ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 3, pp. 276-280. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 3, pp. 339-345.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-189, December 28, 2008.

Hemolytic and Anticoagulant Study of the Neurotoxin Vipoxin and Its Components—Basic Phospholipase A_2 and an Acidic Inhibitor

V. N. Atanasov[1], D. Danchev[2], M. Mitewa[1], and S. Petrova[3]*

[1]*Laboratory of Biocoordination and Bioanalytical Chemistry, Department of Analytical Chemistry, Faculty of Chemistry, Sofia University, J. Bourchier Ave. 1, 1164 Sofia, Bulgaria; fax: +359-2-962-5438; E-mail: vatanasov@chem.uni-sofia.bg; mmitewa@chem.uni-sofia.bg*
[2]*Unit of Hemostasis, Central Clinical Laboratory, Military Medical Academy, G. Sofiiski Str. 3, 1603 Sofia, Bulgaria; fax: +359-2-952-6536; E-mail: dobridanchev@yahoo.com*
[3]*Laboratory of Enzymology, Department of Biochemistry, Faculty of Biology, Sofia University, Dr. Tsankov Ave. 8, 1164 Sofia, Bulgaria; fax: +359-2-865-6641; E-mail: spetrova@biofac.uni-sofia.bg*

Received June 2, 2008
Revision received September 5, 2008

Abstract—In the present study, we demonstrate for the first time that the potent neurotoxin vipoxin from the venom of *Vipera ammodytes meridionalis* exhibits hemolytic and anticoagulant properties. By investigating the effects of phospholipids and calcium ions on hemolysis, we established that the phospholipase A_2 (PLA_2) enzyme activity is responsible for the hemolytic properties. This was confirmed by chemical modification of the PLA_2 active-site histidine residue with *p*-bromophenacylbromide. Applying different clotting assays, we show that the PLA_2 is a weakly anticoagulant enzyme, which affects intrinsic tenase complex by the hydrolysis of procoagulant phospholipids, rather than by nonenzymatic mechanisms (binding to specific coagulation factors). The whole complex—vipoxin—does not affect the coagulation system.

DOI: 10.1134/S0006297909030055

Key words: vipoxin, phospholipase A_2, hemolysis, anticoagulant activity

Phospholipase A_2 enzymes (PLA_2, phosphatide-*sn*-2-acylhydrolase, EC 3.1.1.4) are Ca^{2+}-dependent enzymes that specifically catalyze the hydrolysis of the 2-acylester bond of 1,2-diacyl-3-*sn*-phosphoglycerides releasing fatty acids and lysophospholipids. PLA_2 enzymes (intra- and extracellular) occur ubiquitously in nature and are found in all eukaryotic cell types. The hydrolysis products of the PLA_2 reaction—free fatty acids and lysophospholipids—play important roles in diverse biological processes such as inflammation and signal transduction, remodeling of membrane phospholipids, cell proliferation, and host defense.

The protein complex vipoxin is isolated from the venom of the Bulgarian long-nose viper *Vipera ammodytes meridionalis*. Vipoxin is a heterodimeric ionic complex composed of two protein subunits—a basic and strongly toxic secretory (s)PLA_2 enzyme and an acidic, enzymatically inactive and nontoxic component – inhibitor [1, 2]. The two subunits have the same polypeptide length (122 amino acids) and are closely related, sharing 62% sequence identity [3]. Each subunit has seven disulfide bridges stabilizing the native enzymatically and pharmacologically active conformation. Isolated $sPLA_2$ from vipoxin is one of the most toxic phospholipases with an LD_{100} = 1-3 μg per 20 g mouse [1, 3]. When dissociated, pure $sPLA_2$ subunit looses the toxic activity in 2-3 days and enzyme activity in 10-14 days. Reassociation *in vitro* of the subunits reduces the enzyme activity and toxicity of the complex and increases its stability [1].

