The Basic Phospholipase A_2 from *Naja nigricollis* Venom Inhibits the Prothrombinase Complex by a Novel Nonenzymatic Mechanism†

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ABSTRACT: The three phospholipase A_2 isoenzymes from *Naja nigricollis* venom inhibit blood coagulation with different potencies. The strongly anticoagulant basic isoenzyme CM-IV inhibits the prothrombinase complex, whereas the weakly anticoagulant isoenzymes CM-I and CM-II do not. To determine the role of enzymatic activity of the phospholipases in the inhibition of prothrombinase, we varied the time of incubation of each of these isoenzymes with the prothrombinase complex. The inhibition by CM-IV did not increase with time of incubation. CM-I and CM-II failed to inhibit the complex, even with complete hydrolysis of phospholipids in the assay mixture. After alkylation of its active-site histidine, CM-IV lost 97% of its enzymatic activity but retained 60% of its inhibitory potency on prothrombinase. CM-IV also inhibited prothrombinase activity in the absence of phospholipids, whereas CM-I and CM-II did not. The inhibition of the prothrombinase complex by CM-IV is thus not due to its binding to or hydrolysis of phospholipids. The kinetics of CM-IV inhibition of the prothrombinase complex in both the presence and absence of phospholipids was noncompetitive. This inhibition can be explained by binding of CM-IV to either factor Va or Xa, or both, to inhibit the complex. CM-IV differs from previously described nonenzymatic anticoagulants that are proteinase inhibitors or that inhibit the coagulation complexes by interfering with the binding of clotting factors to phospholipids. We conclude that the basic enzyme, CM-IV, inhibits the prothrombinase complex by a novel mechanism independent of enzymatic activity.

Phospholipases A_2 are ubiquitous enzymes found in abundance in the digestive fluids of most organisms and in animal venoms. In addition to their role in digestion, snake venom PLA_2[1] enzymes can cause a wide variety of pharmacological effects, including an anticoagulant effect (Seegers & Ouyang, 1979). PLA_2 enzymes have been classified according to their anticoagulant potency (Verheij et al., 1980). By comparing sequences of strong, weak, and nonanticoagulant PLA_2 enzymes, we identified a region between residues 54 and 77 in the strongly anticoagulant enzymes that differs from the corresponding region in the weak and nonanticoagulant enzymes (Kini & Evans, 1987). This site has basic residues located in distinct positions forming a cationic amphiphilic surface on the PLA_2 molecule and also forms a part of the interface recognition site. The specific locations of the basic residues in this region appear to be important for the anticoagulant effect, rather than the overall basicity of the protein.

Phospholipids are essential components of coagulation complexes (Jackson & Nemerson, 1980), and it is logical to assume that hydrolysis of the phospholipids by PLA_2 inhibits the coagulation process (Boffa et al., 1980). Previous studies, however, gave contradictory results and led to controversy concerning the role of enzymatic activity in the anticoagulant effect [for details, see Kini and Evans (1988)]. Since most of the earlier studies were performed with whole plasma, the results reflected the overall effect of PLA_2 on several clotting complexes. But all the coagulation complexes are unlikely to have identical phospholipid requirements, and PLA_2 enzymes might affect the complexes differently, depending on this lipid requirement.

Three anticoagulant PLA_2 enzymes with different anticoagulant potencies were purified from the venom of *Naja nigricollis* (Evans et al., 1980; Kini & Evans, 1987). CM-I and CM-II are weak anticoagulants, whereas CM-IV is a strong anticoagulant. Recently, we identified the coagulation complexes in the extrinsic coagulation cascade that are inhibited by these enzymes (Stefansson et al., 1989). The strongly anticoagulant CM-IV inhibits two successive steps, the extrinsic tenase and prothrombinase complexes. In contrast, the weakly anticoagulant isoenzymes inhibit only the tenase complex and not prothrombinase, although the activities of both the complexes are dependent on phospholipid surfaces (Stefansson et al., 1989). Thus we proposed a functional difference between the weakly and strongly anticoagulant PLA_2 enzymes in their ability to inhibit the coagulation complexes. The lack of inhibition of the prothrombinase complex by CM-I and CM-II raised the question of whether hydrolysis of the phospholipids plays any role at all in the inhibition of this complex. In this paper, we examine the role of PLA_2 enzymatic activity in the inhibition of the prothrombinase complex. These studies show that the inhibition of the prothrombinase complex by CM-IV is independent of enzymatic activity.

