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The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities

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The effect of four myotoxins isolated from *Bothrops* snake venoms on the release of peroxidase trapped in large multilamellar liposomes was studied and correlated to their phospholipase A_2, myotoxic and anticoagulant activities. The four myotoxins affected negatively-charged liposomes in a dose-dependent way, having no effect on positively-charged liposomes. Conditions that inhibited phospholipase A_2 activity, i.e., substitution of calcium by EDTA, reduced liposome-disrupting activity of *Bothrops asper* myotoxin I and *Bothrops atrox* myotoxin, both of which have high phospholipase A_2 activity, but did not affect the action of *B. asper* myotoxin II and *Bothrops moojeni* myotoxin II, which have extremely low phospholipase A_2 activity. However, all myotoxins disrupted to some extent negatively-charged liposomes under conditions where phospholipase A_2 activity was abolished. Since these toxins behave as amphiphilic proteins in charge-shift electrophoresis, it is suggested that membrane-disorganization is at least partially due to a non-enzymatic penetration and alteration of bilayers. There was no strict correlation between liposome-disrupting activity and myotoxicity *in vivo*. Thus, although both effects probably depend on the toxins' ability to disturb membranes, it is likely that variation in complexity between skeletal muscle plasma membrane and liposome bilayers are the basis for this difference. The anticoagulant effect seems to depend on the ability of the toxins to enzymatically degrade phospholipids, since only *B. asper* myotoxin I and *B. atrox* myotoxin prolonged the plasma recalcification time.

Introduction

Muscle-damaging toxins, myotoxins, are present in a variety of snake venoms [1,2]. Many of them are basic phospholipases A_2 which, besides myotoxicity, exert other pharmacological activities, such as anticoagulant, neurotoxic, cardiotoxic and inflammatory effects [1,3,4]. Several myotoxic phospholipases A_2 have been isolated from venoms of *Bothrops* species. Some of them are active enzymatically, such as *Bothrops asper* myotoxin I[5,6], *B. asper* myotoxin III [7], *Bothrops atrox* myotoxin [8] and a toxin from *Bothrops jararacussu* venom [9]. In addition, there is a group of myotoxins which lack, or have extremely low, phospholipolytic activity, although biochemical and immunological data indicate that they are phospholipase A_2 analogues. This group consists of bothropstoxin from *B. jararacussu* [9], *Bothrops nummifer* myotoxin [10], myotoxins I and II from *Bothrops moojeni* [8] and *B. asper* myotoxin II [11,12]. The latter has been shown to be a lysine-49 phospholipase A_2 [12].

Experimental evidence suggest that these myotoxins affect skeletal muscle by binding and altering the plasma membrane (Refs. 5, 13 and 14, and Gené, J.A. et al., unpublished data), and that myotoxicity does not depend on enzymatic phospholipid degradation [6,15]. Instead, a membrane-disorganizing activity based on an amphipatic interaction with the membrane has been suggested for *B. nummifer* myotoxin [10]. On the other hand, the anticoagulant effect exerted by some of these toxins on platelet-poor plasma seems to depend on their phospholipase A_2 activity [6,8,16].

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Liposomes constitute a useful model to study the interaction of cytolytic toxins with membranes [17]. In order to gain further insight into the mechanism of action of myotoxins, we have performed a comparative study of the action of four myotoxins isolated from *Bothrops* venoms on liposomes. In addition, phospholipase A_2, myotoxic and anticoagulant effects were quantified, in an attempt to establish the relationship between damage to model membranes and the pharmacological activities of the toxins.

Materials and Methods

Toxins. The following myotoxins were used: *Bothrops asper* myotoxins I and II, *Bothrops atrox* myotoxin and *Bothrops moojeni* myotoxin II. Toxins were isolated as described by Gutiérrez et al. [6], Lomonte and Gutiérrez [11] and Lomonte et al. [8], respectively. Homogeneity was demonstrated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [18] on 12% gels and by urea-PAGE for basic proteins [19] on 12% gels. Proteins were stained with Coomassie blue R-250.