Snake $sPLA_2$ enzymes possess a wide range of pharmacological effects (neurotoxicity cardiotoxicity, myotoxocity, anticoagulant and platelet aggregating activities, etc.) that could not be directly correlated with enzyme activity [4]. Chemical modification studies have provided evidence for existence of a specific pharmacological site usually located at a different place from the catalytic one [4-6]. However, some of the pharmacological effects are results of a complex activity.

Abbreviations: aPTT, activated partial thromboplastin time; pBPB, *p*-bromophenacylbromide; PT, prothrombin time; (s)PLA_2, (secretory) phospholipase A_2; StT, Stypven time; TT, thrombin time.

* To whom correspondence should be addressed.

The anticoagulant properties of $sPLA_2$ have been described for a large number of snake venom enzymes [7]. In general, two main mechanisms are proposed for the anticoagulant action of $sPLA_2$ enzymes—hydrolysis of or binding to procoagulant phospholipids [8-10] and binding to factor Xa (inhibiting formation of prothrombinase complex) [7, 11-14]. The hemolytic activity of $sPLA_2$ enzymes could be a result of direct cell membrane lysis of red blood cells or indirect by formation of hydrolysis products (lysophospholipids and fatty acids), changing the procoagulant properties of the phospholipid mixture [8].

The aim of the present work was to elucidate the role of the enzyme activity of vipoxin and its components on their direct and indirect hemolytic and anticoagulant activities. The role of calcium ions has been investigated in hemolytic experiments. We have modified the catalytically active His48 residue using *p*-bromophenacylbromide (pBPB) to abolish the enzymatic activity of PLA_2 [15].

MATERIALS AND METHODS

Vipoxin was isolated from crude venom of *V. ammodytes meridionalis* (Thracian Herpetological Society, Bulgaria) using ion-exchange chromatography on SP-Sephadex C-50 (Pharmacia, Sweden) [16]. The PLA_2 and the acidic inhibitor component of vipoxin were separated by cation-exchange chromatography on CM-cellulose (Schuchardt, Germany) in the presence of 6 M carbamide (Fluka, Switzerland) [2]. Mono S HR 5/5 (Pharmacia) cation-exchange chromatography was used instead of CM-cellulose for better purification. The homogeneity of the vipoxin components was confirmed by SDS-PAGE. All the chemicals and substrates used were of analytical grade.

Hemolytic activity assay. Human red blood cells separated from heparinized whole blood (collected from healthy volunteers) were used. The number of erythrocytes and the amount of total hemoglobin (determined using the Kampen–Zijlstra method [17]) were used to standardize the blood. The erythrocytes were repeatedly washed and subsequently resuspended (7% v/v) in isotonic saline solution. Aliquots of the cell suspension (1.5 ml) were incubated with the appropriate and comparable amounts of tested toxin forms (0.3 to 0.5 μM final concentrations) at 37°C for 90 min. After the incubation, the suspension was centrifuged for 5 min at 4000 rpm and the absorbance of the supernatant at 540 nm was measured. Control samples with saline (instead of toxin) (0% hemolysis) and 20 μl 100 mM Triton X-100 (Merck, Germany) (100% hemolysis) were prepared at the same time. The indirect hemolysis study was performed using the same scheme but containing 150 μl of 5 mg/ml soybean lecithin (Serva, Germany), emulsified in isotonic saline.

The effect of calcium ions was studied by the addition of different amounts of 100 mM $CaCl_2$ (Merck) to the initial red blood cell suspension. The hemolysis was presented as a percentage of the total hemolysis obtained with the Triton X-100 control sample.

Alkylation of His residue with pBPB. Chemical modification of the catalytic His residue was performed by the following procedure: aliquots (60 μl) of freshly prepared pBPB (20 mM in acetone; Fluka) were added successively to 400 μl enzyme sample (20 μM in 0.05 M Mes/NaOH buffer, pH 7.0) and incubated 2 h at 37°C. The residual enzyme activity was determined according to the PLA_2 assay.

