further fractionated on a DEAE-Sephadex A50 column (2.5 x 50 cm) and eluted with a linear buffer gradient of 250 ml 0.05 Na-citrate, pH 8.5, containing 10% glycerol to 0.2 M Na-citrate pH 8.5. (flow rate 15-20 ml per hour, 4 ml fractions) the separation as illustrated in fig 3 was obtained.

RESULTS

Purified prothrombin, if incubated with Echis carinatus venom, activates to a species with amidolytic activity. This species will not coagulate fibrinogen; clotting activity only arises after prolonged incubation times. As the Echis carinatus venom is known to coagulate plasma readily it is obvious to postulate the requirement of an accessory plasma factor for this action. From figure 1, which shows the generation of (clotting) thrombin from normal plasma and from purified prothrombin upon incubation with Echis carinatus venom under different experimental conditions, it is clear that the accessory plasma factor is not contained in $BaSO_4$ absorbed plasma. The very slow generation of thrombin activity upon incubation of prothrombin with Echis carinatus venom is dramatically increased upon the addition of clotting factor X (frame C); addition of clotting factor X to the same incubation mixture without Echis carinatus venom does not lead to thrombin generation (frame B). These observations point at a direct activation of clotting factor X by Echis Carinatus venom.

From figure 2 it can be seen that factor X does not influence the generation of S2238 splitting activity from prothrombin by Echis Carinatus venom (frame A) unless both PL and Ca^{++} are present. When these reagents are added a significant increase of thrombin generation is seen under conditions where the sample has been preincubated with Ca^{++} for 120 min i.e. under conditons that factor X has been activated by the venom (black circles). From frame B it is clear that clotting activity is hardly generated from prothrombin unless Ca^{++} is present and that it is much more important if factor X is added to the incubation mixture. Optimal conditions are created by the simultaneous presence of Ca^{++}, factor X and phospholipid.

When the generation of factor X_a amidolytic activity upon incubation of factor X with Echis carinatus venom is monitored with S2237, a direct, Ca^{2+}-dependent activation of factor X by Echis carinatus venom can be demonstrated. The addition of phospholipids slows down this generation of amidolytic factor X_a activity. (results not shown)

By the use of column chromatography, it has been possible to separate the factor X activating enzyme from the prothrombin activating species (see methods and fig 3).

When $BaSO_4$ adsorbed plasma is spiked with varying amounts of purified factor II and factor X it becomes clear that factor X is required for the clotting action of the venom on plasma (Figure 4).

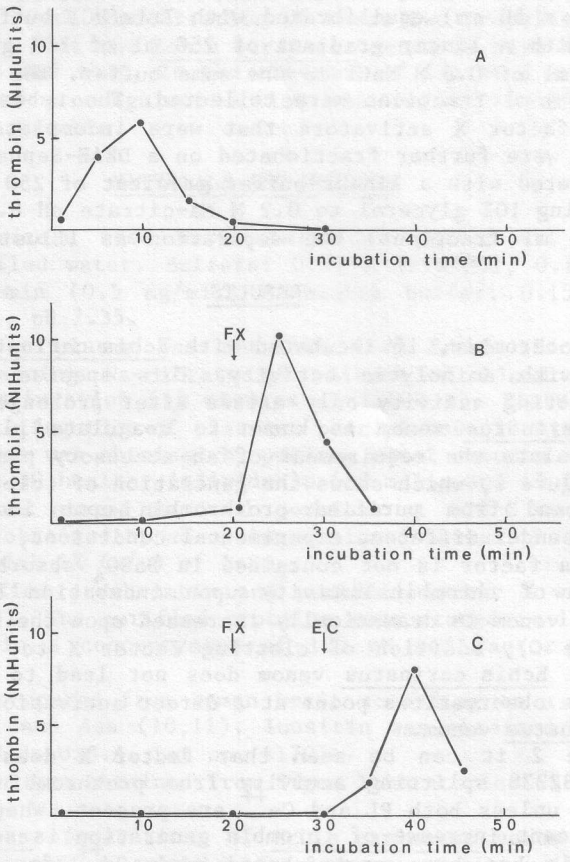

FIG. 1

The generation of thrombin from pooled normal plasma (A) and from purified prothrombin (B and C) upon incubation with Echis carinatus venom (ECV).
The incubation was carried out at room temperature; the thrombin activity was determined by a clotting assay (see Materials and Methods).
Incubation mixtures:

A. 1 ml pooled plasma (defibrinated for 3 minutes at 56°C), 1 ml Michaelis buffer, $CaCl_2$ (final concentration 0.01 M), EVC (final concentration 0.4 g/ml): final volume 3 ml.

