Pergamon

0041-0101(95)00085-2

Toxicon, Vol. 33, No. 11, pp. 1405–1424, 1995

0041-0101/95 $29.00 + 0.00

REVIEW ARTICLE

PHOSPHOLIPASE A_2 MYOTOXINS FROM *BOTHROPS* SNAKE VENOMS

JOSÉ MARÍA GUTIÉRREZ and BRUNO LOMONTE

Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

(*Received* 17 *November* 1994; *accepted* 8 *May* 1995)

J. M. Gutiérrez and B. Lomonte. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. *Toxicon* **33**, 1405–1424.—Several myotoxins have been isolated from *Bothrops* snake venoms during the last 10 years. All of them are group II basic phospholipases A_2, although some lack enzymatic activity (i.e. Lys-49 variants). These myotoxins appear as an antigenically related family of proteins occurring in many, but not all, *Bothrops* venoms, bearing a close structural and antigenic relationship to toxins found in other crotalid venoms of the genera *Agkistrodon* and *Trimeresurus*. Myotoxins are quantitatively important venom components in some *Bothrops* species. Intramuscular injection of *Bothrops* myotoxins leads to a rapid series of drastic degenerative events, probably initiated at the plasma membrane level, which culminate in a selective skeletal muscle necrosis. This *in vivo* specificity contrasts with the ability of myotoxins to lyse many types of cells in culture. Muscle damage, as well as cytolysis and liposome disruption, occur in conditions where phospholipase A_2 activity is inhibited, although enzymatic activity might enhance myotoxin actions. A membrane receptor for *Bothrops* myotoxins has not been identified yet. A working hypothesis on the mechanism of action is proposed. Current evidence suggests that these toxins interact with biological membranes via a molecular region distinct from their known catalytic site. The active region is likely to be formed by a combination of basic and hydrophobic amino acid residues near the C-terminus of the protein, which allow electrostatic interaction and bilayer penetration. These events may lead to membrane destabilization and loss of selective permeability to ions such as calcium, both of which appear to be important mediators in the process of muscle necrosis.

INTRODUCTION

Muscle necrosis is a relevant local effect induced by many snake venoms, as it may lead to permanent tissue loss, disability, and amputation (Rosenfeld, 1971; Kerrigan, 1991; Nishioka and Silveira, 1992). In Latin America, most snakebite envenomations are caused by *Bothrops* spp. and often course with local myonecrosis (Rosenfeld, 1971; Bolaños, 1982; Otero *et al.*, 1992). During the last few years, there has been a growing interest in the study of venom components responsible for myonecrosis and their mode of action. As a result,

several myotoxins from *Bothrops* venoms have been isolated and characterized, and some progress has been made towards understanding their mechanism of action and the pathogenesis of myonecrosis. All of them have phospholipase A_2 (PLA_2) structure, although some lack enzymatic activity. This article summarizes the current knowledge on *Bothrops* myotoxins, 10 years after the first toxin of this group was isolated from the venom of *B. asper* (Gutiérrez *et al.*, 1984*a*).

BOTHROPS MYOTOXINS: ISOLATION AND BIOCHEMICAL CHARACTERIZATION

Isolation strategies

Almost all snake toxins with a direct muscle damaging activity isolated to date are basic proteins (Mebs and Ownby, 1990; Harris, 1991). Therefore, procedures for their isolation usually include a cation-exchange chromatographic step, to separate them from the bulk of acidic venom components. A list of myotoxins isolated from *Bothrops* venoms is presented in Table 1. In many cases, myotoxins are the last components eluting from cation-exchange columns using salt gradients at neutral pH. During purification, fractions may be screened for myotoxicity in mice, by intramuscular injections in the range of 10–100 μg protein. It should be noted that *Bothrops* myotoxins may not be as potent as some elapid counterparts, for which a dose of $\sim$1 mg/kg body weight has been suggested as a guideline (Harris, 1991, 1992). However, the lower potency is compensated by the high amount of venom injected by many *Bothrops* snakes and the relatively high concentration of myotoxins (as much as 25% in the venom of *B. jararacussu*, Moura *et al.*, 1991*a*; 15–25% in *B. asper* venom, unpublished results), suggesting that their activity can be clinically relevant.

Myotoxicity can be assessed by histological evaluation or, more conveniently, by measurement of plasma (or serum) creatine kinase (EC 2.7.3.2) levels 1–3 hr after injection (Gutiérrez *et al.*, 1984*a*, 1991; Moura *et al.*, 1990, 1991*a*; Díaz *et al.*, 1992). Screening for PLA_2 activity is another convenient way to follow the purification of myotoxins, although PLA_2 isoforms devoid of enzymatic activity (i.e. Lys-49 variants) would be missed by this approach, and many PLA_2s lack myotoxic effect. Owing to their high number of intrachain disulfide bridges, PLA_2 myotoxins are generally rigid and stable proteins (Scott *et al.*, 1990), which do not denature under usual handling conditions during purification and assay.

Isoforms

It is common to find isoforms of PLA_2 in snake venoms of a single species or even a single individual (Faure and Bon, 1987; Takasaki *et al.*, 1990; Valiente *et al.*, 1992, Fukagawa *et al.*, 1993). *Bothrops* venoms also present a diversity of myotoxic PLA_2 isoforms. Differences in the expression of myotoxin isoforms at the individual level have been shown in *B. asper*, where at least five variants can be distinguished electrophoretically (Lomonte and Carmona, 1992). Recent cloning studies on *Trimeresurus flavoviridis* myotoxic PLA_2s have shown the existence of six different isozyme genes (Nakashima *et al.*, 1993). There is an interesting ontogenetic regulation of myotoxin expression in *B. asper*, since newborn snakes appear not to express any isoform before 1 month of age (Lomonte *et al.*, 1987*b*; Lomonte and Carmona, 1992).

Bothrops myotoxin isoforms described to date cannot be resolved by separation techniques based on molecular size, such as gel filtration or SDS–PAGE. Thus, the use

of SDS–PAGE as the only criterion for myotoxin homogeneity can be misleading. Ion-exchange columns or charge-based electrophoretic techniques can usually resolve myotoxin isoforms differing in their net charge. An exception may be isoelectric focusing using broad pH gradients (i.e. range 3–9), which fail to resolve the highly basic myotoxins. Reverse-phase HPLC has also been useful in the separation of isoforms (Bruses *et al.*, 1993).

In *B. asper* venom, two sequenced myotoxin isoforms (II and III) differ in primary structure (Kaiser *et al.*, 1990; Francis *et al.*, 1991), therefore being products of related, but distinct genes, probably arising by duplication and divergence (Davidson and Dennis, 1990; Nakashima *et al.*, 1993). Sequence microheterogeneity has been observed in both myotoxins, as well as in bothropstoxin I from *B. jararacussu* (Cintra *et al.*, 1993). Since snake venom PLA_2s described so far are not glycosylated, additional isoform diversity cannot result from glycosylation microheterogeneity. It is possible that other post-translational modifications such as acylation, described for several PLA_2s (Cho *et al.*, 1988; Tomasselli *et al.*, 1989), might add to the molecular diversity of *Bothrops* myotoxins. Recent data demonstrate that *B. asper* myotoxin II can undergo autocatalytic acylation (Pedersen *et al.*, 1995), as discussed in *Structure–function relationships* (pp. 1417–1418).

While all myotoxins in *Bothrops* venoms have PLA_2 structure, some of them lack catalytic activity owing to critical amino acid substitutions in the calcium-binding loop, especially Lys for Asp-49. The discovery of a Ser-49 myotoxic PLA_2, ammodytin L, in the venom of *Vipera ammodytes* (Krizaj *et al.*, 1991) prompts for the possible presence of variants other than Lys-49 replacing Asp-49 in *Bothrops* venoms. Protein engineering studies (Van den Bergh *et al.*, 1989) demonstrated that the low enzymatic activities originally reported for some Lys-49 PLA_2s (Maraganore *et al.*, 1984) are most probably due to contaminant traces of Asp-49 enzymes (Van den Bergh *et al.*, 1989; Scott *et al.*, 1992). The existence and wide distribution of enzymatically inactive Lys-49 myotoxins in

Table 1. *Bothrops* myotoxins

Species	Toxin name	Mol. wt	p*I*	PLA_2 activity*	Reference
B. asper	myotoxin I	10,700†	n.d.	(+)	Gutiérrez *et al.* (1984*a*)
B. asper	myotoxin II	13,300§	n.d.	(−)	Lomonte and Gutiérrez (1989)
B. asper	myotoxin III	13,900§	>9.5	(+)	Kaiser *et al.* (1990)
B. asper	myotoxin IV	15,500‡	n.d.	(−)	Díaz *et al.* (1995)
B. asper	myotoxic PLA_2	14,100	n.d.	(+)	Mebs and Samejima (1986)
B. nummifer	myotoxin	16,000‡	n.d	(−)	Gutiérrez *et al.* (1986*b*)
B. nummifer	myotoxin peak IV	15,000‡	~10.6	n.d.	Brusés *et al.* (1993)
B. jararacussu	S_{III}-SP_{IV} (bothropstoxin I)	13,869§	~8.2	(−)	Homsi-Brandeburgo *et al.* (1988) Cintra *et al.* (1993)
B. jararacussu	bothropstoxin II	15,784§	~7.7	(+)	Homsi-Brandeburgo *et al.* (1988)
B. insularis	myotoxin	15,000‡	n.d.	(+)	Selistre *et al.* (1990)
B. atrox	myotoxin	13,400§	n.d.	(+)	Lomonte *et al.* (1990*b*)
B. moojeni	myotoxin I	13,400§	n.d.	(−)	Lomonte *et al.* (1990*b*)
B. moojeni	myotoxin II	13,400§	n.d.	(−)	Lomonte *et al.* (1990*b*)
B. moojeni	MOO-1	15,000‡	n.d.	(+)	Moura *et al.* (1991*a*)
B. pradoi	PRA-1	15,000	n.d.	(+)	Moura *et al.* (1991*a*)
B. godmani	myotoxin I	14,300§	~8.2	(+)	Díaz *et al.* (1992)
B. godmani	myotoxin II	13,400§	~8.9	(−)	Díaz *et al.* (1992)

Monomeric mol. wts estimated by † gel filtration; ‡ SDS–PAGE; § amino acid composition. n.d., Not determined.