Materials and Methods

Venoms and Reagents. Naja nigricollis crawshawii and *Vipera russelli russelli* venoms, BSA (fatty acid free), *p*-BPB,

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[1] Abbreviations: PLA_2, phospholipase A_2; PC, phosphatidylcholine; PS, phosphatidylserine; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; *p*-BPB, *p*-bromophenacyl bromide; HPTLC, high-performance thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; RVV, Russell's viper venom; Gla, γ-carboxyglutamic acid.

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PMSF, PC from hen's egg, and PS from bovine brain, were purchased from Sigma Chemical Co. S-2238 and STI were purchased from Helena Laboratories and Millipore Corp., respectively.

Purification of Phospholipases. The phospholipases CM-I, CM-II, and CM-IV were purified from *N. nigricollis* venom by ion-exchange chromatography on carboxymethyl Bio-Gel A as described earlier (Evans et al., 1980). The proteins were homogeneous on both native and SDS–PAGE gels.

Phospholipase Assay. PLA_2 was assayed as described by Franson et al. (1974) except that the assay buffer used was 0.05 M Tris and 0.1 M NaCl, pH 7.5. Reaction mixtures in a total volume of 500 μL contained 4.5×10^8 cells of autoclaved *Escherichia coli* corresponding to 10000 cpm and 18 μM phospholipid. The amount of PLA_2 used in the assay was adjusted to produce 20–30% hydrolysis per 15-min incubation at 37 °C. The activities of the native and the His-modified enzymes were determined in two separate experiments performed in triplicate.

Modification of CM-IV with p-BPB. CM-IV, 250 μg, in 0.05 M Tris buffer and 0.1 M NaCl, pH 7.5, was incubated at 37 °C with 100 μM of *p*-BPB dissolved in dimethyl sulfoxide in a total volume of 2 mL. After 120 min the reaction was terminated with 2 mL of 10 mM sodium acetate buffer and 0.15 M NaCl, pH 4.5. Unreacted *p*-BPB was separated from CM-IV by dialysis against 0.05 M Tris buffer and 0.1 M NaCl, pH 7.5.

Purification of Coagulation Factors. Coagulation factor V was purified from bovine plasma according to the method of Nesheim et al. (1981). The purified factor V was stored at −20 °C in 40% glycerol, 1 mM benzamidine, and 1 mM PMSF. Coagulation factor X and prothrombin were purified according to the procedure of Hashimoto et al. (1985) and stored in the assay buffer at 4 °C. The purified coagulation factors did not contain any phospholipid contaminants as determined by phosphate analysis according to the method of McClare (1971).

Phospholipid Vesicle Preparation. Phospholipid vesicles were prepared essentially as described by de Kruijff et al. (1975). Chloroform solutions of equimolar PS and PC were mixed and dried under nitrogen. Lipid vesicles were prepared in 0.05 M Tris buffer and 0.1 M NaCl, pH 7.5, by sonication for 10 min in a Branson sonicator. The vesicles were used within 5 h after preparation. Phospholipid concentrations were determined by phosphate analysis according to the method of McClare (1971).

Analysis of Phospholipids. Phospholipids and their lysoproducts were separated on HPTLC plates by using a solvent system containing a mixture of chloroform, methanol, glacial acetic acid, and water in the ratio 100:45:20:5 (vol/vol) (Rao & Spence, 1976).

