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Targeting of Venom Phospholipases: The Strongly Anticoagulant Phospholipase A_2 from *Naja nigricollis* Venom Binds to Coagulation Factor Xa to Inhibit the Prothrombinase Complex

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The strongly anticoagulant basic phospholipase A_2 (CM-IV) from *Naja nigricollis* venom has previously been shown to inhibit the prothrombinase complex of the coagulation cascade by a novel nonenzymatic mechanism (S. Stefansson, R. M. Kini, and H. J. Evans *Biochemistry* 29, 7742–7746, 1990). That work indicated that CM-IV is a noncompetitive inhibitor and thus it interacts with either factor Va or factor Xa, or both. We further examined the interaction of CM-IV and the protein components of the prothrombinase complex. Isothermal calorimetry studies indicate that CM-IV does not bind to prothrombin or factor Va, but only to factor Xa. CM-IV has no effect on the cleavage of prothrombin by factor Xa in the absence of factor Va. However, in the presence of factor Va, CM-IV inhibits thrombin formation by factor Xa. With a constant amount of CM-IV, raising the concentration of factor Va relieved the inhibition. The phospholipase A_2 enzyme inhibits by competing with factor Va for binding to factor Xa and thus prevents formation of the normal Xa–Va complex or replaces bound factor Va from the complex. Thus factor Xa is the target protein of this anticoagulant phospholipase A_2, which exerts its anticoagulant effect by protein–protein rather than protein–phospholipid interactions.

Snake venom phospholipase A_2 (PLA_2)[4] (EC 3.1.1.4) enzymes induce several pharmacological symptoms including neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, hemolytic, edema-inducing, and platelet effects (1–5). These toxic, pharmacologically active enzymes share with mammalian pancreatic enzymes the common catalytic activity of hydrolysis of phospholipids at the *sn*-2 position. They also share similar primary, secondary, and tertiary structures (6–11). The relationships between structure and pharmacological activity and the mechanisms by which the venom enzymes induce their variety of pharmacological effects are subtle and complex. Because of their ability to hydrolyze phospholipids, the pharmacological activity was initially thought to be related to the substrate specificity and preferential hydrolysis of specific and critical phospholipids, i.e., directed by PLA_2 protein–phospholipid interactions. This model could not explain several contradictory observations (for details, see Ref. 4). To alleviate some of these controversies, we proposed a hypothetical model to explain the pharmacological effects of venom PLA_2 enzymes (4). The essential feature of the model is the targeting of PLA_2 enzymes to specific tissues or cells due to their high affinity for specific "target" proteins (or glycoproteins) on the cell surface. After the initial binding, PLA_2

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[4] Abbreviations used: PLA_2, phospholipase A_2; BSA, bovine serum albumin; S-2238, *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S-2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroaniline hydrochloride and its methyl ester; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PEG, polyethylene glycol; STI, soybean trypsin inhibitor; Gla, γ-carboxyglutamic acid; $hsPLA_2$, human secreted PLA_2. PT, prothrombin.

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enzymes would induce their pharmacological effects by mechanisms either dependent on or independent of phospholipid hydrolysis. Thus, the pharmacological effects of venom PLA_2 enzymes are primarily determined by protein–protein interactions (4). Some studies have shown the interaction of venom PLA_2 enzymes with proteins in their "target" tissues (12–14). Target proteins in neuronal tissue have been isolated and/or characterized based on their binding of neurotoxic PLA_2 enzymes (14–17). A target protein in myotubule membranes that binds two PLA_2 toxins from *Oxyuranus scutellatus* was isolated and cloned (18, 19). The same protein was also shown to be the target of pancreatic PLA_2 and synovial PLA_2 (20, 21). However, target proteins, which determine various other pharmacological effects, have not yet been identified. Such studies will contribute much to our understanding of normal physiological events and their susceptibilities to exogenous agents and will help clarify the mechanisms of toxicity of the venom enzymes.