B. 1 ml defibrinated $BaSO_4$-adsorbed plasma 0.4 ml, Michaelis buffer, purified prothrombin (final concentration 2 M.), $CaCl_2$ (final concentration 0.01 M), inositine (final concentration 0.125 mg/ml), E C V (final concentration 0.4 g/ml), final volume 2.9 ml. After 20 minutes incubation 0.1 ml purified factor X solution was added (final concentration 0.2 M).

C. 1 ml defibrinated $BaSO_4$-adsorbed plasma, 0.4 ml, Michaelis buffer, purified prothrombin (final concentration 2 M), $CaCl_2$ (final concentration 0.01M), inositin (final concentration 0.125 mg/ml): final volume 2.7 ml. After 20 minutes incubation 0.1 ml purified factor X solution was added (final concentration 0.2 M) and after 30 minutes incubation 0.2 ml ECV (final concentration 0.4 g/ml) was added.

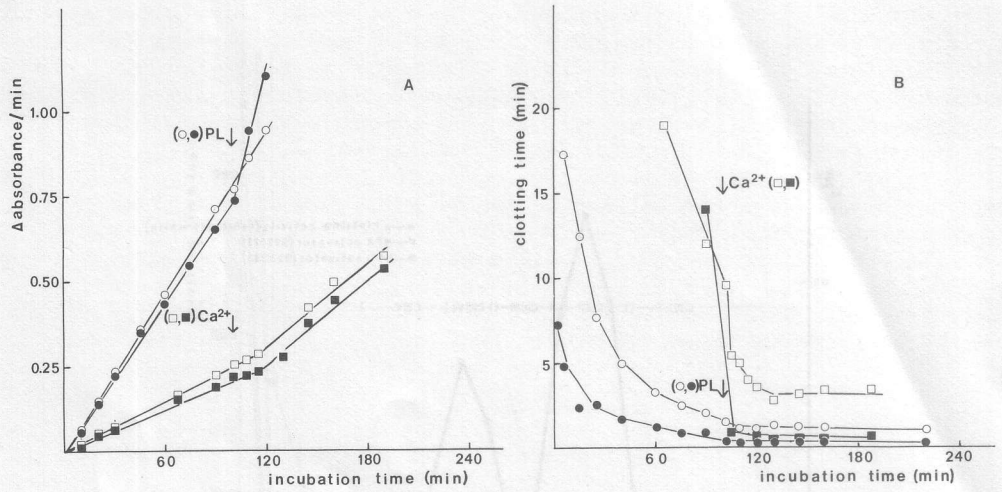

FIG. 2

The generation of amidolytic activity (A) and clotting activity (B) from purified factor II by Echis Carinatus venom in the absence and presence of purified factor X and phospholipid.
The incubation mixtures were prepared in Tris/HCl buffer (pH 7.4 0.05 M, NaCl 0.10 M) containing:

- : prothrombin (2 M), ECV (0.4 g/ml), $CaCl_2$ (0.01 M) after 100 minutes inositine is added (final concentration 0.75 mg/ml)
- : prothrombin (2 M), factor X (0.2 M), ECV (0.4 g/ml), $CaCl_2$ (0.01M); after 100 minutes inositine is added (final concentration 0.75 mg/ml).
- : prothrombin (2 M), ECV (0.4 g/ml), inositine (0.75 mg/ml); after 100 minutes $CaCl_2$ is added (final concentration 0.01M).
- : prothrombin (2 M), factor X (0.2 M), ECV (0.4 g/ml), inositine (0.75 mg/ml) after 100 minutes $CaCl_2$ is added (final concentration 0.01M).