Reconstitution of the Prothrombinase Complex. The prothrombinase complex was reconstituted essentially as described earlier (Stefansson et al., 1989). Factors X and V (final concentrations 2.5 and 2 nM, respectively), 26 μM PS/PC vesicles, 10 mM $CaCl_2$, and 1 μM BSA were incubated in 0.05 M Tris buffer and 0.1 M NaCl, pH 7.5, with a total incubation volume of 2.4 mL. The prothrombinase complex was activated by the addition of 10 μL of 0.01 mg/mL *V. russelli* venom and incubated for 10 min. The *V. russelli* venom did not convert prothrombin to thrombin in the absence of factors V and X nor did it hydrolyze the thrombin substrate. 200-μL aliquots of the prothrombinase complex were then incubated with the PLA_2 enzymes for 5 min, unless otherwise noted, prior to the addition of prothrombin. Prothrombin was added to the mixture to give a final concentration of 60 nM. After a 10-min incubation 40 μL of 1 mg/mL STI was added to inhibit further conversion of prothrombin by factor Xa. STI inhibits the low amidase activity of factor Xa on S-2238 but does not affect the activity of thrombin on the substrate (Rosing et al., 1980). The activity of the complex in the absence of phospholipids was measured after a 30-min incubation with prothrombin. The thrombin activity was measured by transfer of an aliquot of the mixture to a cuvette containing 0.05 M Tris buffer, 0.1 M NaCl, pH 7.5, and 1 mM S-2238. The hydrolysis of S-2238 was measured on a Shimadzu UV-160 recording spectrophotometer at 405 nm.

Results and Discussion

PLA_2 enzymes hydrolyze phospholipids at the *sn*-2 position to yield fatty acids and lysophospholipids. Because of their detergent-like properties, these products may disrupt membranes and membrane-dependent processes [Jain & Zakim, 1987; Senisterra et al. (1988) and references therein]. Since efficient activation of coagulation factors is dependent on phospholipid surfaces, PLA_2 enzymes might cause the anticoagulant effect by phospholipid hydrolysis. Many previous observations support the role of enzymatic activity in the anticoagulant effect of PLA_2 enzymes: (1) nonhydrolyzable ether phospholipid analogues neutralize the anticoagulant effect of the strongly anticoagulant PLA_2 from *Vipera berus* venom (Verheij et al., 1980); (2) alkylation of His-48 of the anticoagulant PLA_2 from *V. berus* results in the complete loss of the anticoagulant activity but does not affect the ability of the enzyme to bind to phospholipids (Verheij et al., 1980); (3) the anticoagulant activity of the PLA_2 enzymes from *N. nigricollis* increases with increasing incubation time as measured by the prothrombin time clotting assay (Kini & Evans, 1988); (4) preincubation with EDTA reduces the anticoagulant effect of the PLA_2 enzymes from *N. nigricollis* as measured by the prothrombin time clotting assay (Kini & Evans, 1988). However, the contention that the enzymatic activity is not essential for the anticoagulant effect is supported by the following observations: (1) anticoagulant potencies of the PLA_2 enzymes do not parallel their catalytic activities (Ouyang et al., 1981); (2) there is no correlation between plasma phospholipid hydrolysis and anticoagulant activity (Condrea et al., 1981, 1982); (3) a polyclonal antibody against the anticoagulant PLA_2 from *V. russelli* venom inhibits its anticoagulant effect without affecting the enzymatic activity (Kasturi & Gowda, 1990); (4) the anticoagulant effect of anticoagulant PLA_2 enzymes is stronger than can be achieved by the addition of lysoproducts to plasma (Ouyang et al., 1981).

An explanation offered to resolve the above controversy is that anticoagulant enzymes preferentially bind to and hydrolyze procoagulant phospholipids such as PS (Boffa et al., 1982). The proponents of a nonenzymatic mechanism argue that strong binding to PS by anticoagulant PLA_2 results in a binding competition between clotting factors and the PLA_2 for the phospholipid surface (Boffa et al., 1980; Prigent-Dachary et al., 1980). However, the basic PLA_2 enzymes, β-bungarotoxin and crotoxin, also bind preferentially to negatively charged phospholipid vesicles (Radvanyi et al., 1987), but these toxins do not have any anticoagulant effect (Verheij et al., 1980). The observed preferential binding to negative phospholipids (Prigent-Dachary et al., 1980) could simply be due to the basic nature of the anticoagulant enzymes and not to a specific affinity.