We are interested in the anticoagulant effects of three PLA_2 enzymes purified from *Naja nigricollis* venom (22). The two neutral isoenzymes, CM-I and CM-II, are weakly anticoagulant, whereas the basic enzyme, CM-IV, is strongly anticoagulant (23). Although blood coagulation requires the assembly of complexes on phospholipid membranes, the differences in anticoagulant potencies of the phospholipases cannot be attributed to their ability to hydrolyze phospholipids, since CM-I and CM-II are catalytically more active than CM-IV. The functional difference appears to be due to the difference in their ability to inhibit various coagulation complexes of the extrinsic pathway of blood coagulation (24). Strongly anticoagulant CM-IV inhibits two successive steps, the extrinsic tenase and the prothrombinase complexes, whereas the weakly anticoagulant enzymes, CM-I and CM-II, inhibit only the extrinsic tenase complex. Moreover, CM-IV is a stronger inhibitor of the extrinsic tenase complex than both CM-I and CM-II (25). Since only the strongly anticoagulant enzyme inhibits the prothrombinase complex, CM-IV seems to be targeted to this complex. The inhibition of the prothrombinase complex by CM-IV is not due to binding to or hydrolysis of phospholipids, but due to its interaction with one or more clotting factors (26). CM-I and CM-II fail to show significant inhibition of the prothrombinase complex, even after complete phospholipid hydrolysis (26). CM-IV does not compete with the substrate prothrombin and therefore the CM-IV inhibition of prothrombinase is not due to its interaction with prothrombin or the active site of the complex. Thus CM-IV was found to be a novel inhibitor which interacts with factor Xa, factor Va, or the Xa–Va complex (26).

In this paper, we examine the interaction of CM-IV with protein factors of the prothrombinase complex using isothermal titration calorimetry. The results presented here indicate that CM-IV binds to factor Xa and interferes with the binding of the natural cofactor, factor Va. Thus, this paper demonstrates targeting of the strongly anticoagulant PLA_2 to factor Xa and confirms that the anticoagulant activity of this PLA_2 is caused by protein–protein interaction.

MATERIALS AND METHODS

Materials. N. nigricollis crawshawii venom, *Vipera russelli* venom, and soybean trypsin inhibitor (STI) were purchased from Sigma Chemical Co. PEG 8000 was purchased from Fisher Scientific. Benzamidine hydrochloride hydrate was purchased from Aldrich Chemical Company, Inc. The chromogenic substrates S-2238 and S-2222 were purchased from DiaPharma Group, Inc. (Franklin, OH).

Purification of phospholipase. The phospholipase CM-IV was purified from *N. nigricollis* venom by ion exchange chromatography on carboxymethyl Bio-Gel A as described earlier (22). The enzyme was homogeneous on both native and SDS–PAGE gels.

Purification of factor-X-activating proteinase from Vipera russelli venom. The factor-X-activating proteinase from Russell's viper venom was isolated according to the method of Kisiel *et al.* (27), except that Bio-Gel P-60 was substituted for Sephadex G-150. The coagulant fractions collected from the QAE-Sephadex A-50 chromatography step were concentrated by ammonium sulfate precipitation, reconstituted in 50 % glycerol, and stored at −20°C.

Purification of coagulation factors. Activated coagulation factor V was purified from bovine plasma according to the method of Esmon (28). To obtain activated factor V, the factor V solution was incubated with thrombin (2 units/ml) in 0.1 M NH_4Cl, 0.02 M Tris–HCl, 0.01 M $CaCl_2$, 0.001 M benzamidine, pH 7.5, for 30 min at 37°C (28). The sample was then applied to a column of QAE-Sephadex A-50 equilibrated in the activation buffer. Factor Va was eluted in a linear gradient from 0.1 to 0.4 M NH_4Cl. The protein was stored in 50% glycerol at −20°C. Bovine coagulation factor X and bovine prothrombin were purified according to the procedure of Hashimoto *et al.* (29). Factor X was converted to activated factor X by incubation with purified factor-X-activating proteinase from Russell's viper venom, and activated factor Xa was isolated by DEAE-Sephadex A-50 chromatography (30).

Isothermal titration calorimetry. All experiments were carried out using an Omega titration calorimeter (Microcal, Inc., Northhampton, MA). The experimental temperature was 25°C and the buffer was 20 mM Hepes, pH 7.4, 0.15 M NaCl, 2.0 mM $CaCl_2$. The reference cell of the calorimeter contained water plus 0.01% sodium azide. In a typical experiment, 12 or 15 aliquots (10 μl) of ligand were injected into 1.396 ml of protein solution rapidly mixing at 400 rpm with 4-min intervals between injections. Protein concentrations are given in figure legends. Numerical integration and data analysis were performed using software supplied by the manufacturer. The proteins used in the studies were dialyzed extensively against identical buffer, filtered to remove particulates, and degassed by vacuum.