*(−) refers to lack of activity or extremely low activity (lower than 2 μEq fatty acid mg^{-1} min^{-1}). Some of these myotoxins have been shown to be Lys-49 variants.

Table 2. Sequences of myotoxic phospholipases A_2 from *Bothrops* venoms

	1			5				10				15					20					25			
(a)	S	L	I	E	F	A	K	M	I	L	E	E	T	K	R	L	P	F	P	Y	—	Y	T	T	Y	G	C
(b)	S	L	F	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	A	K	S	—	Y	G	A	Y	G	C
(c)	S	L	F	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	A	K	S	—	Y	G	A	Y	G	C
(d)	S	L	V	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	L	T	S	—	Y	G	V	Y	G	C
(e)	S	L	V	E	L	G	K	M	I	L	Q	E	T	G	K	N	P	V	T	—	—	Y	G	A			Y
			30					35				40					45				50						
(a)	Y	C	G	W	G	G	Q	G	Q	P	K	D	A	T	D	R	C	C	F	V	H	D	C	C	Y	G	—
(b)	N	C	G	V	L	G	R	G	K	P	K	D	A	T	D	R	C	C	Y	V	H	K	C	C	Y	K	—
(c)	N	C	G	V	L	G	R	G	K	P	K	D	A	T	D	R	C	C	Y	V	H	K	C	C	Y	K	—
(d)	N	C	G	V	G	S	R	H	K	P	K	D	D	T	D	R	C	C	Y	V	H	K	C	C	Y		
	55					60					65					70					75					80	
(a)	—	—	K	L	S	N	C	—	—	—	—	—	—	K	P	K	T	D	R	Y	S	Y	S	R	K	S	G	V
(b)	—	—	K	L	T	G	C	—	—	—	—	—	—	N	P	K	K	D	R	Y	S	Y	S	W	K	D	K	T
(c)	—	—	K	L	T	G	C	—	—	—	—	—	—	N	P	K	K	D	R	Y	S	Y	S	W	K	D	K	T
				85					90					95					100				105				
(a)	I	I	C	—	G	E	G	T	P	C	E	K	Q	I	C	E	C	D	K	A	A	A	V	C	F	R	E
(b)	I	V	C	—	G	E	N	N	S	C	L	K	E	L	C	E	C	D	K	A	V	A	I	C	L	R	E
(c)	I	V	C	—	G	E	N	N	P	C	L	K	E	L	C	E	C	D	K	A	V	A	I	C	L	R	E
		110					115					120					125					130					
(a)	N	L	R	T	Y	K	K	R	Y	M	A	Y	P	D	L/F	L	C	—	K	K	P	A	E/D		K/P		C
(b)	N	L	N	T	Y	N	K	K	Y	R	Y	Y	L	K	P	L/F	C	—	—	K	K	A	D		A		C
(c)	N	L	G	T	Y	N	K	K	Y	R	Y	H	L	K	P	F	C	—	—	K	K	A	D		P		C

(a) *B. asper* myotoxin III (Kaiser *et al.*, 1990); (b) *B. asper* myotoxin II (Francis *et al.*, 1991); (c) *B. jararacussu* bothropstoxin I (Cintra *et al.*, 1993); (d) *B. atrox* PLA_2 (N-terminal 51 residues), (Maraganore *et al.*, 1984); (e) *B. asper* myotoxin IV (N-terminal 24 residues) (Díaz *et al.*, 1995).

Bothrops spp., as well as in other crotalids such as *Agkistrodon* spp. (Maraganore *et al.*, 1984) and *Trimeresurus* spp. (Yoshizumi *et al.*, 1990; Liu *et al.*, 1990, 1991), is intriguing from an evolutionary point of view. One may wonder why the PLA_2-inactive variants, probably originating from active counterparts, have been maintained during evolution, and appear to be quantitatively important components of some venoms. To date, no striking qualitative differences in the *in vivo* pharmacological properties between Lys-49 and Asp-49 isozymes have been reported. In *B. asper* venom it was surprising to detect a Lys-49 isoform (myotoxin II) in every individual, in contrast to enzymatically active isoforms (myotoxins I and III) which varied in their expression among different individuals (Lomonte and Carmona, 1992).

Structural analysis

Bothrops myotoxins are classified as group II PLA_2s, together with all crotalid/viperid venom enzymes and the secreted non-pancreatic mammalian PLA_2 (Davidson and Dennis, 1990). Some *Bothrops* myotoxins have been shown to occur as dimers: *B. asper* myotoxins II and III (Lomonte and Gutiérrez, 1989; Francis *et al.*, 1991), *B. nummifer* myotoxin (Gutiérrez *et al.*, 1986*b*; Bruses *et al.*, 1993) and *B. insularis* myotoxin (Selistre *et al.*, 1990). Amino acid composition analysis indicates that these toxins are rich in basic and hydrophobic amino acids (Gutiérrez *et al.*, 1984*a*, 1989; Homsi-Brandeburgo *et al.*, 1988; Selistre *et al.*, 1990; Lomonte and Gutiérrez, 1989; Lomonte *et al.*, 1990*b*; Díaz *et al.*, 1992, 1995*b*). Complete sequences of three toxins are available (Table 2). *B. asper* myotoxin II and *Bothrops jararacussu* bothropstoxin I are highly homologous Lys-49 PLA_2s, differing by only 4 or 5 out of 121 residues (Francis *et al.*, 1991; Cintra *et al.*, 1993). *Bothrops asper* myotoxin III is an Asp-49 PLA_2 of 122 residues (Kaiser *et al.*, 1990). There is higher

homology between Lys-49 myotoxins of different genera (i.e. *Bothrops*, *Agkistrodon*, and *Trimeresurus* spp.) than between Lys-49 and Asp-49 proteins of the same species, indicating that the gene divergence occurred earlier than the separation of these genera (Francis *et al.*, 1991).

The first crystal structure of a *Bothrops* venom protein, *B. asper* myotoxin II, has been recently reported (Arni and Gutiérrez., 1993; Arni *et al.*, 1995). As expected on the basis of the conserved architecture of PLA_2s (Dijkstra *et al.*, 1981; Renetseder *et al.*, 1985; Westerlund *et al.*, 1992), the three-dimensional structure of myotoxin II closely resembles that of *Agkistrodon p. piscivorus* Lys-49 PLA_2 (Holland *et al.*, 1990; Scott *et al.*, 1992), which shares over 75% sequence identity (Francis *et al.*, 1991). However, in contrast to the latter, myotoxin II is dimeric, both in solution (Lomonte and Gutiérrez, 1989; Francis *et al.*, 1991) and in the crystal state (Arni *et al.*, 1995), and represents a novel dimeric form of PLA_2. The two monomers in the asymmetric unit are related by a nearly perfect two-fold axis, but the dimer differs from that of *Crotalus atrox* PLA_2 (Brunie *et al.*, 1985) in that putative 'catalytic' sites are exposed to the solvent (Arni *et al.*, 1995). When compared to Asp-49 catalytically-active PLA_2s, myotoxin II has an altered local conformation in the calcium-binding region, since the ε-amino group of Lys-49 fills the site normally occupied by the calcium ion in Asp-49 enzymes (Arni *et al.*, 1995). The crystal structure of myotoxin II will be important in future studies attempting to define the toxic site(s) of *Bothrops* myotoxins, so far explored on the basis of sequence comparisons (Francis *et al.*, 1991) and molecular modelling (Lomonte *et al.*, 1994*e*).

Cloning

Moura *et al.* (1993) reported the molecular cloning of a myotoxic PLA_2 from *B. jararacussu*. With this powerful approach, in combination with site-directed mutagenesis and the growing crystallographic information, significant progress on the structure–function relationship of both crotalid (Moura *et al.*, 1993; Ownby and Li, 1993) and elapid (Hodgson *et al.*, 1993) PLA_2 myotoxins is soon to be expected.

NEUTRALIZATION AND ANTIGENIC RELATIONSHIPS

Polyclonal and monoclonal antibodies to *Bothrops* myotoxins have been useful to assess, by neutralization studies, the relative contribution of these toxins in the muscle-damaging activity of crude venoms; to determine the presence and antigenic relationships of myotoxins from different venoms; to study the structure–function relationship in myotoxins; to visualize myotoxin binding to muscle sections *in vitro*; and to assess the distribution of myotoxins and antibodies *in vivo*, in animal models.

Anti-myotoxin sera, and affinity-purified polyclonal antibodies from antivenoms

Monospecific antibodies to myotoxins are readily purified from commercially available antivenoms by affinity chromatography on immobilized myotoxin columns. Equine antibodies to *B. asper* myotoxins I and II obtained by this means were utilized in neutralization studies to demonstrate that basic PLA_2 myotoxins of this venom (all of them are cross-reactive when tested against polyclonal antibodies) are the main factors in the development of myonecrosis: preincubation of crude venom with antibodies to myotoxins I or II abolished 79 and 75%, respectively, of the muscle damage in mice (Lomonte *et al.*, 1985, 1990*c*). This issue was also approached by raising antisera to purified *B. asper*

myotoxins I or II in rabbits: the monospecific antisera inhibited 70–80% of the venom myotoxic effect in preincubation experiments (Lomonte *et al.*, 1987*a*; and unpublished results). In agreement with these findings, Moura *et al.* (1991*b*) showed that a mouse antiserum against *B. jararacussu* myotoxin (JSU-5, corresponding to bothropstoxins described by Homsi-Brandeburgo *et al.*, 1988) neutralized virtually all the myotoxic effect of the homologous crude venom in preincubation tests.