We recently proposed a model to explain the pharmacological effects of venom PLA_2 enzymes (Kini & Evans, 1989).

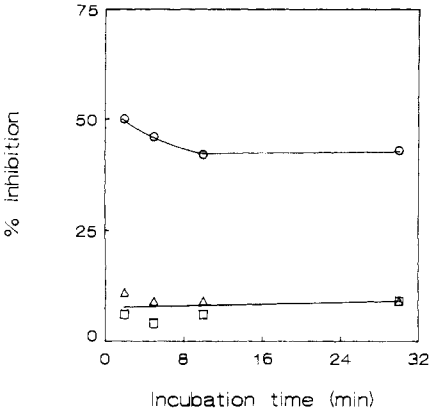

FIGURE 1: Effect of incubation of the prothrombinase complex with the PLA_2 isoenzymes on prothrombinase activity. The isoenzymes were incubated with the prothrombinase complex reconstituted as described under Materials and Methods. At the indicated times, the reaction was terminated by the addition of STI and thrombin formation was measured. Percent inhibition was calculated by comparing the thrombin generated by the samples to that of the controls of the prothrombinase complex with no added PLA_2. The concentration of CM-IV was adjusted to give about 50% inhibition initially. Each point represents the average of three measurements. (□) CM-I (135 μg/mL); (△) CM-II (90 μg/mL); (○) CM-IV (12 μg/mL).

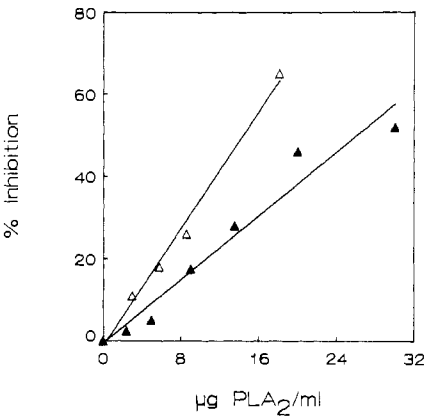

FIGURE 2: Effect of native and His-modified CM-IV on the prothrombinase activity. The percent inhibition was calculated as described in the legend of Figure 1. Each point represents the average of two measurements: (△) native CM-IV; (▲) *p*-BPB-modified CM-IV.

The essential feature of this model is the targeting of venom PLA_2 enzymes to a specific organ or tissue as determined by their high affinity for a target protein rather than for phospholipids. The model explains the contradictory observations made concerning several pharmacological activities of PLA_2 enzymes, including the controversy of the anticoagulant effect. According to the model, a strongly anticoagulant PLA_2 specifically binds to one or more clotting factors and inhibits their interaction. After binding, the PLA_2 may also hydrolyze the phospholipids in the vicinity of the target protein and cause or augment the anticoagulant effect (Kini & Evans, 1989). This model of specific binding of PLA_2 enzymes to target proteins is supported by demonstration of target proteins for several neurotoxic PLA_2 enzymes (Lambeau et al., 1989, Rehm & Betz, 1982, Tzeng et al., 1986, 1989). However, a specific target protein for any anticoagulant PLA_2 has not yet been identified.

As a first step in understanding the mechanism of this anticoagulant effect, we determined the activation steps in the extrinsic cascade that are inhibited by the PLA_2 enzymes from *N. nigricollis* venom (Stefansson et al., 1989). The extrinsic tenase is inhibited by all three isoenzymes. However, only CM-IV strongly inhibits the prothrombinase activity while CM-I and CM-II do not. The inhibition of prothrombinase by CM-IV is not due to the inhibition of the amidolytic activity of thrombin on S-2238 but is due to the inhibition of the activity of the prothrombinase complex (Stefansson et al., 1989).