Enzyme activity. An HPLC method was used to measure the enzyme activity of PLA_2. Dipalmitoylphosphatidylcholine (Sigma, USA) was used as a substrate, and mixed-mode vesicles were formed by methanol injection and subsequent sonication of the sample. Samples of 10 mM dipalmitoylphosphatidylcholine in methanol (80 μl) were injected into buffer solution (50 mM Tris-HCl, pH 8.0) containing 5 mM $CaCl_2$ and 100 μl of internal standard (2.2 mM heptadecanoic acid in methanol; Fluka) in a final volume of 1 ml. After sonication for 3 min at 37°C, 40 μl of enzyme solution (10 μM) was added to the reaction mixture and incubated for 6 min at 37°C. The reaction was stopped with 50 μl sulfuric acid (3 M), and fatty acids released were extracted in 1 ml *n*-heptane–*n*-propanol (9 : 1) mixture for 1 min. The extract was evaporated to dryness under nitrogen flow and dissolved in 200 μl of the mobile phase. Aliquots of 20 μl were injected into the HPLC system. Fatty acids were separated on analytical column Econosphere C18, 5 μm, 150 × 4.6 mm (Alltech, USA), using a mobile phase of 5% (v/v) 20 mM triethylamine (Aldrich, USA) phosphate buffer (pH 2.5) in methanol (Aldrich). The HPLC analyses were performed using an L 6210 (Merck-Hitachi, Germany) isocratic pump combined with Spectra Sys UV 200 (Thermo Electron, USA) UV detector with a flow rate of 1.5 ml/min and detection at 215 nm.

Coagulation studies. The anticoagulant activity of the toxin was studied *in vitro* using human pool plasma and evaluated from the commonly used clotting time assays—PT (prothrombin time), aPTT (activated partial thromboplastin time), TT (thrombin time), and StT (Stypven time). Human pool plasma was obtained from clinically healthy volunteers. Coagulation analyzer STA Compact-Stago (Stago, France) was used. Toxin samples (50 μl) of appropriate concentrations were added to 1 ml human pool plasma and after incubation for 3 min the sample was analyzed. Clotting time was determined after mixing the plasma with the corresponding reagent (STANeoplastin® Plus, STA APTT Kaolin, STA Thrombine Time, purchased from Stago). The accuracy and the reproducibility of the results were controlled using STA PreciClot Plus I (Normal) and STA PreciClot Plus II (Pathological). In each case a control blank assay (without added toxin) was performed (control sample in Table 2; see further). The StT was measured according to

the procedure described by Kini and Evans [6]. Different concentrations of PLA_2 (0.1 to 2 μM) were added to preliminarily incubated (3 min at 37°C) 100 μl of human pool plasma with 10 μl *Vipera russeli* reagent (Stago), and the clotting was initiated with 50 μl 0.06 M $CaCl_2$.

RESULTS

The registered hemolytic activity (Table 1) in the presence of purified phospholipase A_2 and vipoxin was higher, but not more than 10% of the total hemolysis (depending on the toxin concentration and time of incubation). The addition of phospholipids (soybean lecithin) increased the hemolysis of PLA_2 and vipoxin containing samples dramatically (up to 60%). A similar result was obtained when the whole venom sample (containing all the accessory proteins of the snake venom) was used in the hemolytic experiments (Fig. 1).

The indirect hemolysis in the presence of chemically alkylated with pBPB purified PLA_2 was comparable to the blank sample. The hemolysis was dependent on the time of preliminary incubation.

To investigate the role of calcium ions on indirect hemolysis, we performed the assay in the presence of different concentrations of Ca^{2+} (Fig. 2). Figure 2 illustrates that both activities (hemolytic and enzymatic) depend on Ca^{2+} in the reaction mixture following a similar mode.

Anticoagulant studies as PT, aPTT, TT, and StT are presented in Table 2. The most significant changes are registered for aPTT and StT. Alkylated enzyme does not influence the aPTT and StT (compared to the control sample). The aPTT is prolonged for PLA_2 and shortened for the whole venom, but only PLA_2 changed StT (prolonged). The coagulation times were not changed both from the toxic complex vipoxin and chemically modified PLA_2. To identify the mechanism of anticoagulant action, we also measured changes in the clotting times at different PLA_2 concentrations according to Kini and Banerjee [18]. The results presented in Fig. 3 show that aPTT and StT depend on PLA_2 concentration, but PT and TT are not affected.