Anti-myotoxin polyclonal antibodies have revealed cross-reacting components in a variety of crude venoms from different species (Lomonte *et al.*, 1985, 1987*b*, 1990*a*; Moura *et al.*, 1990, 1991*a*, *b*). The conclusion emerging from these studies is that a 'family' of antigenically related myotoxic PLA_2s occurs in many, although not all, *Bothrops* venoms. As a consequence, there is a high degree of cross-reactivity between different equine antivenoms to *Bothrops* spp. venoms produced in Latin America, regarding their ability to recognize these myotoxins in enzyme-immunoassays (Lomonte *et al.*, 1991; and unpublished data). Similar observations were made by preparing monospecific *Bothrops* antivenoms in mice (Moura *et al.*, 1990). Moreover, several of these myotoxins cross-react with structurally related PLA_2s from other crotalid genera, such as *Trimeresurus* and *Agkistrodon* spp. For example, *T. flavoviridis* basic protein I (Yoshizumi *et al.*, 1990) and *A. bilineatus* basic PLA_2 I (Nikai *et al.*, 1993) are strongly recognized by rabbit antibodies to *B. asper* myotoxins I and II, by enzyme-immunoassay (cross-reactivities of 89 and 70%, respectively; unpublished observations by our group and Profs M. Ohno, University of Kyushu, and T. Nikai, University of Meijo, respectively). The conservation of at least some epitopes among several of these crotalid PLA_2s is in agreement with their high sequence homology and similar three-dimensional structure. It would be of interest to assess whether the recognition of these cross-reactive epitopes by antibodies leads to an inhibition of toxic activities.

Polyclonal antibodies to *B. asper* myotoxins were utilized to visualize toxin binding sites on frozen muscle sections exposed *in vitro*, by immunohistochemical staining. Both myotoxin I (Brenes *et al.*, 1987) and II (Lomonte, unpublished data) can be visualized as a homogeneous staining pattern along the periphery of muscle fibres, with no evidence of binding to internal components of the cells.

Monoclonal antibodies (MAbs)

After the isolation of the first *Bothrops* myotoxin (Gutiérrez *et al.*, 1984*a*), the development of MAbs to this toxin provided unequivocal evidence for the presence of several major isoforms in the venom of *B. asper* (Lomonte and Kahan, 1988), now purified and referred to as myotoxins I-IV (Table 1). MAbs have been extremely valuable in neutralization studies, confirming earlier results with polyclonal antibodies. Single neutralizing MAbs, such as MAb-3 or MAb-4, abolished the muscle-damaging effect of the whole venom in preincubation experiments (Lomonte *et al.*, 1992). In addition, MAbs to *B. asper* myotoxins were utilized in a quantitative enzyme-immunoassay to show the immediate *in vivo* disappearance of myotoxins from plasma, in a mouse model (Rovira *et al.*, 1992).

MAbs have also been useful in approaching the structure–function relationship of *B. asper* myotoxins. A clear dissociation between PLA_2 activity and myotoxic effect was observed when myotoxin I was neutralized with MAb-3 or MAb-4 (Lomonte *et al.*, 1992). A precise mapping of the epitopes recognized by MAb-3 and MAb-4 could help to point out the molecular region involved in myotoxicity. These two MAbs recognize different—perhaps overlapping—myotoxin epitopes, as suggested by their different patterns of

isoform recognition and competition binding data (Lomonte and Kahan, 1988). Unfortunately, their reactivity is lost upon antigen denaturation (Lomonte and Kahan, 1988; and unpublished data), indicating the recognition of conformational, discontinuous epitopes. However, the neutralizing ability of these MAbs does not necessarily imply a recognition of the myotoxic site, as other well-known indirect neutralization mechanisms may occur (Ménez *et al.*, 1992). Both neutralizing MAbs have the ability individually to form precipitable complexes with myotoxins, in contrast to non-neutralizing ones (Lomonte and Kahan, 1988). Thus, it remains to be determined whether monovalent Fab fragments of these neutralizing MAbs still retain their ability to inhibit myotoxins, in the absence of a macromolecular complex formation mechanism.

Non-immunological neutralizing agents

Despite the idea of utilizing heparin in the treatment of certain snakebites—aiming at the coagulation disorders—being old (Ahuja *et al.*, 1946), it was shown only recently that heparin may be therapeutically useful against the myotoxicity of some venoms, being able to neutralize this effect (Melo and Suarez-Kurtz, 1988). Heparin, and other sulfated glycosaminoglycans and polyanions, have been shown to form complexes with PLA_2 myotoxins isolated from *B. jararacussu* (Melo *et al.*, 1993) and *B. asper* (Lomonte *et al.*, 1994*c*) venoms, blocking their toxic activity when preincubated *in vitro*. The fact that these complexes are held, at least partially, by electrostatic forces, and that several types of glycosaminoglycans can bind (Melo *et al.*, 1993; Lomonte *et al.*, 1994*c*, *e*), suggests a 'non-specific' type of interaction. However, two observations raise the possibility that a specific recognition element might additionally be involved: first, not all structural types of highly basic myotoxins appear to be inhibited by heparin (Lomonte *et al.*, 1994*c*). Second, the overall charge density of different glycosaminoglycans does not correlate with their myotoxin-binding ability. For example, the binding of chondroitin sulfate and dermatan sulfate to *B. asper* myotoxin II is weaker than the binding of heparan sulfate, although the latter is less sulfated (Lomonte *et al.*, 1994*e*).

The secreted non-pancreatic human PLA_2, which has a similar molecular architecture and shares significant homology with crotalid class II PLA_2s (Wery *et al.*, 1991), was recently shown to interact with heparin *in vitro*, under physiological conditions (Dua and Cho, 1994). As both heparin (Ekre *et al.*, 1992) and secreted PLA_2 (Pruzanski and Vadas, 1991) are released during inflammatory responses, their interaction is probably not fortuitous, but may have evolved as a physiological regulatory mechanism.

The ability of heparin to neutralize the toxic actions of PLA_2 myotoxins is independent of its anticoagulant activity (Lomonte *et al.*, 1994*c*, *e*). This makes the non-anticoagulant forms of heparin (isolated by their low affinity for antithrombin, and comprising as much as two-thirds of conventional heparin preparations; Roden *et al.*, 1992), attractive candidates for the *in vivo* neutralization of myotoxins. However, preliminary observations suggest that, *in vivo*, high-affinity heparin-binding factors may compete strongly with myotoxins, thus affecting the neutralizing ability of heparin (Lomonte *et al.*, 1994*c*).

The blood serum of *Clelia clelia*, an ophiophagous colubrid snake distributed in Latin America, neutralizes the myotoxic activity of *B. asper* venom in mice (Lomonte *et al.*, 1982), and should therefore contain an inhibitor of its myotoxic PLA_2s. This putative inhibitor(s) has not been purified yet. The blood sera of two marsupials, *Didelphis marsupialis aurita* and *Philander oppossum*, have also been shown to neutralize the myotoxic effect of *B. jararacussu* venom *in vitro* and *in vivo* (Melo and Suarez-Kurtz, 1988).

This serum factor is an α-glycoprotein with acidic properties. An extract from the plant *Eclipta prostrata* is able to neutralize the myotoxic effect of *Bothrops* spp. (Melo *et al.*, 1988) and of *Crotalus durissus terrificus* (Mors *et al.*, 1989) venoms. In these studies, wedelolactone was identified as one of the active components of the plant extracts.

PATHOGENESIS OF MUSCLE DAMAGE

Bothrops myotoxins induce prominent muscle damage of rapid onset after i.m. injection. The pathogenesis of this effect has been investigated by following the morphological and biochemical alterations in affected muscle.

Macroscopic observations

A few minutes after i.m. injection of myotoxins, mice have difficulties in moving the hind leg (Gutiérrez *et al.*, 1989, 1991). After approximately 10 min there is a moderate swelling of the injected muscle, lasting for about 6 hr (Gutiérrez *et al.*, 1989, 1991). No haemorrhage is observed in mice injected with myotoxins.

Histological analysis

The first morphological alterations induced by myotoxins in muscle fibres are focal, peripheral, wedge-shaped lesions which are observed as early as 15 min after injection (Gutiérrez *et al.*, 1984*a*, *b*, 1989, 1991). These are identical to the 'delta lesions', originally described in Duchenne muscular dystrophy (Mokri and Engel, 1975), as well as in detergent-induced myonecrosis (Pestronk *et al.*, 1982), which are focal areas of degeneration located beneath portions of the cell where the plasma membrane is discontinuous or lost. After this initial alteration, there is a hypercontraction of myofibrils, with clumping of myofilaments (Gutiérrez *et al.*, 1984*a*, *b*, 1989, 1990, 1991). Similar alterations have been described for other myotoxic agents present in snake venoms, such as for elapid cardiotoxins (Duchen *et al.*, 1974; Ownby *et al.*, 1993) and elapid PLA_2s (Harris *et al.*, 1975; Harris and Maltin, 1982; Sharp *et al.*, 1993), an indication that structurally different venom components can induce a similar pattern of pathological changes. Myonecrosis is widespread 3 hr after injection of *Bothrops* myotoxins. In the case of myotoxin I from *B. asper*, between 3 and 6 hr there is a change in the morphological pattern of necrotic muscle fibres, as they shift from the clumped, hypercontracted pattern to a more hyaline pattern where myofibrillar material has a more uniform distribution (Gutiérrez *et al.*, 1984*b*, 1990). Inflammatory infiltrate, composed of polymorphonuclear leucocytes and macrophages, is observed after the 6th hr and becomes abundant by 24–48 hr (Gutiérrez *et al.*, 1984*b*, 1989, 1990, 1991). No histological alterations have been found in blood vessels or nerves after injection of these myotoxins (Gutiérrez *et al.*, 1984*b*, 1989, 1991).