Since efficient activity of the prothrombinase complex is dependent on a phospholipid surface (Nesheim et al., 1979), we examined what effect phospholipid hydrolysis by the PLA_2 enzymes has on the activity of the prothrombinase complex. Figure 1 shows inhibition of the complex by CM-I, CM-II, and CM-IV with increasing incubation time. At all time intervals CM-IV showed greater inhibition than CM-I and CM-II, consistent with our earlier observations (Stefansson et al., 1989). No increase in the inhibition by any of the PLA_2 enzymes was observed with incubation up to 30 min. Although CM-I and CM-II have higher catalytic activity than CM-IV (unpublished observations), they did not show more than 8–10% inhibition with either increasing incubation time (Figure 1) or increasing protein concentration (Stefansson et al., 1989). If enzymatic activity caused the inhibition, the inhibition should have increased with continuing incubation, as more phospholipids were hydrolyzed. However, after a 30-min incubation of the isoenzymes, no unhydrolyzed PC or PS was detected in the assay mixtures by HPTLC (data not shown). Total hydrolysis of phospholipids by CM-I and CM-II did not cause more than 10% inhibition of the activity of the complex. In addition, our recent results indicate that the fully active prothrombinase complex can be reconstituted on equimolar mixtures of lyso PC and lyso PS, with or without fatty acids. The level of thrombin generated by the prothrombinase complex on lyso PS/lyso PC vesicles was 100 ± 7% (n = 5) of that generated by the complex on PS/PC vesicles (unpublished observations). Indeed, the lipid requirement of prothrombinase is quite flexible as evidenced by the reconstitution of the complex on a variety of surfaces such as taurocholate vesicles (Bajwa & Hanahan, 1976) and PS/PC membranes containing 15% stearylamine (Rosing et al., 1988) and in solution with no phospholipids at all (Rosing et. al., 1980; Nesheim et al., 1979). All the above results considered together make it unlikely that inhibition of the prothrombinase by CM-IV is due to enzymatic hydrolysis of the phospholipid surface.

To verify the nonenzymatic mechanism of inhibition of the prothrombinase complex, we modified CM-IV with *p*-BPB as described under Materials and Methods. Under these conditions, *p*-BPB alkylates only His-48 in the active site and not other amino acid residues of the basic PLA_2 from *N. nigricollis* venom (Yang & King, 1980). After the modification CM-IV retained only 3% of its catalytic activity (data not shown). The effect of the His-alkylated enzyme on the activity of the prothrombinase complex is shown in Figure 2. The modified enzyme retains about 60% of the inhibitory activity of the native enzyme. Since the percent inhibition was retained over a range of protein concentrations, the 3% of enzymatic activity remaining cannot explain the retention of 60% of the inhibitory effect on the prothrombinase complex. Furthermore, since the percent inhibition was calculated by comparison with controls that also contained RVV, the small amounts of contaminating PLA_2 from the RVV cannot account for the observed inhibition. The loss of 40% of the activity could be explained by the likely conformational changes of the enzyme caused by the modification. The active-site histidine is close enough to the predicted anticoagulant region (Kini & Evans, 1987) for its modification to affect the conformation of that region. Other researchers have shown that after His-alkylation, the

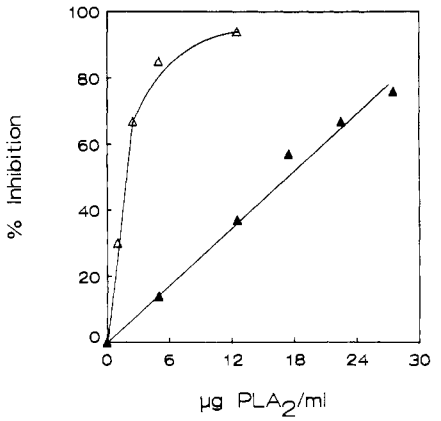

FIGURE 3: Effect of CM-IV on the prothrombinase complex reconstituted with and without phospholipids. The percent inhibition was calculated as described in the legend of Figure 1. Each point represents the average of two measurements: (▲) with 26 μM PS/PC; (△) no added phospholipids.

basic PLA_2 from *N. nigricollis* venom lost its anticoagulant effect (Condrea et al., 1983; Chwetzoff et al., 1989). However, in these cases the anticoagulant effect was determined by recalcification time assays, which reflect the effects of the PLA_2 on the activities of all the clotting complexes of the intrinsic pathway. The contribution of phospholipid hydrolysis in the inhibition of other clotting complexes cannot be ruled out (discussed later).