Effect of myotoxins on liposomes. Positively charged (L-α-phosphatidylcholine, 63 μmoles; stearylamine, 18 μmoles; cholesterol, 9 μmoles) and negatively charged (phosphatidylcholine, 63 μmoles; diacetyl phosphate, 18 μmoles; cholesterol, 9 μmoles) liposomes were purchased from Sigma (St. Louis, MO, U.S.A.), kits L-4137 and L-4262, respectively. After solvent evaporation under a nitrogen stream, 30 μmoles of lipids were mixed with 2 ml of a 0.5 mg/ml horseradish peroxidase (type VI, Sigma) solution. The mixture was sonicated at 25% power for 5 min at 4 °C in a Fisher sonic dismembrator (Fisher Co., MA, U.S.A.). Then, liposomes were gel filtered on a Sepharose 6B column (Pharmacia, Sweden) previously equilibrated with 0.145 M NaCl-KCl [10]. Peroxidase-containing liposomes eluted in the void volume were pooled and stored at 4 °C to be used within 24 h. Liposomes were multilamellar, as observed by negative staining in a Hitachi HU-12A electron microscope. The effect of myotoxins on liposomes was studied in microtiter plates by incubating 20 μl of liposome suspension and 20 μl of solutions of varying concentrations of myotoxins dissolved in phosphate-buffered saline solution (PBS), (pH 7.2). Incubation was carried out for 30 min at 37 °C and then, 40 μl of peroxidase substrate (2.5 mM 5-aminosalycilic acid and 0.025% H_2O_2, pH 6.0) were added to each well. The color reaction was stopped by the additon of 25 μl of 4 M H_2SO_4 followed by 8 μl of 10% Triton X-100. Absorbances were recorded at 492 nm in a microplate reader. Peroxidase release was expressed as percentage, taking as 100% the absorbances of samples in which liposomes were incubated with 20 μl of 0.2% Triton X-100 instead of toxin. To correct for spontaneous release, controls where liposomes were incubated with PBS were run in parallel, and the absorbance was subtracted from sample readings.

In order to determine whether phospholipase A_2 activity was relevant for liposome disruption by myotoxins, a similar series of experiments was carried out by incubating 40 μl of liposomes with 40 μl of toxin dissolved in 0.14 M NaCl, 0.01 M Tris (pH 7.2). Then, 20 μl of 5 mM $CaCl_2$ were added to a series of wells, whereas 20 μl of 5 mM EDTA were added to the other series. Incubations and detection of peroxidase release were performed as described above.

Phospholipase A_2 activity. Enzymatic activity was determined by titration with a Radiometer PHM 82 pHmeter using L-α-phosphatidylcholine as substrate, in a reaction mixture containing Triton X-100 (2:1 molar ratio of Triton X-100/phospholipid), as described by Aird and Kaiser [20].

Myotoxic activity. Groups of four Webster mice (18–20 g) were injected intramuscularly in the right gastrocnemius with either 40 μg or 80 μg of each myotoxin, dissolved in 100 μl of PBS. Controls received 100 μl of PBS. After 2 h, mice were bled from the tail and blood was collected in heparinized capillary tubes and centrifuged. The creatine kinase (CK, EC 2.7.3.2) activity of plasma was determined by using the Sigma Kit No. 520. Creatine kinase activity was expressed in Units/ml, one unit resulting in the phosphorylation of one nanomole of creatine per min at 25 °C.

Anticoagulant activity. Sheep platelet-poor plasma was prepared by centrifuging citrated blood twice at 1000 × *g* at 5 °C. For the assay, 0.5 ml of platelet-poor plasma was incubated with 0.1 ml of various toxin concentrations dissolved in PBS. Incubations were carried out for 10 min at 37 °C and then 0.1 ml of 0.25 M $CaCl_2$ was added and coagulation time was recorded. In control tubes, plasma was incubated with PBS instead of toxin. Observations were carried out for a maximum period of 45 min.