Intravital microscopy

The immediate stages of muscle damage induced by *B. asper* myotoxin II were analysed by intravital microscopy in the mouse cremaster muscle (Lomonte *et al.*, 1994*a*). Muscle fibres exposed to myotoxin II respond within seconds with strong and slow contractions, which cause pronounced distortions of the local microvasculature, and cease in 1–2 min This phenomenon is compatible with the proposed action of *Bothrops* myotoxins at the plasma membrane level, causing a rapid calcium influx. Subsequently, 3–4 min after

exposure, muscle fibres develop a narrow transverse band with local loss of striation, followed by a slow retraction of myofibrils in opposite directions until a transverse rupture of the fibre occurs. This phenomenon is repeated at several points along the muscle fibre, finally leaving rows of hypercontracted fibre fragments separated by spaces apparently devoid of myofilaments (Lomonte *et al.*, 1994*a*).

In addition to the effects on muscle fibres, the local oedema-inducing activity of myotoxin II (Lomonte and Gutiérrez, 1989; Lomonte *et al.*, 1993) was confirmed intravitally, by visualizing a rapid leakage of fluoresceine-labelled dextran marker from the muscle microvasculature, particularly at small venules and at their adjoining capillary segments (Lomonte *et al.*, 1994*a*).

Ultrastructural analysis

The earliest alterations after i.m. injection of myotoxins from *B. asper* (Gutiérrez *et al.*, 1984*b*) and *B. jararacussu* (Gutiérrez *et al.*, 1991) are focal disruptions or discontinuities at the plasma membrane. These focal lesions are also characterized by hypercontraction of myofilaments. Cells in more advanced degenerative stages are characterized by prominent hypercontraction of myofilaments, leaving cytosolic spaces apparently devoid of myofilaments. At later time periods, myofilaments shift to a more uniform pattern of distribution, no longer showing a clumped appearance (Gutiérrez *et al.*, 1984*b*, 1990, 1991).

Necrotic cells exhibit conspicuous alterations in all organelles. Mitochondria show high-amplitude swelling, dense intracrystal spaces, flocculent densities and vesiculated cristae. In some of them the membranes are disrupted (Gutiérrez *et al.*, 1984*b*, 1991). A large population of small vesicles appears in necrotic cells, probably as a consequence of intracellular membrane alterations, since no sarcoplasmic reticulum or T tubules are observed. Moreover, *B. asper* myotoxin I inhibits calcium-ATPase activity of rabbit sarcoplasmic reticulum and hydrolyses phospholipids of this organelle (Gutiérrez *et al.*, 1987). Necrotic fibres show myonuclei with clumped chromatin and disrupted membranes. Throughout the process of necrosis, the basal lamina remains apparently intact at the periphery of the cells. However, there may be changes in specific basal lamina components, such as those reported in other muscle pathologies (Gulati *et al.*, 1983; Gulati, 1985). Polymorphonuclear leucocytes and macrophages are abundant after the 6th hr, both in the interstitial space and inside necrotic fibres (Gutiérrez *et al.*, 1984*b*, 1991).

Biochemical alterations

A prominent increase in plasma creatine kinase levels is observed after injection of *Bothrops* myotoxins (Gutiérrez *et al.*, 1984*a*, 1989, 1991; Lomonte and Gutiérrez, 1989; Lomonte *et al.*, 1990*b*; Moura *et al.*, 1990, 1991*b*; Díaz *et al.*, 1992). Creatine kinase levels peak between 1 and 3 hr, decreasing afterwards. Simultaneously, there is a reduction in creatine content and creatine kinase activity in the injected gastrocnemius, but not in the contralateral muscle (Moreno and Gutiérrez, 1988). An increment in muscle calcium levels occurs after administration of *B. asper* myotoxin I (Gutiérrez *et al.*, 1984*a*) and *B. nummifer* myotoxin (Gutiérrez *et al.*, 1989).

Changes in myofibrillar proteins after injection of *B. asper* myotoxin I were studied by Gutiérrez *et al.* (1990). The initial stage of hypercontraction is not associated with drastic myofilament degradation, with the exception of desmin, which disappears as early as 15 min after injection. After the 3rd hr, myofilaments shift from a hypercontracted to a hyaline pattern, a change associated with the removal of α-actinin. Finally, at later time

periods, there is a widespread degradation of all myofibrillar proteins, probably as a consequence of the activity of proteases from inflammatory phagocytic cells.

Histological, ultrastructural and biochemical evidence strongly indicate that muscle cell plasma membrane is the first cellular structure to be affected by *Bothrops* myotoxins. Immunohistochemical staining of muscle sections exposed to *B. asper* myotoxins I (Brenes *et al.*, 1987) and II (Lomonte, unpublished results) *in vitro* showed the binding of these toxins only at the periphery of muscle cells.

Skeletal muscle regeneration

Muscle regeneration after myonecrosis induced by *B. asper* myotoxin I (Gutiérrez *et al.*, 1984*c*), bothropstoxins I and II from *B. jararacussu* (Homsi-Brandeburgo *et al.*, 1988; Gutiérrez *et al.*, 1991) and *B. nummifer* myotoxin (Gutiérrez *et al.*, 1989) proceeds normally and successfully. Small regenerating muscle cells, with centrally located nuclei, are observed 1 week after injection. By 28 days, the regenerative process is complete, as the diameter of regenerating muscle fibres does not differ from the diameter of adult normal muscle fibres, although regenerating cells still have centrally located nuclei. Nerves and blood vessels remain intact throughout the process of regeneration and no fibrosis or proliferation of fibroblasts is observed. Very similar findings have been described with other myotoxic PLA_2s isolated from elapid snake venoms, such as notexin (Harris *et al.*, 1975) and taipoxin (Maltin *et al.*, 1983). It was proposed that the regenerative process is adequate because myotoxins do not affect blood vessels or nerves, and the basal lamina remains at the periphery of necrotic cells (Gutiérrez *et al.*, 1984*c*, 1989, 1991), playing the role of a scaffold for muscle regeneration (Vracko and Benditt, 1972).

In contrast to the observations described, muscle regeneration after injection of crude venoms with myotoxic and haemorrhagic activities is severely impaired (Gutiérrez *et al.*, 1984*c*; Queiroz *et al.*, 1984; Arce *et al.*, 1991). In these cases there is abundant fibroblast proliferation and fibrosis, and regenerating fibres are of a very small diameter. It was suggested that the drastic microvascular alterations induced by haemorrhagic toxins are responsible for the poor regeneration, as an adequate blood supply is a key condition for muscle regeneration (Allbrook, 1981; Grounds, 1991).

STUDIES ON THE MECHANISM OF ACTION

Effects on liposomes

Liposomes are useful models for studying the interaction of cytolysins with membranes. Various studies have demonstrated that *Bothrops* myotoxins disrupt multilamellar and unilamellar vesicles, releasing entrapped enzymatic or fluorescent markers (Díaz *et al.*, 1991; Rufini *et al.*, 1992). Electron-spin resonance measurement of intravesicular TEMPOcholine reduction by external ascorbate was also used to monitor liposome leakage (Rufini *et al.*, 1992). Negatively charged multilamellar liposomes are affected by these myotoxins, whereas no effect is observed on positively charged vesicles (Díaz *et al.*, 1991; Bultrón *et al.*, 1993*a*). Unilamellar vesicles made of phosphatidylcholine and phosphatidic acid are more susceptible to *B. asper* myotoxin II than vesicles made only of phosphatidylcholine (Rufini *et al.*, 1992). These studies indicate that *Bothrops* myotoxins affect preferentially negatively charged bilayers, suggesting the involvement of basic amino acids in the course of the membrane-perturbing mechanism, perhaps in the binding step. Interestingly, liposome disruption by *B. asper* myotoxin III is inhibited at 4°C (Bultrón

et al., 1993*a*), suggesting that membrane fluidity is an important factor in the mechanism of action.

The liposome-disrupting effect of two myotoxins which lack PLA_2 activity (*B. asper* myotoxin II and *B. moojeni* myotoxin II) is not affected when the assay is performed in the absence of calcium, and hence of enzymatic activity (Díaz *et al.*, 1991; Rufini *et al.*, 1992). In contrast, the liposome-disrupting effect of two enzymatically active myotoxins (*B. asper* myotoxin I and *B. atrox* myotoxin) is significantly decreased, although not completely abolished, by elimination of calcium (Díaz *et al.*, 1991). These results suggest that *Bothrops* myotoxins are capable of disrupting bilayers by a mechanism independent of enzymatic phospholipid hydrolysis, and that PLA_2 activity in enzymatically active variants enhances liposome disruption. Pedersen *et al.* (1994) clearly confirmed this hypothesis, by demonstrating that *B. asper* myotoxin II causes rapid membrane leakage of liposomes made of non-hydrolysable ether-linked phospholipids.

Effects on muscle preparations in vitro

When *B. asper* myotoxins I and III, *B. nummifer* myotoxin, and *B. jararacussu* bothropstoxin are incubated with mouse gastrocnemius and extensor digitorum longus muscles, a dose-dependent release of creatine kinase occurs, indicating a membrane-disrupting activity (Gutiérrez *et al.*, 1986*a*, *b*; Melo *et al.*, 1993; Bultrón *et al.*, 1993*a*). In the case of *B. asper* myotoxin III, membrane damage is inhibited if the muscles are incubated at 4°C (Bultrón *et al.*, 1993*a*). When calcium is eliminated and EDTA added to the bathing solution, there is a significant reduction in creatine kinase release induced by *B. asper* myotoxin III, although a residual myotoxicity is observed (Bultrón *et al.*, 1993*a*). In contrast, there is no reduction in myotoxic activity when enzymatically inactive *B. nummifer* myotoxin is incubated in the absence of calcium (Gutiérrez *et al.*, 1986*b*).

Bothrops jararacussu bothropstoxin I inhibits muscle twitch tension evoked either directly or indirectly through stimulation of the motor nerve in the mouse phrenic nerve–diaphragm preparation (Heluany *et al.*, 1992). Moreover, the toxin induces membrane depolarization, which is inhibited in the presence of 10 mM Ca^{2+}, and contracture (Heluany *et al.*, 1992).