DISCUSSION

The experiments shown in the figures 1 and 2 demonstrate that normal factor X is activated by Echis carinatus venom. From the literature it is clear that a purified fraction from this snake venom activates prothrombin directly into meizothrombin, i.e. prothrombin cleaved at the site that links the A- and B-chain of the thrombin moiety. Meizothrombin has no (or a neglectible) procoagulant action on fibrinogen. For this activity to arise meizothrombin has to be degraded further. In various articles the direct formation of thrombin from purified prothrombin by Echis carinatus venom has been reported (7,8). Most authors prefer to explain the formation of thrombin from purified prothrombin by autolysis of meizothormbin (7,13). In view of our results it is clear that thrombin can also be produced by the action of factor X activated by the venom and that one only can arrive at conclusions if the presence of contaminating traces of factor X in the prothrombin preparations can be rigorously excluded. In any case the generation of thrombin from prothrombin by Echis carinatus venom is slow and therefore this venom hardly brings about coagulation in plasma devoid of factor X (fig. 4).

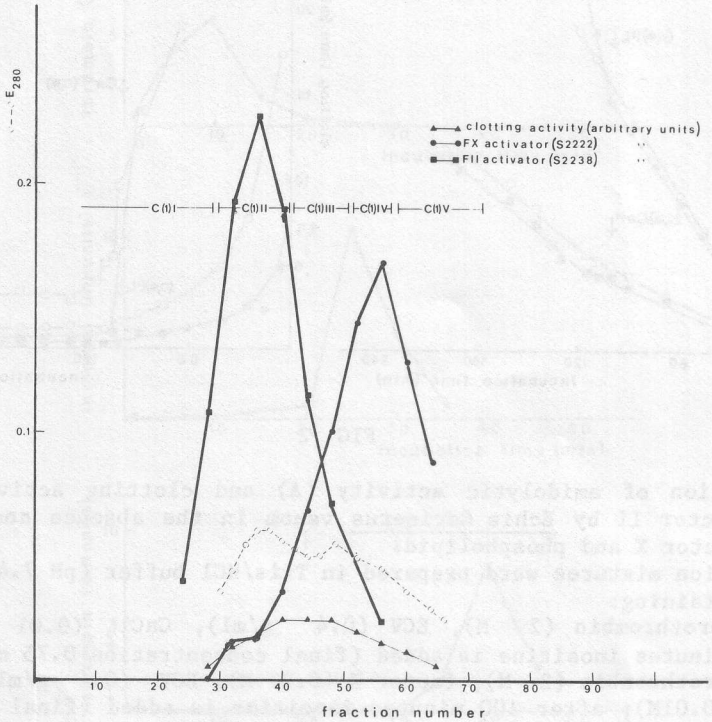

FIG. 3

Separation of factor II and factor X activators from Echis carinatus venom on a DEAE-Sephadex A50 column.
The material obtained as described in the methods section was applied to a 2.5x30 cm DEAE, Sephadex A50 column and eluted at a rate of 15-20 ml per hour with a gradient of 250 ml 0.05 Na-citrate pH 8.5 containing 10% glycerol to 250 ml of 0.2 M Na citrate, pH 8.5. The factor II and factor X splitting activity is measured as described in the methods section and rendered in arbitrary units.

If factor X is present the generation of clotting activity is rapid. We therefore conclude that whole plasma is clotted by Echis carinatus venom via factor X. The fact that phospholipid is necessary for this action can be accepted as supportive evidence for this view (the activation of factor X by Echis carinatus venom is slightly inhibited by phospholipids).

The main conclusion from the chromatography experiments is that there exist separate prothrombin and factor X activators in Echis Carinatus venom.

In a series of parallel experiments we used Boomslang (Dyspholidus types) venom. In all experiments it could replace Echis carinatus venom and showed the same results, also on the clotting time of plasmas containing varying amounts of factor II and X (results not shown).