Table 1. Comparison between direct and indirect hemolysis

Sample	Hemolysis in the absence of PL, % (± SD)	Hemolysis in the presence of PL, % (± SD)
Blank	0.3 (± 0.02)	1.2 (± 0.04)
PLA_2	0.9 (± 0.04)	66.4 (± 7.72)
Inhibitor	0.4 (± 0.01)	1.3 (± 0.01)
Vipoxin	0.5 (± 0.02)	68.3 (± 7.31)
Alkylated PLA_2	–	1.0 (± 0.39)
Whole venom	–	67.0 (± 3.43)

Note: Equal final concentrations of the tested compounds (7 μg, corresponding to 0.4 μM in the case of PLA_2) were used. Each test was performed in triplicate in parallel with the control sample. PL, phospholipid from soybean (0.5 mg/ml lecithin).

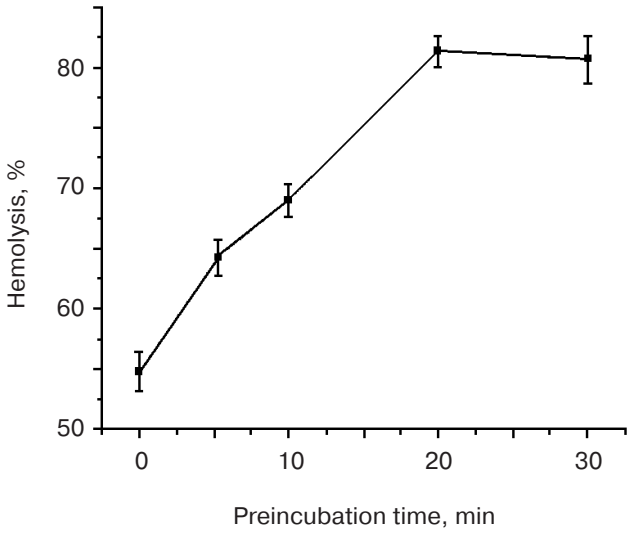

Fig. 1. Indirect hemolysis as a function of lecithin preincubation time. Each point represents the average of three measurements ± standard deviation (0.3 μM final concentration of the PLA_2 is used; $n = 3$).

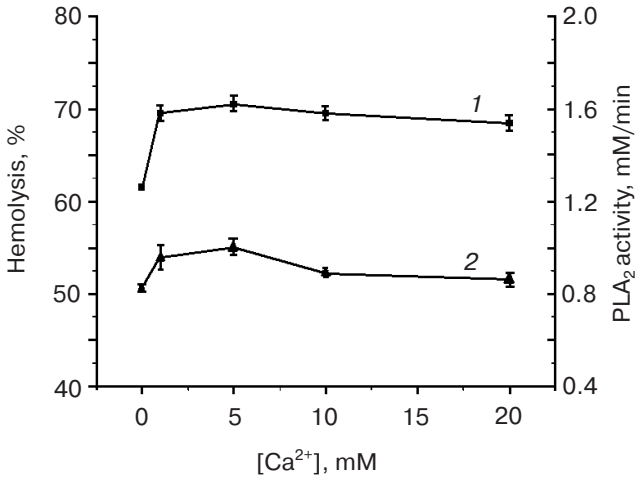

Fig. 2. Effect of calcium concentration on indirect hemolysis (*1*) and phospholipase activity (*2*). Each point represents the average of three measurements ± standard deviation (0.3 μM final concentration of PLA_2 is used).