Effects on cells in culture

Despite the observation that *Bothrops* myotoxins seem to affect only skeletal muscle *in vivo* (Gutiérrez *et al.*, 1986*a*; Moreno and Gutiérrez, 1988), studies with cultured cells clearly indicate that they affect not only myoblasts and myotubes, but also other cell types. Myotoxins from *B. nummifer* are cytotoxic for cultured muscle cells, neurones, macrophages and fibroblasts, although they are more active towards myotubes (Brusés *et al.*, 1993). Moreover, *B. asper* myotoxins II (Lomonte *et al.*, 1994*d*) and III (Bultrón *et al.*, 1993*b*) induce membrane damage in a variety of cell types in culture. Cytotoxicity is higher if cells are incubated in solutions devoid of calcium, decreasing when calcium is added (Bultrón *et al.*, 1993*b*). Similar observations have been made with other membrane-disrupting agents (Bashford *et al.*, 1986). It has been observed that divalent cations protect cells against the action of a variety of cytolytic agents (Bashford *et al.*, 1989). As in the case of liposomes, myotoxins II and III lack cytotoxicity at 4°C, suggesting that this effect is influenced by membrane fluidity (Bultrón *et al.*, 1993*b*; Lomonte *et al.*, 1994*d*). The only cells that appear to be resistant to the membrane-damaging activity of *B. asper* myotoxins I, II, and III are erythrocytes from several species (Gutiérrez *et al.*, 1986*a*; Bultrón *et al.*,

1993*b*; Gené, unpublished results), despite that myotoxin II has been reported to bind mouse erythrocytes (Rovira *et al.*, 1992).

These studies clearly indicate that at least some *Bothrops* myotoxins affect *in vitro* many cell types in addition to skeletal muscle myoblasts, thus behaving more as cytotoxins than as myotoxins. Nevertheless, cultured myotubes are more susceptible than myoblasts to *B. asper* myotoxins I and II (V. Arce, personal communication), suggesting that the observed specificity of myotoxins for skeletal muscle *in vivo* may be due to a much higher susceptibility of mature muscle cells as compared to other cell types.

Since *B. asper* myotoxin II does not damage target cells at 4°C, Lomonte *et al.* (1994*d*) showed that washing with cold culture medium easily removed the toxin from cells, resulting in a loss of cytolytic effect when cultures were subsequently transferred to 37°C. Moreover, even after 30 min of incubation with cells at 4°C, myotoxin II was still susceptible to neutralization by a monoclonal antibody (MAb-3) or by heparin with low affinity for antithrombin. These results indicate that the strength of interaction of myotoxin II with its putative membrane target is greatly diminished at 4°C, and suggest that in addition to an electrostatic interaction, hydrophobic penetration is probably required in the cytolytic mechanism (Lomonte *et al.*, 1994*d*).

The broad cytolytic specificity of *Bothrops* myotoxins evaluated so far is in contrast to the lack of *in vitro* cytolytic activity of basic PLA_2 myotoxins isolated from *Notechis s. scutatus* (notexin) and *Vipera russelli* venoms (Lomonte *et al.*, 1994*d*), suggesting that different molecular pathways of muscle damage caused by PLA_2 myotoxins might exist.

Other pharmacological activities

Lethal activity has been reported for *B. nummifer* myotoxin, *B. asper* myotoxin I, *B. godmani* myotoxin II, and *B. jararacussu* bothropstoxin I, with i.v. LD_{50}s of 3.9, 5.6, 4.2, and 4.8 mg/kg, respectively (Gutiérrez *et al.*, 1986*a*, *b*; Homsi-Brandeburgo *et al.*, 1988; Díaz *et al.*, 1992) In addition, *B. asper* myotoxin I has an intraventricular LD_{50} of 0.025 mg/kg (Gutiérrez *et al.*, 1986*a*) and bothropstoxin I has an i.p. LD_{50} of 7.5 mg/kg (Homsi-Brandeburgo *et al.*, 1988). Thus, *Bothrops* myotoxins have low toxicity when tested by the i.v. route. This is in contrast to other myotoxic PLA_2s from elapid and viperid venoms, which are neurotoxic and highly lethal (Rosenberg, 1990).

Bothrops myotoxins induce oedema in the mouse footpad assay (Gutiérrez *et al.*, 1986*a*, *b*; Lomonte and Gutiérrez, 1989; Lomonte *et al.*, 1993, 1994*c*; Díaz *et al.*, 1992). Oedema induced by *B. asper* myotoxins I (Gutiérrez *et al.*, 1986*a*) and II (Lomonte *et al.*, 1993) is of rapid onset, reaching its peak by 1 hr and remaining high for several hours. The early oedema (30 min) is inhibited by pretreatment with cyproheptadine, indicating that histamine and/or serotonin are involved. The late oedema (5 hr) is inhibited by pretreatment with aspirin, suggesting the participation of prostaglandins (Gutiérrez *et al.*, 1986*a*). Although the oedema-inducing effect of PLA_2s has usually been attributed to their ability to hydrolyse phospholipids (Cirino *et al.*, 1989; Vishwanath *et al.*, 1988; Lloret and Moreno, 1993), the finding that *B. asper* myotoxin II can induce oedema in the absence of PLA_2 activity (Lomonte *et al.*, 1993, 1994*a*) implies a different mechanism of action for this pharmacological effect. An indirect oedema response to muscle damage seems unlikely, since there are highly myotoxic venoms (e.g. from several *Micrurus* spp.) that do not induce significant oedema (Gutiérrez *et al.*, 1983) in the mouse. The possible direct action of myotoxin II on endothelial cells or tissue mast cells has been speculatively suggested (Lomonte *et al.*, 1994*b*, *c*). *Bothrops asper* myotoxin II also induces a rapid systemic

interleukin-6 response in mice, presumably as an indirect consequence of muscle necrosis (Lomonte *et al.*, 1993), leading to an acute-phase response.

All the enzymatically active variants of myotoxins are anticoagulant *in vitro*, prolonging recalcification time of platelet-poor plasma. In contrast, *Bothrops* myotoxins that lack PLA_2 activity fail to induce anticoagulation (Gutiérrez *et al.*, 1986*a*, *b*; Lomonte and Gutiérrez, 1989; Lomonte *et al.*, 1990*b*; Kaiser *et al.*, 1990; Díaz *et al.*, 1992) The addition of phosphatidylserine prevents the anticoagulant effect of *B. godmani* myotoxin I, suggesting that anticoagulation might be due to an interference with the role of phospholipids involved in the coagulation cascade. The fact that only enzymatically active variants induce this effect strongly suggests that phospholipid hydrolysis is required for anticoagulation (Díaz *et al.*, 1991, 1992). *In vivo*, *B. asper* myotoxin I does not prolong clotting time (Alvarado and Gutiérrez, 1988), indicating that myotoxins are not involved in the haemostatic alterations observed in *Bothrops* envenomations.

Structure–function relationships

Kini and Iwanaga (1986) proposed that a cationic site around residues 79–87 is responsible for myotoxicity in presynaptic PLA_2 neurotoxins with myotoxic activity. Such a cationic site is not present in *B. asper* myotoxins II (Francis *et al.*, 1991) and III (Kaiser *et al.*, 1990), and *B. jararacussu* bothropstoxin I (Cintra *et al.*, 1993). When sequences of myotoxic and non-myotoxic PLA_2s are compared, the following amino acids are present in several myotoxic variants: Lys-38, Thr-112 and Tyr-113, in addition to three or four tyrosines between residues 112 and 121 (Francis *et al.*, 1991). In the three-dimensional structure of *B. asper* myotoxin II, Lys-38 lies close to the C-terminus (Arni *et al.*, 1995). Interestingly, the C-terminal region has been recently implicated in the cytolytic activity of *B. asper* myotoxin II (Lomonte *et al.*, 1994*e*).

Treatment of *B. asper* myotoxin II with cyanogen bromide releases its N-terminal octapeptide, resulting in the reduction of myotoxicity and liposome-disrupting activities (Díaz *et al.*, 1994*a*). A similar decrease in cytotoxicity after cyanogen bromide digestion was observed in the case of nigexin, a toxic PLA_2 from *Naja nigricollis* venom (Chwetzoff, 1990). The N-terminal domain in PLA_2s participates in the interfacial binding surface (Scott *et al.*, 1990), a region involved in the interaction with micellar substrates. Therefore, the observations made with *B. asper* myotoxin II may indicate that the N-terminal region plays a role in myotoxicity. Alternatively, such treatment may induce conformational changes affecting other regions of the molecule (Díaz *et al.*, 1994*a*).

Alkylation of *B. asper* myotoxin III with *p*-bromophenacyl bromide (BPB) reduces enzymatic activity by more than 95%, whereas the membrane-damaging effect on myoblasts was reduced by 70% (Bultrón *et al.*, 1993*b*), suggesting a dissociation of both activities. BPB modification of enzymatically inactive *B. asper* myotoxin II reduces its myotoxic and liposome-disrupting activities (Díaz *et al.*, 1993). This suggests that, in addition to the covalent modification of histidine, there may be conformational changes affecting pharmacological activities. Renetseder *et al.* (1988) described alterations in the crystal structure of a PLA_2 as a consequence of BPB modification.