Measurement of prothrombinase activity in the absence of phospholipid. Assays were performed in the presence of 20 mM Hepes, 0.15 M NaCl, 2 mM $CaCl_2$, 0.1% PEG, pH 7.4. Protein concentrations are given in figure legends. In the absence of the other prothrombinase components, the factor Xa concentration was 50 nM. In the presence of factor Va, the factor Xa concentration was reduced to 1 nM to give comparable rates of thrombin production as for factor Xa alone. Prothrombin was incubated with factor Va and inhibitor (CM-IV), if included, for 15 min prior to the addition of factor Xa. At various time points, aliquots were either measured immediately or the reaction stopped by addition of 1/10th volume of 20 mM Hepes, 0.15 M NaCl, 0.1% PEG, 50 mM EDTA, 1 μg/μl STI. STI inhibits the low amidase

activity of factor Xa on S-2238 but does not affect the activity of thrombin on the substrate (31). Spectroscopic measurements of dA/min at 405 nm were performed on a Shimadzu UV-160 recording spectrophotometer and initiated by addition of an aliquot to a cuvette containing 20 mM Hepes, 0.15 M NaCl, 0.1% PEG, 50 mM EDTA, 0.24 mM S-2238 in a final volume of 200 μl. Measured rates of change in absorbance over time were related to the concentration of thrombin using a standard curve showing the rate of S-2238 hydrolysis by known amounts of bovine thrombin in an identical assay.

Factor Xa amidolytic assay. Factor Xa (25 nM) in 0.05 M Tris buffer, 0.1 M NaCl, 5.0 mM $CaCl_2$, pH 7.5, was incubated with various concentrations of CM-IV from 0 to 8 μM for 10 min. The factor Xa amidolytic activity was measured by transfer of an aliquot of the mixture to a cuvette containing 0.05 M Tris buffer, 0.1 M NaCl, 5 mM $CaCl_2$, pH 7.5, and 250 μM S-2222. The hydrolysis of S-2222 was measured on a Shimadzu UV-160 recording spectrophotometer at 405 nm.

RESULTS AND DISCUSSION

Specificity of Inhibition of the Prothrombinase Complex

In our previous studies, we used a systematic dissection approach to determine the specificity of inhibition of different coagulation complexes by *N. nigricollis* PLA_2 enzymes (24). The prothrombinase complex is inhibited by only the strongly anticoagulant CM-IV, whereas the weakly anticoagulant enzymes, CM-I and CM-II, do not significantly inhibit this complex (24, 26). Subsequently, we determined that the basic PLA_2 from *N. nigricollis* venom, CM-IV, inhibits the prothrombinase complex by a novel nonenzymatic mechanism, even in the absence of phospholipids (26). The results also indicate that the inhibition of the prothrombinase complex by CM-IV is not due to either binding to or hydrolysis of phospholipids, but due to its interaction with the protein clotting factor(s) (26).