To determine whether CM-IV interaction with phospholipids is essential for the inhibitory effect, we reconstituted the prothrombinase complex without phospholipids. This complex showed about 800-fold lower activity than the complex formed on PS/PC vesicles, in agreement with earlier observations (Rosing et al., 1980; Nesheim et al., 1979). As shown in Figure 3, CM-IV is a more potent inhibitor of prothrombinase activity in the absence of phospholipids. Inhibition of the prothrombinase complex by CM-IV is dependent on the phospholipid concentration, and the inhibition decreases with increasing phospholipid concentrations (data not shown). This is consistent with the results of other workers, who found that the addition of phospholipids to plasma inhibits the anticoagulant effects of several PLA_2 enzymes (Ouyang et al., 1981). Without phospholipids CM-IV has an IC_{50} value of 1.7 μg/mL, compared to 14 μg/mL with phospholipids (Figure 3). The higher IC_{50} in the presence of phospholipids is probably due to binding of CM-IV to the phospholipid vesicles, since phospholipids are the substrate of the enzyme. Such binding would reduce the effective inhibitor concentration. The higher IC_{50} value could also reflect the increased affinity between Xa and Va in the presence of phospholipids (Lindhout et al., 1982). CM-I and CM-II, however, even at 50 μg/assay, did not inhibit the prothrombinase complex in the absence of phospholipids (data not shown).

The inhibition of the prothrombinase by CM-IV cannot be correlated with either phospholipid binding or penetrating ability, as suggested earlier (Boffa et al., 1980). It appears likely that CM-IV interacts directly with one or more unidentified clotting factors, supporting the target hypothesis (Kini & Evans, 1987). CM-I and CM-II, on the other hand, do not inhibit the prothrombinase complex because (1) hydrolysis of the phospholipids does not inhibit the complex and (2) they do not interact with the clotting factors.

To characterize the type of inhibition by CM-IV, we measured the rate of thrombin formation with two concentrations of CM-IV in the presence or absence of phospholipids. In the absence of phospholipids, using Va, Xa, and Ca^{2+} at 32 nM, 20 nM, and 4 mM, respectively, we obtained a K_m value of 3.0 μM for the complex, which is in good agreement with previously published values (Higgins et al., 1985). CM-IV exhibited noncompetitive inhibition with a K_i value of 0.25 μM. This implies that CM-IV does not inhibit the complex by binding to the active site of the prothrombinase or to the substrate prothrombin but rather binds to a distal site, possibly interfering in the interaction of Xa and Va. In the presence of 23 μM PS/PC, with 3.2 nM Va, 2 nM Xa, and 10 mM Ca^{2+}, a K_m value of 0.13 μM was obtained for the complex, which corresponds to those obtained by Rosing et al. (1980). The K_i value for the inhibition is 0.25 μM without phospholipids as compared to 26 μM when they are present. Although CM-IV is a less potent inhibitor of the complex when phospholipids are present, it still exhibits noncompetitive inhibition. The 100-fold increase in K_i can be explained by the binding of CM-IV to the phospholipids or the tighter binding between Xa and Va, as mentioned above.

Although CM-IV inhibits the prothrombinase complex by a nonenzymatic mechanism, other clotting complexes can be inhibited by PLA_2 enzymes due to phospholipid hydrolysis. Earlier, by studying the effects of these PLA_2 enzymes on plasma clotting by the prothrombin time clotting assay, which is initiated by the tissue factor–factor VIIa activation of factor X (extrinsic tenase), we showed that the inhibition had contributions from both enzymatic and nonenzymatic mechanisms (Kini & Evans, 1988). The prothrombin clotting time determines the combined effect on the extrinsic tenase reaction, the prothrombinase reaction, and the conversion of fibrinogen to fibrin. We found that none of the isoenzymes inhibit the formation of the fibrin clot by thrombin (Stefansson et al., 1989). The results presented here indicate that the prothrombinase complex is inhibited by a nonenzymatic mechanism. Therefore we expect that the hydrolysis of phospholipids contributes to the inhibition of the extrinsic tenase reaction. Unlike factor Va of the prothrombinase complex, tissue factor is an intrinsic membrane protein that requires an intact phospholipid surface for its activity. The tenase complex is inactive when the tissue factor is reconstituted with lyso PC (Pitlick & Nemerson, 1970). Thus inhibition by enzymatic cleavage of the intact phospholipid is consistent with the lipid requirements of the extrinsic tenase complex.