Charge-shift electrophoresis. In order to study whether myotoxins behave as amphiphilic proteins, the procedure of Helenius and Simons [21], with some modifications, was followed. Briefly, electrophoresis of myotoxins was performed in 1% agarose gels in three different systems: (i) 0.05 M glycine-NaOH, 0.1 M NaCl (pH 9.0), containing 0.5% Triton X-100; (ii) the same buffer but containing in addition 0.05% of the cationic detergent cetyltrimethylammonium bromide (CTAB); and (iii) the same buffer as (i), but containing also 0.25% of the anionic detergent deoxycholate. In each case, the buffers in the electrode chambers were identical to the ones of the agarose gels. Electrophoreses were run at 48 mA for 50 V · h. Then, gels were fixed in methanol/acetic acid/water (50:7:43, v/v) for 30 min, stained with Coomassie blue R-250 for 60 min, and destained with methanol/ethanol/acetic acid/water (20:10:5:65, v/v). Migration of the toxins

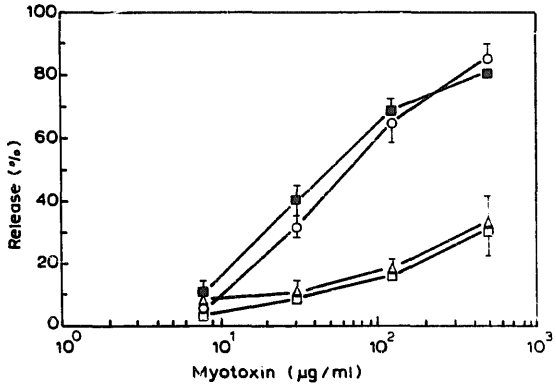

Fig. 1. Release of peroxidase trapped in negatively-charged liposomes incubated with myotoxins isolated from *Bothrops* snake venoms. Release is expressed as percentage, taking as 100% the peroxidase release from liposomes incubated with 0.2% Triton X-100. Results are presented as means ± S.E. ($n = 4$). ■, *Bothrops asper* mytoxin I; ○, *B. atrox* myotoxin; △, *B. asper* mytoxin II; □, *B. moojeni* mytoxin II.

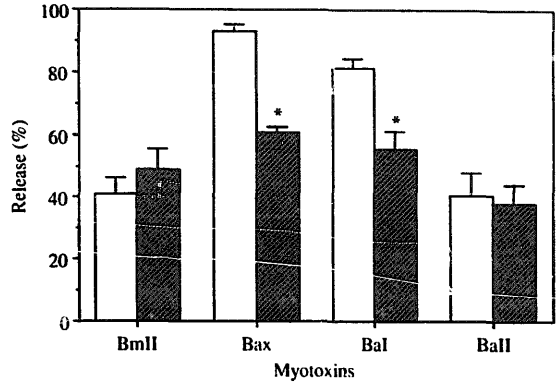

Fig. 2. Release of peroxidase trapped in negatively-charged liposomes incubated with myotoxins (400 μg/ml) in solutions containing either $CaCl_2$ or EDTA. Peroxidase release is expressed as for Fig. 1. Results are presented as means + S.E. ($n = 8$). □, in the presence of calcium; ▨, in the presence of EDTA. * $P < 0.001$.

was then measured. Experiments were repeated three times.

Statistical analysis. Analysis of variance was used to determine the significance of the differences. Then, comparison between mean values of pairs of experimental groups was performed by Student's *t*-test.

Results

Phospholipase A_2 activity

B. asper myotoxin I and *B. atrox* myotoxin had high phospholipase A_2 activity when L-α-phosphatidylcholine was used as substrate. The activities of these toxins were 63 and 11 μmol fatty acid per mg per min, respectively. In contrast, *B. asper* myotoxin II and *B. moojeni* myotoxin II had extremely low phospholipase A_2 activities of 0.3 and 0.2 μmol fatty acid released per mg per min, respectively.