To establish whether the inhibition of the prothrombinase complex is specific to CM-IV or merely due to its basic nature, we examined the effect of some other basic PLA_2 enzymes from snake venoms and other basic proteins. They were caudoxin from *Bitis caudalis*, notexin from *Notechis scutatus scutatus*, pseudexin B from *Pseudechis porphyriacus* (all kindly provided by Dr. John Middlebrook, U.S. Army Medical Research Center, Fort Dietrich, Maryland), lysozyme, and cytochrome *c*. The isoelectric points and the inhibitory potencies of these proteins as compared with CM-IV are shown in Table I. Only CM-IV strongly inhibits the prothrombinase complex. Caudoxin shows about 50% of the potency of CM-IV. We compared the amino acid sequences of caudoxin (37), notexin (38), and pseudexin B (39) with the predicted anticoagulant site of venom PLA_2 enzymes (23). This region in caudoxin contains three of the four Lys residues of the predicted anticoagulant region, unlike the other basic PLA_2 enzymes tested. This probably explains the ability of caudoxin to inhibit the prothrombinase complex. There is no correlation between the isoelectric points and the inhibitory potencies of the proteins (Table I). Thus, the inhibition of the prothrombinase complex is a specific effect of CM-IV and is independent of the overall basicity of the protein. The specific location of the basic residues, and not the overall basicity of the PLA_2, determines the pharmacological effects of the enzyme (23). This idea was confirmed by site-directed mutagenesis (40). Inada *et al.* replaced Asp 59 and Ser 60 of pig pancreatic PLA_2 with Arg and Gly, respectively. The mutant enzyme showed 5- to 10-fold increased inhibition of the prothrombinase reaction, whereas the catalytic activity was unaffected (40). Thus, the basic residue at position 59 appears to have an essential role in binding of the PLA_2 to the prothrombinase complex. Recent studies by our group (32) as well as Mounier *et al.* (41) have shown that the synthetic peptides based on the predicted region exhibit anticoagulant effects.

TABLE I

Effect of Basic Venom PLA_2 Enzymes and Other Basic Proteins on the Activity of the Prothrombinase Complex[a]

Protein	p*I*	% Inhibition[b]
N. nigricollis CM-IV	10.1	78
B. caudalis caudoxin	10.2	44
N. scutatus scutatus notexin	9.2	0
P. porphyriacus pseudexin B	9.9	0
Lysozyme	10.8	0
Cytochrome *c*	10.6	0

[a] Data from Ref. (45).

[b] The prothrombinase complex was reconstituted in 0.05 M Tris–Cl, 2 mM $CaCl_2$, 2% polyethylene glycol, pH 7.5. The percentage of inhibition was compared with 5 μg of each basic protein added to 22 nM of factor Va, 23 nM of factor Xa, and 1.23 μM prothrombin.

Interaction of CM-IV with Factor Xa

Previous kinetic studies indicated that CM-IV binds to either factor Va or factor Xa, or both, to inhibit the prothrombinase complex (26). To identify the protein target of CM-IV, we utilized isothermal titration calorimetry, which detects macromolecular interactions by directly measuring changes in heat due to these interactions. At first we examined for the binding of factor Xa to factor Va as a positive control. As shown in Table II, we obtained a K_d value of 1.2×10^{-6} and a stoichiometry of 0.98. This is in agreement with the published K_d values, which range between 4.0×10^{-7} and 2.7×10^{-6} M (42, 43). Earlier kinetic studies (26) showed that CM-IV does not bind to prothrombin and thus it is a good negative control. As expected, no significant changes in heat could be measured by the addition of prothrombin, indicating that it did not interact with CM-IV (data not shown). We then titrated individual factors, factor Va or factor Xa, with CM-IV. CM-IV showed interaction with factor Xa (Fig. 1), but it did

TABLE II

Isothermal titration calorimetry data for the detection of macromolecular interactions

Protein	Ligand	K_d (M)	ΔH (kcal/mol)	*n*
Factor Va	Factor Xa	1.2×10^{-6}	18.9	0.98
Prothrombin	CM-IV	$>10^{-4}$	n.d.[a]	n.d.
Factor Va	CM-IV	$>10^{-4}$	n.d.	n.d.
Factor Xa	CM-IV	5.0×10^{-7}	34.4	1.3

Note. Protein concentration was typically 5 to 20 μM. Ligand concentration was typically 50 to 300 μM. *n*, stoichiometry of protein to ligand.

[a] n.d., not detectable.

not bind to Factor Va (data not shown). As shown in the binding isotherm of the titration of factor Xa with CM-IV, the binding was saturable. We obtained a K_d value for the interaction of 5.0×10^{-7} M and a stoichiometry of 1.3 (Table II). These experiments directly demonstrate that CM-IV binds to factor Xa but not factor Va or prothrombin. Human secreted PLA_2 ($hsPLA_2$) also interacts with factor Xa with a K_d of 2.3×10^{-7} M and a stoichiometry of 0.95 (41). Thus, CM-IV, similar to $hsPLA_2$ (41), targets factor Xa in the prothrombinase complex. The interaction of factor Xa with CM-IV does not affect its enzymatic activity on both chromogenic substrate S-2222 and macromolecular substrate prothrombin (data not shown).