DISCUSSION

The main purpose of the study was to investigate the correlation between enzymatic, hemolytic, and anticoagulant activity of the toxin vipoxin and its components. It is

Table 2. Coagulation tests

Sample	PT, sec	aPTT, sec	TT, sec	StT, sec
Control	11.5	27.6	16.4	28
PLA_2	7.6	64.1	16.5	77
Inhibitor	10.5	27.6	16.7	29
Vipoxin	9.9	27.4	16.2	26
Whole venom	9.1	<20.0	19.6	31
Alkylated PLA_2	10.1	30.0	16.7	27

Note: Equal final concentrations of the tested compounds (20 μg, corresponding to 1.3 μM in the case of PLA_2) were used. Each test was performed in triplicate in parallel with the control sample (n = 3; maximum SD is less than 1 sec for PT, aPTT, TT and 3 sec for StT).

generally thought that the enzyme activity of a toxin and its pharmacological potential are determined by different sites on the protein structure [4-6]. The hemolytic activity of the enzyme could be a result of direct disruption of the membranes of red blood cells (similarly to cardiotoxins) [19] or indirect – the result of formation of hydrolysis products that change the coagulation complexes [19-22]. The anticoagulant activity of the $sPLA_2$ toxins is associated with binding or hydrolysis of the procoagulant phospholipids or interaction with specific blood coagulation proteins (factor Xa) [4, 6, 7, 13, 18]. The role of the enzyme activity and the mechanism of anticoagulant effects of PLA_2 enzymes are not entirely explained so far due to conflicting results.

The hemolytic activity results (Table 1) in the absence and presence of phospholipids clearly indicate that hemolysis is strongly phospholipid-dependent (more than 50% increase) when PLA_2, vipoxin, and whole venom are used in the samples. This suggests an indirect mechanism of action, i.e. the toxins initially hydrolyze the phospholipids, and the lysophospholipids obtained act as surface-active compounds and physically destroy the erythrocyte membranes. The proposed explanation is confirmed after alkylation of the catalytically active His48 of the enzyme. The hemolytic activity of the modified enzyme was abolished.

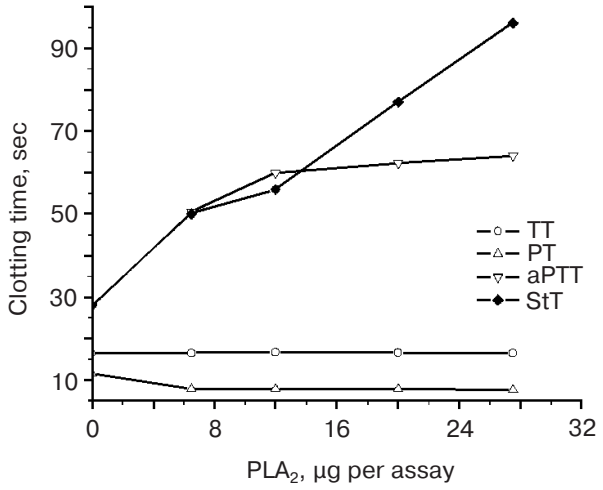

Fig. 3. Coagulation assays as a function of PLA_2 concentration (0.2 to 2 μM) (n = 3; maximum SD is less than 1 sec for PT, aPTT, TT and 3 sec for StT).

Another result supporting the indirect mechanism of action is presented in Fig. 1—time dependence of hemolysis. The hemolysis increases with the time of preliminary incubation of the enzyme and phospholipids. The amount of liberated lysophospholipids increases during the preincubation, which determines the higher hemolysis.

The effect of Ca^{2+} on hemolysis (Fig. 2) also supports enzyme-correlated hemolysis. Measuring the hemolytic and enzyme activities at different Ca^{2+} concentrations allow us to compare the calcium dependence of the processes. The maximum of both activities is practically the same, which confirms the hydrolytic type of action.