Effects of myotoxins on liposomes

The four myotoxins induced a dose-dependent release of peroxidase from negatively-charged multilamellar liposomes (Fig. 1). *B. asper* myotoxin I and *B. atrox* myotoxin induced a higher release than *B. asper* myotoxin II and *B. moojeni* myotoxin II. In contrast, neither myotoxin caused disruption of positively-charged liposomes, since even at toxin concentrations as high as 500 μg/ml peroxidase release did not reach 15% (results not shown).

In the case of myotoxins having high phospholipase A_2 activity (*B. asper* myotoxin I and *B. atrox* myotoxin) liposome-disruption effect was significantly higher under conditions that allowed phospholipase A_2 activity, i.e., when calcium was present, than under conditions where this activity was inhibited, i.e., when calcium was absent and EDTA was added (Fig. 2). In order to corroborate that phospholipolytic activity was negligible when liposomes were incubated in the presence of EDTA, the release of free fatty acids was assessed by extracting and titrating them according to Dole et al. [22]. Results indeed showed that phospholipase A_2 activity was eliminated under these experimental conditions (results not shown). In the case of *B. asper* myotoxin II and *B. moojeni* myotoxin II, both of which have extremely low phospholipase A_2 activity, no significant difference was observed between experiments performed in the presence and in the absence of calcium ions (Fig. 2).

Myotoxic activity

Fig. 3 shows that *B. asper* myotoxin I induced the highest increase in plasma CK levels. No significant difference ($P > 0.1$) was observed in the CK levels of mice injected with *B. atrox* myotoxin and *B. moojeni* myotoxin II. On the other hand, both *B. atrox* my-

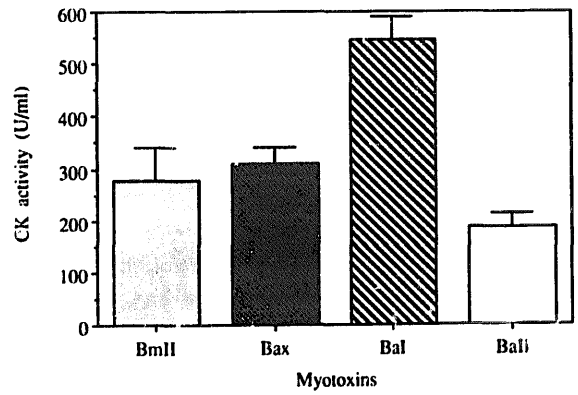

Fig. 3. Plasma creatine kinase (CK) levels in mice injected intramuscularly with 80 μg of *Bothrops* myotoxins and bled 2 h after injection. Plasma CK activity is expressed in units/ml, one unit resulting in the phosphorylation of one nanomole of creatine per min at 25 °C. Control mice injected with saline solution had plasma CK levels of 10 ± 6.2 units/ml ($n = 4$). Results are presented as means ± S.E. ($n = 5$).

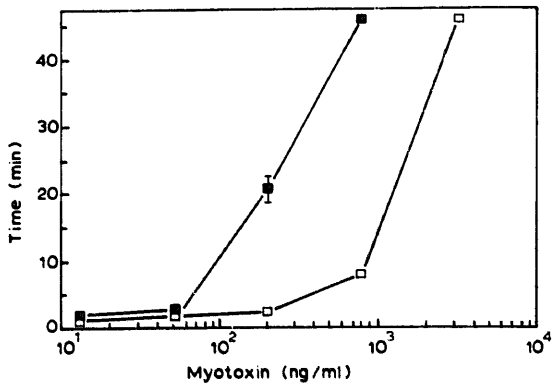

Fig. 4. Coagulation time of sheep, platelet-poor plasma recalcified after incubation with *Bothrops* myotoxins. Observations were carried out for a maximum period of 45 min. Results represent mean values of three independent experiments. ■, *B. asper* mytoxin I; □, *B. atrox* mytoxin.

otoxin and *B. moojeni* myotoxin II induced significantly higher ($P < 0.01$) increases in CK levels than did *B. asper* myotoxin II (Fig. 3). These differences in myotoxic activity were observed in samples collected from mice injected with either 40 μg (data not shown) or 80 μg of toxins.