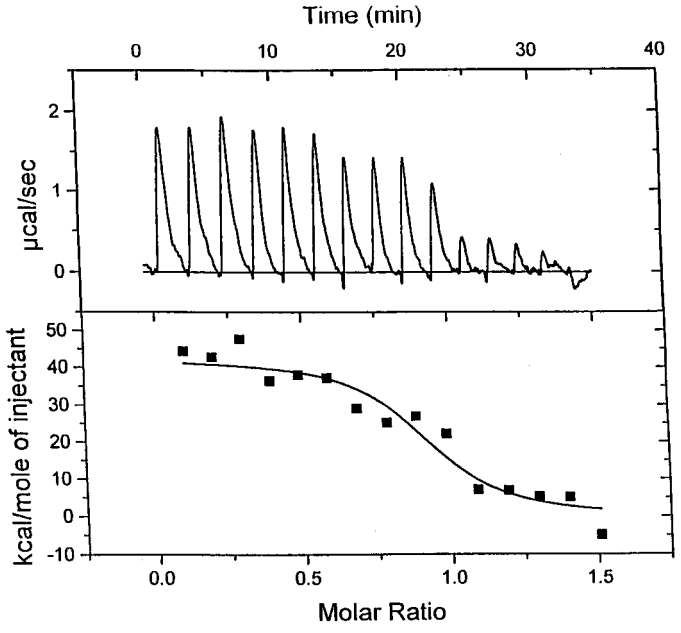

FIG. 1. Isothermal titration calorimetry trace showing the binding of CM-IV to factor Xa at 298 K. (Top) The heat signals for 15 injections of 10-μl aliquots of CM-IV (100 μM) in 20 mM Hepes, pH 7.4, 0.15 M NaCl, 2.0 mM $CaCl_2$. The reaction vessel contained 1.396 ml of 10 μM factor Va in the same buffer stirred rapidly at 400 rpm. (Bottom) Integrated heat of each injection after correction for the heat of dilution. The molar ratio is calculated as the ratio of ligand (CM-IV) to protein (factor Xa). This figure was produced using Microcal ORIGIN for ITC software provided by the manufacturer.

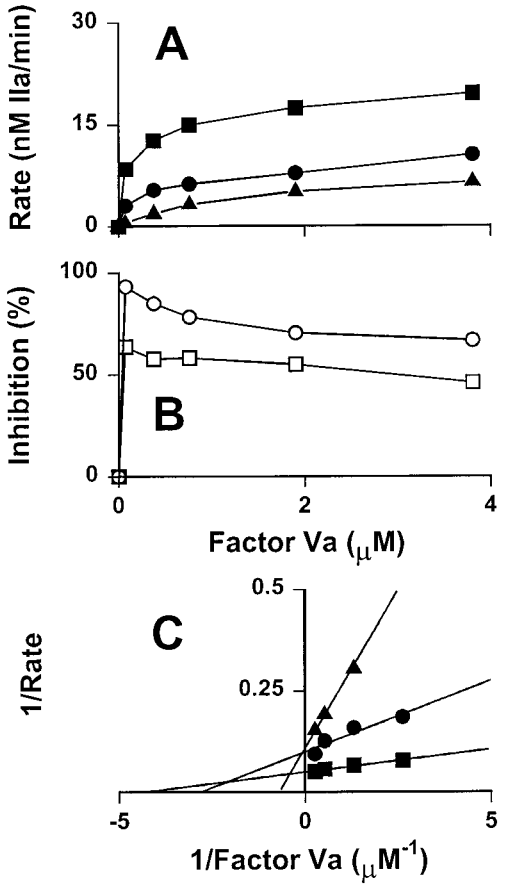

FIG. 2. (A) Effect of factor Va on thrombin formation at various concentrations of CM-IV. The data is representative of five separate experiments. The concentration of CM-IV was 0 μM (■), 0.72 μM (●), or 3.5 μM (▲). The concentrations of prothrombin and factor Xa were 1.0 μM and 1 nM, respectively. (B) Effect of factor Va on inhibition of thrombin formation by CM-IV. Inhibition was calculated by comparing the rate of thrombin formation in the presence of CM-IV to that in its absence at the same concentration of factor Va. The concentration of CM-IV was 0.72 μM (□) or 3.5 μM (○). (C) Effect of CM-IV on the binding interaction of factor Va with factor Xa. The lines are drawn according to values obtained by linear regression analysis of the data. The concentration of CM-IV was 0 μM (■), 0.72 μM (●), or 3.5 μM (▲).