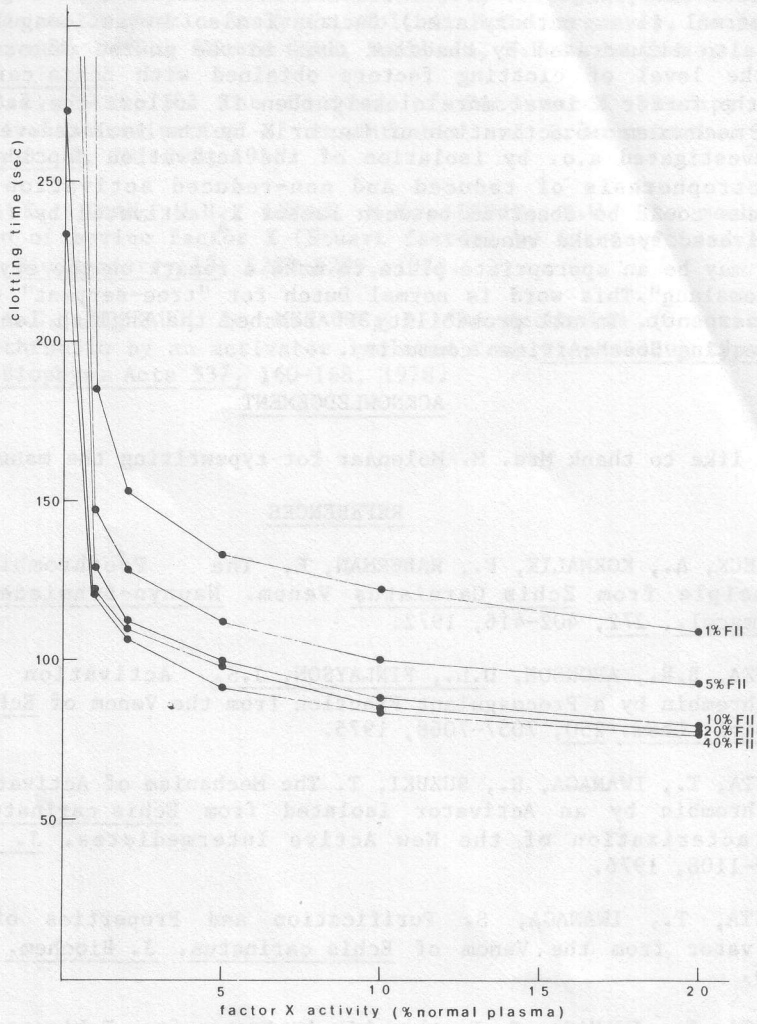

FIG. 4

The Echis carinatus clotting time as a function of the factor II and factor X content of $BaSO_4$-adsorbed plasma.
To $BaSO_4$-adsorbed plasma varying amounts of purified factor II and factor X were added. The clotting time of the resulting mixture is determined as described in the section Materials and Methods.

From our results it becomes likely that the factor X activating fraction in both venoms is the one responsable for the procoagulant action and that the main procoagulant pathway is:
venom factor X factor II fibrinogen.
This explains why the venom clotting time is dependent upon both the factor X and the factor II concentration. Relatively low concentrations of factor X are sufficient to obtain a seizable coagulation velocity. The concentrations of factor II have to be somewhat higher (figure 4). The action of the venoms on PIVKA-X has yet to be investigated. The fact that phospholipids are

necessary for the coagulation to occur indicates that the main pathway has to be via normal (i.e. carboxylated) factor X also in anticoagulated plasma. This is also illustrated by the fact that in the course of oral anticoagulation the level of clotting factors obtained with Echis carinatus venom follows the factor X level more closely then it follows the factor II level (9). The mechanism of activation of factor X by the isolated venom has still to be investigated a.o. by isolation of the activation peptides. On S.D.S. gel electrophoresis of reduced and non-reduced activation mixtures no differences could be observed between factor X_a activated by factor IX_a and that activated by snake venom.

This may be an appropriate place to make a remark on the etymology of the name "Boomslang".This word is normal Dutch for "tree-serpent" (Boom = tree, slang = serpent). In all probability it reached the English language via the Dutch speaking South-African community.

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