The mechanism of CM-IV inhibition of prothrombinase differs from that of other nonenzymatic anticoagulant proteins, such as the family of Ca^{2+}-dependent phospholipid-binding proteins that includes lipocortins, calelectrins, and endonexin (Funakoshi et al., 1987; Chap et al., 1988). These proteins bind to anionic phospholipids in the presence of Ca^{2+} and inhibit the activity of the prothrombinase complex by competing for the phospholipid surface. They do not inhibit the prothrombinase in the absence of phospholipids (Chap et al., 1988). Similarly, the anticoagulant properties of peptides containing the Gla domain of factors X and IX have been attributed to their phospholipid-binding properties (Nawroth et al., 1986). Nonenzymatic inhibitor proteins isolated from the venoms of *Agkistrodon acutus* and *Trimeresurus flavoviridis* bind to the Gla domain of factor Xa and interfere in its binding to phospholipids (Teng & Seegers, 1981; Atoda & Morita, 1989). CM-IV, however, inhibits the prothrombinase complex even in the absence of phospholipids and hence does not interfere with the interactions between clotting factors and phospholipids. Finally, inhibitors such as extrinsic pathway inhibitor (Warn-Cramer et al., 1988), antithrombin III, and α_2-macroglobulin inhibit coagulation by inhibiting the proteinases of the clotting cascade [for review, see Bikfalvi and

Beress (1987)]. Unlike proteinase inhibitors, CM-IV noncompetitively inhibits the prothrombinase complex. We therefore conclude that the inhibition by CM-IV is due to a novel nonenzymatic mechanism.

Registry No. PLA_2, 9001-84-7; prothrombinase, 72162-96-0.

REFERENCES

Atoda, H., & Morita, T. (1989) *J. Biochem. (Tokyo) 106*, 808–813.

Bajwa, S. S., & Hanahan, D. J. (1976) *Biochim. Biophys. Acta 444*, 118–130.

Bikfalvi, A., & Beress, L. (1987) *Comp. Biochem. Physiol. 87B*, 435–441.

Boffa, M. C., Rothen, C., Verheij, H. M., Verger, R., & de Haas, G. H. (1980) in *Natural Toxins* (Eaker, D., & Wadstrom, R., Eds.) pp 131–138, Pergamon Press, Oxford.

Boffa, M. C., Dachary, J. P., Verheij, H. M., Rothen, C., Dufourcq, J., Verger, R., & de Haas, G. H. (1982) *Toxicon 20*, 305.

Chap, H., Comfurius, P., Bevers, E. M., Fauvel, J., Vicendo, P., Douste-Blazy, L., & Zwaal, R. F. A. (1988) *Biochem. Biophys. Res. Commun. 150*, 972–978.

Chwetzoff, S., Couderc, J., Frachon, P., & Menez, A. (1989) *FEBS Lett. 248*, 1–4.

Condrea, E., Yang, C. C., & Rosenberg, P. (1981) *Thromb. Haemostasis 45*, 82–85.

Condrea, E., Yang, C. C., & Rosenberg, P. (1982) *Thromb. Haemostasis 47*, 298.

Condrea, E., Rapuano, B. E., Soons, K. R., Yang, C. C., & Rosenberg, P. (1983) *Life Sci. 32*, 1455–1461.

de Kruijff, B., Cullis, P. R., & Radda, G. K. (1975) *Biochim. Biophys. Acta 406*, 6–20.

Evans, H. J., Franson, R., Qureshi, G. P., & Moo-Penn, W. F. (1980) *J. Biol. Chem. 255*, 3793–3797.

Franson, R., Patriarca, P., & Elsbach, P. (1974) *J. Lipid Res. 15*, 380–388.