Inhibition of Thrombin Formation by CM-IV in a Factor Va-Dependent Manner

In the presence of factor Va, CM-IV inhibits the prothrombinase reaction (Fig. 2A). CM-IV causes a concentration-dependent decrease in the rate of thrombin formation in the presence of factor Va (see also, Ref. (26)). Thus, CM-IV has no direct effect on the catalytic properties of factor Xa, but it inhibits the cofactor

activity of factor Va. Although there is no inhibition of thrombin formation in the absence of factor Va, at a constant concentration of CM-IV, the percentage of inhibition decreases as the factor Va concentration increases (Fig. 2B). This suggests that CM-IV and factor Va compete for binding to factor Xa. Therefore, we examined the effect of CM-IV on the affinity between factor Xa and factor Va. We calculated the K_d value from the *x*-intercept of the plot of reciprocal rate versus reciprocal cofactor concentration (Fig. 2C) (44). In the absence of CM-IV, the K_d value for interaction between factor Va and factor Xa was found to be 8.3×10^{-8} M. CM-IV increases the K_d for factor Xa–factor Va interaction to 1.3×10^{-7} and 8.1×10^{-7} M at 0.72 and 3.5 μM concentrations, respectively (Fig. 2C). Thus a 10-fold decrease in the affinity for factor Xa–factor Va interaction was observed in the presence of CM-IV. Since CM-IV binds to factor Xa (Fig. 1) without any significant effect on prothrombinase reaction, CM-IV–factor Xa interaction results in inactive complexes. This will invariably reduce the apparent V_{max}. As shown Fig. 2C, a two- to threefold reduction in the apparent V_{max} was observed in the presence of CM-IV. This suggests that CM-IV binds to factor Xa and either prevents factor Va binding or disrupts an existing Xa–Va binary complex. Since increasing amounts of factor Va (Fig. 2B) or factor Xa (45) neutralizes the inhibition of the prothrombinase complex by CM-IV, we suggest that CM-IV competes with factor Va for binding to factor Xa and reduces the effectiveness of the cofactor.

Novel Mechanism of Inhibition of the Prothrombinase Complex by CM-IV

A schematic model (Fig. 3A) summarizes the mechanism of inhibition of the prothrombinase complex by the strongly anticoagulant PLA_2 enzyme. During normal coagulation, factor Xa binds to factor Va to form the prothrombinase complex. The prothrombinase inhibitor, CM-IV, binds to factor Xa at a domain that is involved in the interaction between factor Xa and factor Va. Because of the competition, CM-IV retards the interaction of factor Xa and factor Va. By binding to factor Xa in the complex, CM-IV disrupts the binding of factor Va and thus the prothrombinase complex itself. Unlike factor Va (K_d value 8.8×10^{-6} M; Ref. 43), CM-IV does not interact with prothrombin (K_d value $> 10^{-4}$ M, Figs. 3B and 3C). This could explain the inability of CM-IV to enhance the prothrombinase activity of factor Xa. Thus, factor Xa is the target protein in the coagulation cascade to which only the strongly anticoagulant PLA_2 binds with a high specificity. Therefore protein–protein interactions, rather than protein–phospholipid interactions, cause the anticoagulant effect of CM-IV. Since CM-IV and factor Va compete to bind to the same site, we expect that these interaction sites on CM-IV and factor Va are similar, if not identical in their properties. Thus, further studies of the domain of CM-IV that interacts with factor Xa should provide insight into a specific domain in factor Va that interacts with factor Xa. The use of the anticoagulant region of CM-IV to gain information about the factor Xa–factor Va interaction offers the advantages that CM-IV is much smaller than factor Va, contains only one rather than two chains, and does not interact with the prothrombin substrate as does factor Va. These advantages coupled with the known crystal structures of related PLA_2 molecules should help in understanding the structure–function relationship of the interaction.