Molecular analyses of the interaction between heparin and *B. asper* myotoxin II provided valuable insights on the structure–function relationships of this protein. In contrast to neutralizing MAbs (see *Monoclonal antibodies*, p. 1410), heparin was found to interact with a continuous stretch of amino acids, near the C-terminus of myotoxin II (Lomonte *et al.*, 1994*e*). This lysine-rich heparin-binding region includes residues 115–129

(numbering of Renetseder *et al.*, 1985), and is clearly separated from the 'catalytic' site (Scott *et al.*, 1990). Molecular modelling studies revealed that two lysine residues, Lys-36 and Lys-38, come in close vicinity to this region in the native structure of the protein (Arni *et al.*, 1995), enhancing its cationic character and, presumably, its heparin-binding ability (Lomonte *et al.*, 1994*e*). Interestingly, a synthetic peptide of residues 115–129 was able specifically to reproduce the cytolytic effect of myotoxin II on cultured cells, although with a 10–15-fold lower efficiency than the whole protein. The data support the conclusion that heparins neutralize myotoxin II by binding to a region directly involved in its cytolytic activity, providing the first experimental evidence of a cytotoxic region in a myotoxic PLA_2 (Lomonte *et al.*, 1994*e*). However, the role of this molecular region in myotoxicity remains to be investigated. While this putative cytotoxic region of myotoxin II is different from that predicted by Kini and Iwanaga (1986), it is of interest to note that it forms a hydrophobic/cationic combination, a structural feature that seems to be common to many cytotoxins (Kini and Evans, 1989; Bilwes *et al.*, 1994; Chien *et al.*, 1994).

Neutralization studies using heparins have also confirmed the dissociation of enzymatic and myotoxic activities of *B. asper* myotoxin III: myotoxicity was inhibited whereas PLA_2 activity was not affected (Lomonte *et al.*, 1994*c*). This observation is in agreement with the hypothesis that proposes the existence of a cytolytic toxin region distinct from the catalytic site.

Bothrops asper myotoxin II was found to undergo autocatalytic acylation, binding covalently long-chain fatty acids after *in vitro* incubation (Pedersen *et al.*, 1995). Acylated myotoxin binds to the surface of unilamellar liposomes, with the fatty acid moiety inserted into the lipid bilayer. Thus fatty acids may play a role as an anchor in the membrane and, perhaps, participating in the membrane perturbation induced by these myotoxins, a hypothesis that needs to be tested. Lugtigheid *et al.* (1993) recently demonstrated that acylation of porcine pancreatic PLA_2 influences its monolayer penetrating ability.

There is no information on the presence of membrane receptors for *Bothrops* myotoxic PLA_2s. The observation that negatively charged, but not positively charged, liposomes are disrupted by *Bothrops* myotoxins suggests that negatively charged phospholipids may play a role in the binding of these myotoxins to membranes (Díaz *et al.*, 1991; Rufini *et al.*, 1992). It would be relevant to study whether cell susceptibility correlates with the amount of negatively charged phospholipids present in the outer monolayer of plasma membranes. In an attempt to define whether anionic acceptor sites, different from phospholipids, play a role in the cytolytic mechanism of *B. asper* myotoxin II, cells were pretreated with neuraminidase, heparitinase, tunicamycin, and protamine, and subsequently assayed for cytotoxicity. However, none of these treatments modified the susceptibility of cells to the toxin, indicating that its action is not affected by removal of sialic acid or cell surface heparan sulfate, by inhibition of N-glycosylation of proteins, or by the presence of high concentration of protamine, a polycationic peptide, in the medium (Lomonte *et al.*, 1994*c*, *d*).

Lambeau *et al.* (1990, 1994) have purified, cloned and characterized a receptor for elapid PLA_2s from the membrane of muscle cells. It is a membrane protein of 180,000 mol. wt, with an N-terminal cysteine-rich domain, a fibronectin type II domain, eight repeats of a carbohydrate recognition domain, a transmembrane domain and an intracellular C-terminal domain (Lambeau *et al.*, 1994). It would be important to study whether this receptor participates in the interaction between *Bothrops* myotoxins and muscle plasma membrane.

CONCLUDING REMARKS: A WORKING HYPOTHESIS ON THE MECHANISM OF ACTION

The data collected on the effect of *Bothrops* myotoxins on cells, liposomes, and skeletal muscle *in vivo* may be unified in the following hypothetical mechanism of action: myotoxins bind to a still unidentified site in the muscle cell plasma membrane. This site may be a protein, such as the receptor characterized by Lambeau *et al.* (1994), a negatively charged phospholipid domain, or another type of membrane component. In any case, an electrostatic interaction between cationic sites of the toxins and negatively charged groups in the membrane seems to be involved. After this initial binding, myotoxins penetrate the bilayer by a hydrophobic interaction mediated by a cytotoxic region of the molecule, different from the catalytic site. It is suggested that this region combines basic and hydrophobic amino acid residues, such as the proposed cytolytic region of *B. asper* myotoxin II (Lomonte *et al.*, 1994*e*). In addition, acylation may contribute to the interaction and penetration of the cell membrane. Such hypothetical toxic domain would be present in both enzymatically active and inactive variants, although it may not be structurally identical in all myotoxins of this group. It is very likely, however, that the presence of hydrophobic residues flanked by cationic residues constitutes a common molecular design for the cytolytic region in these myotoxins. The penetration of this region in the hydrophobic core of the bilayer is responsible for membrane destabilization, with the consequent impairment in the regulation of the permeability to ions and macromolecules. Membrane penetration probably requires a fluid membrane, as myotoxins fail to induce cytotoxicity at 4°C. Besides this initial membrane perturbation, enzymatically active variants induce further membrane damage by hydrolysing bilayer phospholipids, an effect not observed with Lys-49 enzymatically inactive myotoxins. A prominent calcium influx is probably the most relevant consequence of membrane disturbance, being responsible for the onset of a variety of degenerative mechanisms such as cytoskeletal alterations (e.g myofilament hypercontraction), mitochondrial damage, and activation of calcium-dependent proteases and phospholipases which, in turn, cause further cell damage.

Although some progress has been made towards understanding the structure and mechanism of action of *Bothrops* myotoxins, our knowledge is still fragmentary. It is expected that new findings will provide a more clear picture on the way these toxins bind and interfere with membrane homeostasis, as well as on their structure–function relationships. Hopefully, such knowledge will reveal mechanisms of cellular damage relevant for muscle pathology in addition to snakebite, paving the way for the development of more efficient therapeutic strategies.

Acknowledgements—The important contributions by different collaborators and graduate students to the studies on myotoxins are gratefully acknowledged, as well as the financial support from Vicerrectoría de Investigación (Universidad de Costa Rica), International Foundation for Science, Third World Academy of Sciences, Swedish Agency for Research Cooperation with Developing Countries—SAREC, and Costa Rican National Council for Scientific and Technological Research (CONICIT).

REFERENCES

Ahuja, M. L., Veeraraghavan, N. and Menon, I. G. K. (1946) Action of heparin on the venom of *Echis carinatus*. *Nature* **158**, 878.

Allbrook, D. (1981) Skeletal muscle regeneration. *Muscle Nerve* **4**, 234–245.

Alvarado, J. and Gutiérrez, J. M. (1988) Anticoagulant effect of myotoxic phospholipase A_2 isolated from the venom of the snake *Bothrops asper* (Viperidae). *Rev. Biol. Trop.* **36**, 563–565.

Arce, V., Brenes, F. and Gutiérrez, J. M. (1991) Degenerative and regenerative changes in murine skeletal muscle after injection of venom from the snake *Bothrops asper*: a histochemical and immunocytochemical study. *Int. J. exp. Path.* **72,** 211–226.

Arni, R. K. and Gutiérrez, J. M. (1993) Crystallization and preliminary diffraction data of two myotoxins isolated from the venoms of *Bothrops asper* (terciopelo) and *Bothrops nummifer* (jumping viper). *Toxicon* **31,** 1061–1064.

Arni, R. K., Ward, R. J., Gutiérrez, J. M. and Tulinsky, A. (1995) Crystal structure of a calcium-independent phospholipase-like myotoxic protein from *Bothrops asper* venom. *Acta Crystallographica* **D51,** 311–317.

Bashford, C. L., Alder, G. M., Menestrina, G., Micklem, K. J., Murphy, J. J. and Pasternak, C. A. (1986) Membrane damage by hemolytic viruses, toxins, complement, and other cytotoxic agents. *J. biol. Chem.* **261,** 9300–9308.

Bashford, C. L., Rodrigues, L. and Pasternak, C. A. (1989) Protection of cells against membrane damage by haemolytic agents: divalent cations and protons act at the extracellular side of the plasma membrane. *Biochim. biophys. Acta* **983,** 56–64.

Bilwes, A., Rees, B., Moras, D., Ménez, R. and Ménez, A. (1994) X-ray structure at 1.55 Å of toxin γ, a cardiotoxin from *Naja nigricollis* venom. Crystal packing reveals a model for insertion into membranes. *J. molec. biol.* **239,** 122–136.

Bolaños, R. (1982) Las serpientes venenosas de Centroamérica y el problema del ofidismo. Primera parte: aspectos zoológicos, epidemiológicos y biomédicos. *Rev. Cost. Cienc. Méd.* **3,** 165–184.

Brenes, F., Gutiérrez, J. M. and Lomonte, B. (1987) Immunohistochemical demonstration of the binding of *Bothrops asper* myotoxin to skeletal muscle sarcolemma. *Toxicon* **25,** 574–577.

Brunie, S., Bolin, J., Gewirth, D. and Sigler, P. B. (1985) The refined crystal structure of dimeric phospholipase A_2 at 2.5 Å. Access to a shielded catalytic center. *J. biol. Chem.* **260,** 9742–9749.

Brusés, J. L., Capaso, J., Katz, E. and Pilar, G. (1993) Specific *in vitro* biological activity of snake venom myotoxins. *J. Neurochem.* **60,** 1030–1042.

Bultrón, E., Gutiérrez, J. M. and Thelestam, M. (1993*a*) Effects of *Bothrops asper* (terciopelo) myotoxin III, a basic phospholipase A_2, on liposomes and mouse gastrocnemius muscle. *Toxicon* **31,** 217–222.

Bultrón, E., Thelestam, M. and Gutiérrez, J. M. (1993*b*) Effects on cultured mammalian cells of myotoxin III, a phospholipase A_2 isolated from *Bothrops asper* (terciopelo) venom. *Biochim. biophys. Acta* **1179,** 253–259.