Funakoshi, T., Hendrickson, L. E., McMullen, B. A., & Fujikawa, K. (1987) *Biochemistry 26*, 8087–8092.

Hashimoto, N., Morita, T., & Iwanaga, S. (1985) *J. Biochem. (Tokyo) 97*, 1347–1355.

Higgins, D. L., Callahan, P. J., Prendergast, F. G., Nesheim, M. E., & Mann, K. G. (1985) *J. Biol. Chem. 260*, 3604–3612.

Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 49*, 765–811.

Jain, M. K., & Zakim, D. (1987) *Biochim. Biophys. Acta 906*, 33–68.

Kasturi, S., & Gowda, T. V. (1990) *Toxicon 28*, 91–99.

Kini, R. M., & Evans, H. J. (1987) *J. Biol. Chem. 262*, 14402–14407.

Kini, R. M., & Evans, H. J. (1988) *Thromb. Haemostasis 60*, 170–173.

Kini, R. M., & Evans, H. J. (1989) *Toxicon 27*, 613–635.

Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., & Lazdunski, M. (1989) *J. Biol. Chem. 264*, 11503–11510.

Lindhout, T., Govers-Riemslag, J. W. P., van de Wart, P., Hemker, H. C., & Rosing, J. (1982) *Biochemistry 21*, 5494–5502.

McClare, C. W. F. (1971) *Anal. Biochem. 39*, 527–539.

Nawroth, P. P., Kisiel, W., & Stern, D. M. (1986) *Thromb. Res. 44*, 625–637.

Nesheim, M. E., Taswell, J. B., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 10952–10962.

Nesheim, M. E., Katzman, J. A., Tracy, P. B., & Mann, K. G. (1981) *Methods Enzymol. 80*, 249–274.

Ouyang, C., Jy, W., Zan, Y. P., & Teng, C. M. (1981) *Toxicon 19*, 113–120.

Pitlick, F. A., & Nemerson, Y. (1970) *Biochemistry 9*, 5105–5113.

Prigent-Dachary, J., Boffa, M. C., Boisseau, M. R., & Dufourcq, J. (1980) *J. Biol. Chem. 255*, 7734–7739.

Radvanyi, F., Saliou, B., Bon, C., & Strong, P. N. (1987) *J. Biol. Chem. 262*, 8966–8977.

Rao, B. G., & Spence, M. W. (1976) *J. Lipid Res. 17*, 506–515.

Rehm, H., & Betz, H. (1982) *J. Biol. Chem. 257*, 10015–10022.

Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., & Hemker, C. (1980) *J. Biol. Chem. 255*, 274–283.

Rosing, J., Speijer, H., & Zwaal, R. F. A. (1988) *Biochemistry 27*, 8–11.

Seegers, W. H., & Ouyang, C. (1979) in *Handbook for Experimental Pharmacology, Vol. 52, Snake Venoms* (Lee, C. Y., Ed.) pp 684–749, Springer-Verlag, Berlin.

Senisterra, G. A., Disalvo, E. A., & Gagliardino, J. J. (1988) *Biochim. Biophys. Acta 941*, 264–270.

Stefansson, S., Kini, R. M., & Evans, H. J. (1989) *Thromb. Res. 55*, 481–491.

Teng, C. M., & Seegers, W. H. (1981) *Thromb. Res. 23*, 255–263.

Tzeng, M. C., Hseu, M. J., Yang, J. H., & Guillory, R. J. (1986) *J. Protein Chem. 5*, 221–228.

Tzeng, M. C., Hseu, M. J., & Yen, C. H. (1989) *Biochem. Biophys. Res. Commun. 165*, 689–694.

Verheij, H. M., Boffa, M. C., Rothen, C., Bryckaert, M. C., Verger, R., & de Haas, G. H. (1980) *Eur. J. Biochem. 112*, 25–32.

Warn-Cramer, B. J., Rao, L. V. M., Maki, S. L., & Rapaport, S. I. (1988) *Thromb. Haemostasis 60*, 453–456.

Yang, C. C., & King, K. (1980) *Biochim. Biophys. Acta 614*, 373–388.