Blood coagulation is a complicated combination of sequential processes resulting in a clot formation, preventing loss of blood. In fact, there are two mechanisms of initiation—by the contact system, which initiates the intrinsic pathway, and the tissue factor-inducible or extrinsic pathway, which is the primary triggering pathway for the clotting cascade. The aPTT assay involves activation of the contact system and it is characteristic for so-called "intrinsic" tenase complex. The test itself requires the addition of negatively charged phospholipids, because the platelets have been removed from the plasma. The PT clotting time is characteristic for the "extrinsic" complex and shows formation of normal prothrombin, whereas TT defines the last stage of the cascade, which measures the time of fibrinogen-to-fibrin conversion. Additionally, StT was measured according to the dissection approach proposed by Kini and Banerjee [18]. The StT is initiated by the addition of diluted *Vipera russeli* venom and provides information about the last two stages of the clotting cascade by activation of factor X to Xa, which are common for both pathways (intrinsic and extrinsic). This approach is used for testing of eventual protein binding to factor Xa.

The $sPLA_2$ enzymes from viperid venoms are usually characterized as weak or non-anticoagulant venoms and are classified as group III according to Boffa et al. [8, 23].

Strongly anticoagulant $sPLA_2$ enzymes inhibit blood coagulation at concentrations below 2 μg/ml, and weakly anticoagulant PLA_2 enzymes are effective up to 10 μg/ml. The third group of $sPLA_2$ enzymes acts in concentrations higher than 15 μg/ml and have little or no effect on the clotting times [13]. The weak anticoagulants exhibit their action through hydrolysis of procoagulant phospholipids [6, 8, 13] or inhibit activation of factor X to factor Xa by extrinsic tenase complex [13]. In contrast, the strong anticoagulants act affecting target proteins or interfere with the prothrombinase complex [13]. According to the structure–function relationship characteristics [24], the investigated $sPLA_2$ from vipoxin is a potential anticoagulant—it is a basic protein (p*I* 10.5) and possesses only one pair of lysine residues at position 54-77 in its sequence.

Our results indicate that $sPLA_2$ from vipoxin belongs to the group of weakly anticoagulant $sPLA_2$ enzymes, showing their effects (prolonged aPTT and StT) at enzyme concentration higher than 5 μg/ml (more than 0.5 μM) (Fig. 3). The PT and TT are not changed compared to the initial control sample.

The intrinsic tenase complex is affected, before factor X to Xa activation (Table 2). Both of the clotting times (aPTT and StT) depend on the toxin concentration (Fig. 3). However, StT increases if preliminary pre-incubation with the PLA_2 is performed (more than two-fold after 3 min pre-incubation), which indicates enzymatic (hydrolytic) mode of action and at the same time it excludes protein-dependent mechanism of anticoagulation (most strongly anticoagulant $sPLA_2$ enzymes (CM-IV from *Naja nigricollis* venom) act as factor X-binding proteins [13, 14, 18]). The PT, which is changed usually in the case of $sPLA_2$-induced anticoagulant activity, here is not affected. The TT is also unchanged—none of the anticoagulant PLA_2 enzymes has been shown to affect this assay [6].

The phospholipid-dependent mechanism is typical for the weakly anticoagulant $sPLA_2$ enzymes, which act through enzymatic cleavage of the negatively charged phospholipids [6]. It is suggested that positive charges of the basic enzymes facilitate binding to phospholipids and subsequent hydrolysis obstructs their normal procoagulant role in the coagulation complex. This usually blocks the coagulation cascade before X to Xa activation and/or prothrombin-to-thrombin transformation.

The role of PLA_2 enzymatic hydrolysis of phospholipids is demonstrated by alkylation of the active-site histidine residue. The alkylated enzyme does not affect the coagulation system (Table 2). The modified enzyme lost catalytic, hemolytic, and anticoagulant activities, which confirmed enzyme-dependent mechanism of its anticoagulant activity. Paradoxically, the whole venom of *V. ammodytes meridionalis* showed procoagulant effect resulting in shortened aPTT (Table 2). The same results for *V. ammodytes ammodytes* venom were obtained earlier [25].