Chien, K. Y., Chiang, C. M., Hseu, Y. C., Vyas, A. A., Rule, G. S. and Wu, W. G. (1994) Two distinct types of cardiotoxin as revealed by the structure and activity relationship of their interaction with zwitterionic phospholipid dispersions. *J. biol. Chem.* **269,** 14,473–14,483.

Cho, W., Tomasselli, A., Heinrikson, R. L. and Kezdy, F. J. (1988) The chemical basis for interfacial activation of monomeric phospholipases A_2. *J. biol. Chem.* **263,** 11,237–11,241.

Chwetzoff, S. (1990) On the mode of action of basic phospholipase A_2 from *Naja nigricollis* venom. *Biochim. biophys. Acta* **1045,** 285–290.

Cintra, A. C. O., Marangoni, S., Oliveira, B. and Giglio, J. R. (1993) Bothropstoxin-I: amino acid sequence and function. *J. Prot. Chem.* **12,** 57–64.

Cirino, G., Peers, S. H., Wallace, J. L. and Flower, R. J. (1989) A study of phospholipase A_2-induced oedema in rat paw. *Eur. J. Pharmac.* **166,** 505–510.

Davidson, F. and Dennis, E. A. (1990) Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venom to human secreted forms. *J. molec. Evol.* **31,** 228–238.

Díaz, C., Gutiérrez, J. M., Lomonte, B. and Gene, J. A. (1991) The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities. *Biochim. biophys. Acta* **1070,** 455–460.

Díaz, C., Gutiérrez, J. M. and Lomonte, B. (1992) Isolation and characterization of basic myotoxic phospholipases A_2 from *Bothrops godmani* (Godman's pit viper) snake venom. *Arch. Biochem. Biophys.* **298,** 135–142.

Díaz, C., Gutiérrez, J. M., Lomonte, B. and Nuñez, J. (1993) *p*-Bromophenacyl bromide modification of *Bothrops asper* myotoxin II, a lysine-49 phospholipase A_2, affects its pharmacological activities. *Toxicon* **31,** 1202–1206.

Díaz, C., Alape, A., Lomonte, B., Olamendi, T. and Gutiérrez, J. M. (1994) Cleavage of the NH_2-terminal octapeptide of *Bothrops asper* myotoxic lysine-49 phospholipase A_2 reduces its membrane-destabilizing effect. *Arch. Biochem. Biophys.* **312,** 336–339.

Díaz, C., Lomonte, B., Zamudio, F. and Gutiérrez, J. M. (1995) Purification and characterization of myotoxin IV, a phospholipase A_2 variant, from *Bothrops asper* snake venom. *Natural Toxins* **3,** 26–31.

Dijkstra, B. W., Kalk, K. H., Hol, W. G. and Drenth, J. (1981) Structure of bovine pancreatic phospholipase A_2 at 1.7 Å resolution. *J. molec. Biol.* **147,** 97–123.

Dua, R. and Cho, W. (1994) Inhibition of human secretory class II phospholipase A_2 by heparin. *Eur. J. Biochem.* **221,** 481–490.

Duchen, L. W., Excell, B. J., Patel, R. and Smith, B. (1974) Changes in motor endplates resulting from muscle fibre necrosis and regeneration. A light and electron microscopic study of the effects of the depolarizing fraction (cardiotoxin) of *Dendroaspis jamesoni* venom. *J. neurol. Sci.* **21,** 391–417.

Ekre, H. P., Naparstek, Y., Lider, O., Hyden, P., Hägermark, Ö., Nilsson, T., Vlodavsky, I. and Cohen, I. (1992) Anti-inflammatory effects of heparin and its derivatives: inhibition of complement and of lymphocyte migration. In: *Heparin and Related Polysaccharides*, pp. 329–340 (Lane, D. A., Björk, I. and Lindahl, U., Eds). New York: Plenum Press.

Faure, G. and Bon, C. (1987) Several isoforms of crotoxin are present in individual venoms from the South American rattlesnake *Crotalus durissus terrificus*. *Toxicon* **25,** 229–234.

Francis, B., Gutiérrez, J. M., Lomonte, B. and Kaiser, I. I. (1991) Myotoxin II from *Bothrops asper* (terciopelo) venom is a lysine-49 phospholipase A_2 *Arch. Biochem. Biophys.* **284,** 352–359.

Fukagawa, T., Nose, T., Shimohigashi, Y., Ogawa, T., Oda, N., Nakashima, K., Chang, C. and Ohno, M. (1993) Purification, sequencing and characterization of single amino acid-substituted phospholipase A_2 isozymes from *Trimeresurus gramineus* (green habu snake) venom. *Toxicon* **31,** 957–967.

Grounds, M. D. (1991) Towards understanding skeletal muscle regeneration. *Path. Res. Pract.* **187,** 1–22.

Gulati, A. K. (1985) Basement membrane component changes in skeletal muscle transplants undergoing regeneration or rejection. *J. cell. Biochem.* **27,** 337–346.

Gulati, A. K., Reddi, A. H. and Zalewski, A. A. (1983) Changes in the basement membrane zone components during skeletal muscle fiber degeneration and regeneration. *J. Cell Biol.* **97,** 957–962.

Gutiérrez, J. M., Lomonte, B., Portilla, E., Cerdas, L. and Rojas, E. (1983) Local effects induced by coral snakes (*Micrurus*) venoms: evidence of myonecrosis after experimental inoculation of venom from five species. *Toxicon* **21,** 777–783.

Gutiérrez, J. M., Ownby, C. L. and Odell, G. V. (1984*a*) Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon* **22,** 115–128.

Gutiérrez, J. M., Ownby, C. L. and Odell, G. V. (1984*b*) Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Exp. molec. Path.* **40,** 367–379.

Gutiérrez, J. M., Ownby, C. L. and Odell, G. V. (1984*c*) Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops asper* (fer-de-lance). *Toxicon* **22,** 719–731.

Gutiérrez, J. M., Lomonte, B., Chaves, F., Moreno, E. and Cerdas, L. (1986*a*) Pharmacological activities of a toxic phospholipase A isolated from the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* **84C,** 159–164.

Gutiérrez, J. M., Lomonte, B. and Cerdas, L. (1986*b*) Isolation and partial characterization of a myotoxin from the venom of the snake *Bothrops nummifer*. *Toxicon* **24,** 885–894.

Gutiérrez, J. M., Rojas, G., Lomonte, B., Gené, J. A. and Cerdas, L. (1987) Effects of a myotoxic phospholipase A_2 isolated from *Bothrops asper* venom on skeletal muscle sarcoplasmic reticulum. *Toxicon* **25,** 1244–1248.

Gutiérrez, J. M., Chaves, F., Gené, J. A., Lomonte, B., Camacho, Z. and Schosinsky, K. (1989) Myonecrosis induced by a basic myotoxin isolated from the venom of the snake *Bothrops nummifer* (jumping viper) from Costa Rica. *Toxicon* **27,** 735–746.

Gutiérrez, J. M., Arce, V., Brenes, F. and Chaves, F. (1990) Changes in myofibrillar components after skeletal muscle necrosis induced by a myotoxin isolated from the venom of the snake *Bothrops asper*. *Exp. molec. Path.* **52,** 25–36.

Gutiérrez, J. M., Nuñez, J., Díaz, C., Cintra, A. C. O., Homsi-Brandeburgo, M. I. and Giglio, J. R. (1991) Skeletal muscle degeneration and regeneration after injection of bothropstoxin II, a phospholipase A_2 isolated from the venom of the snake *Bothrops jararacussu*. *Exp. molec. Path.* **55,** 217–229.

Harris, J. B. (1991) Phospholipases in snake venoms and their effects on nerve and muscle. In: *Snake Toxins* pp. 91–129 (Harvey, A. L., Ed.). New York: Pergamon Press.

Harris, J. B. (1992) Natural toxins in study of degeneration and regeneration of skeletal muscle. In: *Methods in Neurosciences*, Vol. 8, *Neurotoxins* pp. 298–310 (Conn, P. M., Ed.). San Diego: Academic Press.

Harris, J. B. and Maltin, C. A. (1982) Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. *Br. J. Pharmac.* **76,** 61–75.

Harris, J. B., Johnson, M. A. and Karlsson, E. (1975) Pathological responses of rat skeletal muscle to a single subcutaneous injection of a toxin isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin. exp. Pharmac. Physiol.* **2,** 383–404.

Heluany, N. F., Homsi-Brandeburgo, M. I., Giglio, J. R., Prado-Franceschi, J. and Rodrigues-Simioni, L. (1992) Effects induced by bothropstoxin, a component from *Bothrops jararacussu* snake venom, on mouse and chick muscle preparations. *Toxicon* **30,** 1203–1210.

Hodgson, D., Gasparini, S., Drevet, P., Ducancel, F., Bouet, F., Boulain, J. C., Harris, J. B. and Ménez, A. (1993) Production of recombinant notechis 11'2L, an enzymatically active mutant of a phospholipase A_2 from *Notechis scutatus scutatus* venom, as directly generated by cleavage of a fusion protein produced in *Escherichia coli*. *Eur. J. Biochem.* **212,** 441–446.

Holland, D. R., Clancy, L. L., Muchmore, S. W., Ryde, T. J., Einspahr, H. M., Finzel, B. C., Heinrikson, R. L. and Watenpaugh, K. D. (1990) The crystal structure of a lysine 49 phospholipase A_2 from the venom of the cottonmouth snake at 2.0-Å resolution. *J. biol. Chem.* **265,** 17,649–17,656.

Homsi-Brandeburgo, M. I., Queiroz, L. S., Santi-Neto H., Rodrigues-Simioni, L. and Giglio, J. R. (1988) Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. *Toxicon* **26,** 615–627.

Kaiser, I. I., Gutiérrez, J. M., Plummer, D., Aird, S. D. and Odell, G. V. (1990) The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. *Arch. Biochem. Biophys.* **278,** 319–325.

Kerrigan, K. R. (1991) Venomous snakebite in Eastern Ecuador. *Am. J. trop. Med. Hyg.* **44,** 93–99.