Anticoagulant activity

The toxins having high phospholipase A_2 activity, *B. asper* myotoxin I and *B. atrox* myotoxin, prolonged recalcification times of platelet-poor plasma in a dose-response manner (Fig. 4), the former being more active than the latter. On the other hand, plasma incubated with *B. asper* myotoxin II and *B. moojeni* myotoxin II showed recalcification times which were similar to those of control samples (3.3 ± 0.1 min), even at toxin concentrations of 50 μg/ml. Thus, these toxins did not exert an anticoagulant effect.

Charge-shift electrophoresis

The four myotoxins studied behaved as amphiphilic proteins in charge-shift electrophoresis, as evidenced by their different migration depending upon the charge of the detergent present in the gels (Table I).

Discussion

Several myotoxins have been isolated from *Bothrops* snake venoms [5,6,8,9,11]. Some of them are active phospholipases A_2, whereas others lack, or have extremely low catalytic activity, although they are closely related structurally to phospholipase A_2. One of these toxins, *B. asper* myotoxin II, is a lysine-49 phospholipase A_2 [12] with extremely low catalytic activity. Similar proteins have been isolated from *Agkistrodon piscivorous* and *Trimeresurus flavoviridis* snake venoms [23–25]. In our study we used two myotoxins having high phospholipase A_2 activity and two which have extremely low activity in order to correlate phospholipid degradation with the pharmacological effects induced by these toxins.

Previous pathological studies have suggested that some of these myotoxins affect skeletal muscle cells by first binding and then affecting the integrity of the plasma membrane. This hypothesis is based on histological and ultrastructural observations [5,10,13], as well as on the early release of cytosolic markers such as creatine, creatine kinase [5,6,10] and tritiated uridine (Gené, J.A., et al., unpublished data). A similar conclusion has been reached by other workers when studying the action of toxic phospholipases such as crotoxin [26], notexin [27] and taipoxin [28]. Since liposomes represent a valuable model to study the interaction of cytolytic toxins with membranes [17], the effect of *Bothrops* myotoxins on liposomes was studied. Our results indicate that the four toxins tested interact with and disrupt multilamellar liposomes, as evidenced by the release of trapped horseradish peroxidase. The interaction of myotoxins with liposomes strongly depends on the net charge of the phospholipid vesicles, since only negatively-charged liposomes were significantly disrupted. This agrees with recent observations by Gené et al. (unpublished data) with *B. asper* myotoxin II, as well as with the conclusions reached by Radvanyi et al. [29] on the interactions of neurotoxic phospholipases with micelles of different charge. Since the four myotoxins used in this study are highly basic, it is very

TABLE I

Electrophoretic migration of Bothrops myotoxins in charge-shift electrophoresis agarose gels containing either Triton X-100, Triton plus CTAB or Triton plus deoxycholate

Electrophoresis was carried out at 48 mA for 50 V·h. Migration of each toxin towards the anode (A) or the cathode (C) is expressed in mm (mean ± S.E.; $n = 3$). BmII, *B. moojeni* myotoxin II; Bax, *B. atrox* myotoxin; BaI, *B. asper* mytoxin I; BaII, *B. asper* mytoxin II.