The inhibitor component of vipoxin displays no anticoagulant effect, as well as no enzymatic activity. More interestingly, the whole vipoxin complex is also anticoagulant ineffective, although it has phospholipase activity. This effect could be explained with the occupation of positively charged PLA_2 contact sites (vipoxin is an ionic complex between basic PLA_2 and acidic protein – inhibitor), which results in the absence and/or weakening of the contact with negatively charged phospholipids of the coagulation complex.

REFERENCES

1. Aleksiev, B., and Tchorbanov, B. (1976) *Toxicon*, **14**, 477-485.
2. Tchorbanov, B., Grishin, E., Aleksiev, B., and Ovchinnikov, Y. (1978) *Toxicon*, **16**, 427-437.
3. Mancheva, I., Kleinshmidt, T., Aleksiev, B., and Braunitzer, G. (1987) *Biol. Chem. Hoppe-Seyler*, **368**, 343-352.
4. Kini, R. M., and Evans, H. J. (1989) *Toxicon*, **27**, 613-635.
5. Rosenberg, P., Ghassemi, A., Condrea, E., Dhillon, D., and Yang, Ch. (1989) *Toxicon*, **27**, 137-159.
6. Kini, R. M., and Evans, H. J. (1997) in *Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism* (Kini, R. M., ed.) John Wileys & Sons, Chichester, pp. 353-368.
7. Mounier, C., Bon, C., and Kini, R. M. (2001) *Haemostasis*, **31**, 279-287.
8. Verheij, H. M., Boffa, M. C., Rothen, C., Bryckaert, M. C., Verger, R., and de Haas, G. H. (1980) *Eur. J. Biochem.*, **112**, 25-32.
9. Andriao-Escarso, S. H., Soares, A. M., Rodrigues, V. M., Angulo, Y., Diaz, C., Lomonte, B., Gutierrez, J. M., and Giglio, J. R. (2000) *Biochimie*, **82**, 755-763.
10. Zhong, X., Liu, J., Wu, X., and Zhou, Y. (2001) *J. Nat. Toxins*, **10**, 17-25.
11. Kerns, R. T., Kini, R. M., Steffanson, S., and Evans, H. (1999) *Arch. Biochem. Biophys.*, **36**, 107-113.
12. Steffanson, S., Kini, R. M., and Evans, H. J. (1990) *Biochemistry*, **29**, 7742-7746.
13. Kini, R. M. (2006) *Biochem. J.*, **397**, 377-387.
14. Kini, R. M. (2005) *Toxicon*, **45**, 1147-1161.
15. Soares, A. M., and Giglio, J. R. (2003) *Toxicon*, **42**, 855-868.
16. Tchorbanov, B., and Aleksiev, B. (1981) *J. Appl. Biochem.*, **3**, 558-561.
17. Van Kampen, E. J., and Zijlstra, W. C. (1961) *Chin. Chim. Acta*, **6**, 538-544.
18. Kini, R. M., and Banerjee, Y. (2005) *J. Thromb. Haemost.*, **3**, 170-171.
19. Jiang, M. S., Fletcher, J. E., and Smith, L. A. (1989) *Toxicon*, **27**, 247-257.
20. Ibarra-Alvarado, C., Alejandro Garcia, J., Aguilar, M. B., Rojas, A., Falcon, A., Heimer, and de la Cotera, E. P. (2007) *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.*, **146**, 511-518.
21. Adhikari, D., Samanta, S. K., Dutta, A., Roy, A., Vedasiromoni, J. R., and Sen, T. (2007) *Ind. J. Pharmacol.*, **39**, 155-159.
22. Nikai, T., Komori, Y., Yagihashi, S., Ohara, A., Ohizumi, Y., and Sugihara, H. (1993) *Int. J. Biochem.*, **25**, 879-884.
23. Boffa, M. C., and Boffa, G. A. (1976) *Biochim. Biophys. Acta*, **429**, 839-852.
24. Kini, R. M., and Evans, H. J. (1987) *J. Biol. Chem.*, **262**, 14402-14407.
25. Tan, N. G., and Ponnudurai, G. (1990) *Comp. Biochem. Physiol.*, **96B**, 683-688.