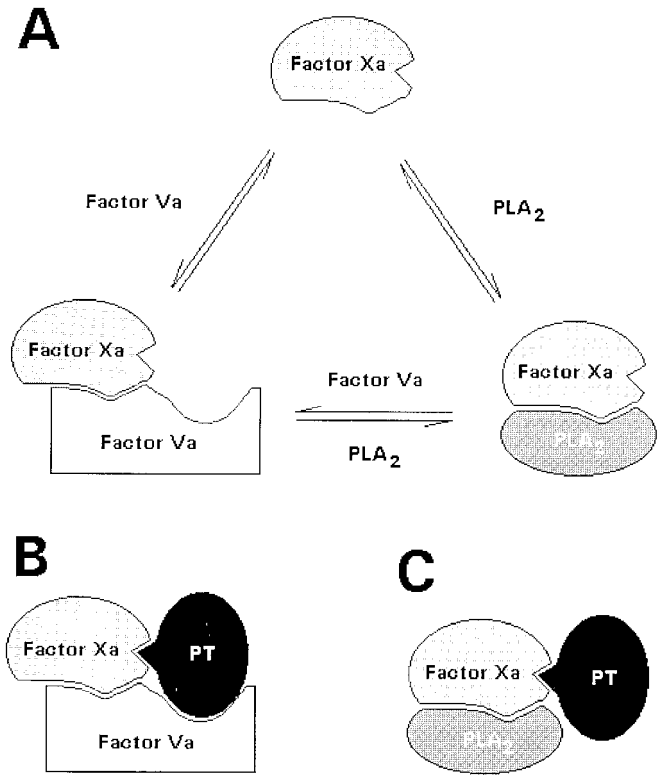

FIG. 3. A model showing the mechanism of inhibition of the prothrombinase complex by the strongly anticoagulant PLA_2, CM-IV. CM-IV binds to factor Xa with a K_d value 5×10^{-7} M. It can replace factor Va in the normal prothrombinase complex (bottom left), resulting in an inactive CM-IV-factor Xa complex (bottom right). Interaction of the substrate prothrombin (PT) with normal prothrombinase complex (B) or with factor Xa–PLA_2 complex (C) is shown. Factor Xa–PLA_2 complex binds to prothrombin with the same affinity as factor Xa and PLA_2 does not interact with prothrombin. This explains the inability of CM-IV to interfere in the prothrombinase activity of factor Xa.

The inhibition of prothrombinase by $hsPLA_2$ was shown to be independent of the presence of phospholipids but required the presence of factor Va (46). The anticoagulant effect of the enzyme was not observed on

factor V-deficient plasma, but only on normal and factor X-deficient plasma. Thus, the authors initially concluded that the inhibition of the prothrombinase complex by hsPLA$_2$ involves the inhibition of factor Va. However, their recent studies indicate that hsPLA$_2$ binds to factor Xa (41). These results are consistent with the model of inhibition presented above.

Several anticoagulant proteins have been isolated from venom sources and the mechanisms of their anticoagulant effects investigated (47–49). Some of these proteins bind to Gla domain of factors X and IX and interfere in their binding to phospholipid surfaces (48, 49). Bothrojaracin, a protein closely related to Ca^{2+}-dependent lectins, is a thrombin-specific inhibitor (50). It binds to exosite 1 and perhaps to exosite 2, but not to the active site of thrombin (51). Other anticoagulant proteins are proteinase inhibitors that inhibit an active proteinase in the coagulation cascade. For example, antistasins and hirudins from leeches inhibit factor Xa (52) and thrombin (53), respectively. The hookworm *Ancylostoma caninum* has very specific anticoagulants that bind to factor Xa and the factor VIIa/tissue factor complex. These inhibitors belong to the category of serine proteinase inhibitors (54). Unlike all these anticoagulants, CM-IV binds to factor Xa and competes with the natural cofactor, factor Va, for the same site on factor Xa. This anticoagulant inhibits blood coagulation by interfering in the formation of a coagulation complex. Because of its novelty in inhibiting the strategic prothrombinase complex, further studies of this inhibitor should contribute to our understanding of the interactions involved in the formation of the normal prothrombinase complex.

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