Detergents in the gel	Myotoxin migration (mm)			
	BmII	Bax	BaI	BaII
Triton X-100 plus CTAB	16.8 ± 0.6 (C)	19.3 ± 0.6 (C)	21.5 ± 0.6 (C)	22.8 ± 0.2 (C)
Triton X-100	14.0 ± 0.4 (C)	17 0 ± 0.3 (C)	18.3 ± 0.5 (C)	18.5 ± 0.6 (C)
Triton X-100 plus deoxycholate	14.5 ± 0.4 (A)	13.2 ± 0.2 (A)	11.2 ± 0.1 (A)	11.7 ± 0.1 (A)

likely that electrostatic interaction between a cationic site of the toxin and the negatively-charged phospholipids is required for toxin binding.

What role does phospholipase A_2 activity have in the liposomal disruption by myotoxins? Elimination of calcium and addition of EDTA significantly reduced the effect of the two myotoxins having high phospholipase A_2 activity, but did not affect the action of *B. asper* myotoxin II and *B. moojeni* myotoxin II, both of which have extremely low phospholipase A_2 activity. Thus, in the case of myotoxic phospholipases A_2, phospholipid hydrolysis plays a role in the liposomal-disruption effect. However, even in the absence of phospholipid degradation, the four myotoxins induced peroxidase release from liposomes, a clear indication that phospholipase A_2 activity is not strictly required to exert this effect.

These findings agree with previous observations which indicate that the myotoxic activities of *B. asper* myotoxin I and *B. nummifer* myotoxin do not depend on phospholipid hydrolysis [6,10]. Furthermore, our results support the most general hypothesis that at least some pharmacological effects induced by toxic phospholipases A_2 are not due to enzymatic phospholipid degradation, but rather to the presence of a pharmacological active site [4,30,31]. In the case of *Bothrops* myotoxins, charge-shift electrophoresis indicated that they behave as amphiphilic proteins, as is also the case with *B. nummifer* myotoxin [10]. Thus, these myotoxins apparently possess a hydrophobic region which might be responsible for penetration and disorganization of bilayers. Further studies on the nature of membrane damage induced by these toxins are required in order to test this hypothesis.

Although there was a relationship between liposomal-disruption activity and the myotoxic effect *in vivo*, the correlation is not strict, as there was a significant difference in myotoxic activity between *B. asper* myotoxin I and *B. atrox* myotoxin, whereas no such difference was observed on liposomal-disruption effect. Thus, although both effects probably depend on the ability of the toxins to disorganize membranes, there are differences in the two test systems, probably due to the fact that skeletal muscle plasma membrane is much more complex than liposomal membranes. In addition, the interaction of toxins and muscle membranes might be affected by membrane components other than phospholipids.

In contrast to myotoxicity, the anticoagulant effect of myotoxins seems to depend on their phospholipase A_2 activity, since only myotoxins having high phospholipolytic activity prolonged the recalcification time in platelet-poor plasma. A variety of phospholipases A_2 are anticoagulant [4,32], and it has been shown that many potent anticoagulant phospholipases are basic and have a high penetrating power on monomolecular phospholipids [32,33]. The anticoagulant effect of *Bothrops* myotoxins seems to depend on both their ability to penetrate bilayers and their ability to hydrolyze phospholipids. Membrane penetration and disorganization, without phospholipid degradation, does not induce incoagulability, as was shown for *B. asper* myotoxin II and *B. moojeni* myotoxin II. It would be important to test this hypothesis with other lysine-49 phospholipases A_2.

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References

1 Harris, J.B., Johnson, M.A. and Mac Donell, C.A. (1980) in Natural Toxins (Eaker, D. and Wadström, T., eds.), pp. 569-578, Pergamon Press, Oxford.
2 Gutiérrez, J.M. and Cerdas, L. (1984) Rev. Biol. Trop. 32, 213-222.
3 Chang, C.C. (1979) in Snake Venoms (Lee, C.Y., ed.), pp. 309-376, Springer-Verlag, Berlin.
4 Rosenberg, P. (1990) in Handbook of Toxinology (Shier, W. and Mebs, D., eds.), pp. 67-277, Marcel Dekker, New York and Basel.
5 Gutiérrez, J.M., Ownby, C.L. and Odell, G.V. (1984) Toxicon 22, 115-128.
6 Gutiérrez, J.M., Lomonte, B., Chaves, F., Moreno, E. and Cerdas, L. (1986) Comp. Biochem. Physiol. 84C, 159-164.
7 Kaiser, I.I., Gutiérrez, J.M., Plummer, D., Aird, S.D. and Odell, G.V. (1990) Arch. Biochem. Biophys. 278, 319-325.
8 Lomonte, B., Gutiérrez, J.M., Furtado, M.F., Otero, R., Rosso, J.-P., Vargas, O., Carmona, E. and Rovira, M.E. (1990) Toxicon 28, 1137-1146.
9 Homsi-Brandeburgo, M.I., Queiroz, L.S., Santo-Neto, H., Rodrígues-Simioni, L. and Giglio, J.R. (1988) Toxicon 26, 615-627.
10 Gutiérrez, J.M., Chaves, F., Gené, J.A., Lomonte, B., Camacho, Z. and Schosinsky, K. (1989) Toxicon 27, 735-745.
11 Lomonte, B. and Gutiérrez, J.M. (1989) Toxicon 27, 725-733.
12 Francis, B., Gutiérrez, J.M., Lomonte, B. and Kaiser, I.I. (1991) Arch. Biochem. Biophys. 284, 352-359.
13 Gutiérrez, J.M., Ownby, C.L. and Odell, G.V. (1984) Exp. Mol. Pathol. 40, 367-379.
14 Brenes, F., Gutiérrez, J.M. and Lomonte, B. (1987) Toxicon 25, 574-577.
15 Lomonte, B., Gutiérrez, J.M., Moreno, E. and Cerdas, L. (1987) Toxicon 25, 443-449.

16 Alvarado, J. and Gutiérrez, J.M. (1988) Rev. Biol. Trop. 36, 563–565.
17 Freer, J.H. (1980) in Natural Toxins (Eaker, D. and Wadström, T. eds.), pp. 183–188, Pergamon Press, Oxford.
18 Laemmli, U.K. (1970) Nature 227, 680–685.
19 Traub, P., Mizushima, S., Lowry, C.V. and Nomura, M. (1971) Meth. Enzymol. 20, 391–417.
20 Aird, S.D. and Kaiser, I.I. (1985) Toxicon 23, 361–374.
21 Helenius, A. and Simons, K. (1977) Proc. Natl. Acad. Sci. USA 74, 529–532.
22 Dole, V.P. (1956) J. Clin. Invest. 35, 150–154.
23 Maraganore, J.M. and Heinrikson, R.L. (1986) J. Biol. Chem. 261, 4797–4804.
24 Yoshizumi, K., Liu, S.-Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S. and Kihara, H. (1990) Toxicon 28, 43–54.
25 Liu, S.-Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S. and Kihara, H. (1990) J. Biochem. (Tokyo) 107, 400–408.
26 Gopalakrishnakone, P., Dempster, D.W., Hawgood, B.J. and Elder, H.Y. (1984) Toxicon 22, 85–98.
27 Harris, J.B., Johnson, M.A. and Karlsson, E. (1975) Clin. Exp. Pharm. Physiol. 2, 383–404.
28 Harris, J.B. and Maltin, C.A. (1982) Br. J. Pharmac. 76, 061–075.
29 Radvanyi, F., Saliou, B. and Bon, C. (1987) J. Biol. Chem. 262, 8966–8974.
30 Condrea, E., Fletcher, J.E., Rapuano, B.E., Yang, Ch.-Ch. and Rosenberg, P. (1981) Toxicon 19, 705–720.
31 Kini, R.M. and Evans, H.J. (1989) Toxicon 27, 613–635.
32 Boffa, M.C., Rothen, C., Verheij, H.M., Verger, R. and De Haas, G.H. (1980) in Natural Toxins (Eaker, D. and Wadström, T. eds.), pp. 131–138, Pergamon Press, Oxford
33 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.