Kini, R. M. and Evans, H. J. (1989) A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. *Int. J. Peptide Prot. Res.* **34,** 277–286.

Kini, R. M. and Iwanaga, S. (1986) Structure–function relationships of phospholipases. II: Charge density distribution and the myotoxicity of presynaptically neurotoxic phospholipases. *Toxicon* **24,** 895–905.

Krizaj, I., Bieber, A. L., Ritonja, A. and Gubenšek, F. (1991) The primary structure of ammodytin L, a myotoxic phospholipase A_2 homologue from *Vipera ammodytes* venom. *Eur. J. Biochem.* **202,** 1165–1168.

Lambeau, G., Schmid-Alliana, A., Lazdunski, M. and Barhanin, J. (1990) Identification and purification of a very high affinity binding protein for toxic phospholipases A_2 in skeletal muscle. *J. biol. Chem.* **265,** 9526–9532.

Lambeau, G., Ancian, P., Barhanin, J. and Lazdunski, M. (1994) Cloning and expression of a membrane receptor for secretory phospholipases A_2. *J. biol. Chem.* **269,** 1575–1578.

Liu, C. S., Chen, J. M., Chang, C. H., Chen, S. W., Teng, C. M. and Tsai, I. H. (1991) The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochim. biophys. Acta* **1077,** 362–370.

Liu, S Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S. and Kihara, H. (1990) Purification and amino acid sequence of basic protein II, a Lysine-49 phospholipase A_2 with low activity, from *Trimeresurus flavoviridis* venom. *J. Biochem.* **107,** 400–408.

Lloret, S. and Moreno, J. J. (1993) Oedema formation and degranulation of mast cells by phospholipase A_2 purified from porcine pancreas and snake venoms. *Toxicon* **31,** 949–956.

Lomonte, B. and Carmona, E. (1992) Individual expression patterns of myotoxin isoforms in the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* **102B,** 325–329.

Lomonte, B. and Gutiérrez, J. M. (1989) A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27,** 725–733.

Lomonte, B. and Kahan, L. (1988) Production and partial characterization of monoclonal antibodies to *Bothrops asper* (terciopelo) myotoxin. *Toxicon* **26,** 675–689.

Lomonte, B., Cerdas, L., Gené, J. A and Gutiérrez, J. M. (1982) Neutralization of local effects of the terciopelo (*Bothrops asper*) venom by blood serum of the colubrid snake *Clelia clelia*. *Toxicon* **20,** 571–579.

Lomonte, B., Gutiérrez, J. M. and Mata, E. (1985) Isolation from a polyvalent antivenom of antibodies to a myotoxin in *Bothrops asper* snake venom. *Toxicon* **23,** 807–813.

Lomonte, B., Gutiérrez, J. M., Moreno, E. and Cerdas, L. (1987*a*) Antibody neutralization of a myotoxin from the venom of *Bothrops asper* (terciopelo). *Toxicon* **25,** 443–449.

Lomonte, B., Moreno, E. and Gutiérrez, J. M. (1987*b*) Detection of proteins antigenically-related to *Bothrops asper* myotoxin in crotaline snake venoms. *Toxicon* **25,** 947–955.

Lomonte, B., Furtado M. F., Rovira, M. E., Carmona, E., Rojas, G., Aymerich, R. and Gutiérrez, J. M. (1990*a*) South American snake venom proteins antigenically-related to *Bothrops asper* myotoxins. *Braz. J. med. Biol. Res.* **23,** 427–435.

Lomonte, B., Gutiérrez, J. M., Furtado, M. F., Otero, R., Rosso, J. P., Vargas, O., Carmona, E. and Rovira, M. E. (1990*b*) Isolation of basic myotoxins from *Bothrops moojeni* and *Bothrops atrox* snake venoms. *Toxicon* **28,** 1137–1146.

Lomonte, B., Gutiérrez, J. M., Carmona, E. and Rovira, M. E. (1990*c*) Equine antibodies to *Bothrops asper* myotoxin II: isolation from polyvalent antivenom and neutralizing ability. *Toxicon* **28,** 379–384.

Lomonte, B., Gutiérrez, J. M., Rojas, G. and Calderón, L. (1991) Quantitation by enzyme-immunoassay of antibodies against *Bothrops* myotoxins in four commercially-available antivenoms. *Toxicon* **29,** 695–702.

Lomonte, B., Gutiérrez, J. M., Ramirez, M. and Díaz, C. (1992) Neutralization of myotoxic phospholipases A_2 from the venom of the snake *Bothrops asper* by monoclonal antibodies. *Toxicon* **30,** 239–245.

Lomonte, B., Tarkowski, A. and Hanson, L. Å. (1993) Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* **17,** 93–105.

Lomonte, B., Lundgren, J., Johansson, B. and Bagge, U. (1994*a*) The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. *Toxicon* **32,** 41–55.

Lomonte, B., Gutiérrez, J. M., Borkow, G., Ovadia, M., Tarkowski, A. and Hanson, L. Å. (1994*b*) Activity of hemorrhagic metalloproteinase BaH-1 and myotoxin II from *Bothrops asper* snake venom on capillary endothelial cells *in vitro*. *Toxicon* **32,** 505–510.

Lomonte, B., Tarkowski, A., Bagge, U. and Hanson, L. Å. (1994*c*) Neutralization of the cytolytic and myotoxic activities of phospholipases A_2 from *Bothrops asper* snake venom by glycosaminoglycans of the heparin/heparan sulfate family. *Biochem. Pharmac.* **47,** 1509–1518.

Lomonte, B., Tarkowski, A. and Hanson, L. Å. (1994*d*) Broad cytolytic specificity of myotoxin II, a lysine-49 phospholipase A_2 of *Bothrops asper* snake venom. *Toxicon* **32,** 1359–1369.

Lomonte, B., Moreno, E., Tarkowski, A., Hanson, L. Å. and Maccarana, M. (1994*e*) Neutralizing interaction between heparins and myotoxin II, a Lys-49 phospholipase A_2 from *Bothrops asper* snake venom. Identification of a heparin-binding and cytolytic toxin region by the use of synthetic peptides and molecular modeling. *J. biol. Chem.* **269,** 29,867–29,873.

Lugtigheid, B., Nicolaes, G. A. F., Veldhuizen, E. J. A., Slotboom, A. J., Verheij, H. M. and De Haas, G. H. (1993) Acylation of porcine pancreatic phospholipase A_2 influences penetration and substrate head-group binding, depending on the position of the acylated lysine in the enzyrne molecule. *Eur. J. Biochem.* **216,** 519–525.

Maltin, C. A., Harris, J. B. and Cullen, M. J. (1983) Regeneration of mammalian skeletal muscle following the injection of the snake-venom toxin, taipoxin. *Cell Tissue Res.* **232,** 565–577.

Maraganore, J. M., Merutka, G., Cho, W., Welches, W., Kezdy, F. J. and Heinrikson, R. L. (1984) A new class of phospholipases A_2 with lysine in place of aspartate 49. *J. biol. Chem.* **259,** 13,839–13,843.

Mebs, D. and Ownby, C. L. (1990) Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmac. Ther.* **48,** 223–236.

Mebs, D. and Samejima, Y. (1986) Isolation and characterization of myotoxic phospholipases A_2 from crotalid venoms. *Toxicon* **24,** 161–168.

Melo, P. A. and Suarez-Kurtz, G. (1988) Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: antagonism by heparin and by the serum of South American marsupials. *Toxicon* **26,** 87–95.

Melo, P. A., Suarez-Kurtz, G., Do Nascimento, M. C., Parente, J. P. and Mors, W. B. (1988) Antagonism of the myotoxic and hemorrhagic effects of *Bothrops* venoms by extracts of *Eclipta prostrata L.*, Asteraceae, and their components. *Ann. Acad. brasil. Ciênc.* **60,** 263.

Melo, P. A., Homsi-Brandeburgo, M. I., Giglio, J. R. and Suarez-Kurtz, G. (1993) Antagonism of the myotoxic effect of *Bothrops jararacussu* venom and bothropstoxin by polyanions. *Toxicon* **31,** 285–291.

Ménez, A., Pillet, L., Leonetti, M., Bontems, F. and Maillere, B. (1992) Snake toxins as antigens. In: *Structure of Antigens*, pp. 293–319 (Van Regenmortel, M. H. V., Ed.). Boca Raton: CRC Press.

Mokri, B. and Engel, A. G. (1975) Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. *Neurology* **25,** 1111–1120.

Moreno, E. and Gutiérrez, J. M. (1988) Body distribution of *Bothrops asper* (terciopelo) snake venom myotoxin and its relationship to pathological changes. *Toxicon* **26,** 403–409.

Mors, W. B., Do Nascimento, M. C., Parente, J. P., Da Silva, M. H., Melo, P. and Suarez-Kurtz, G. (1989) Neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of the plant *Eclipta prostrata* (Asteraceae). *Toxicon* **27,** 1003–1009.

Moura, A. M., Cardoso, D. F. and Tanizaki, M. M. (1990) Differences in distribution of myotoxic proteins in venoms from different *Bothrops* species. *Toxicon* **28,** 1293–1301.

Moura, A. M., Desmond, H., Laing, G. and Theakston, R. D. G. (1991*a*) Isolation and comparison of myotoxins isolated from venoms of different species of *Bothrops* snakes. *Toxicon* **29,** 713–723.

Moura, A. M., Cardoso, D. F., Tanizaki, M. M. and Mota, I. (1991*b*) Neutralization of myotoxic activity of *Bothrops* venoms by antisera to purified myotoxins and to crude venoms. *Toxicon* **29,** 1471–1480.

Moura, A. M., Paine, M. J. I., Diniz, M. R. V., Crampton, J. M. and Theakston, R. D. G. (1993) Molecular cloning of a novel myotoxic phospholipase A_2 from the venom of *Bothrops jararacussu*. Abstracts of the XI European Meeting of the International Society on Toxinology, p. 66, Arezzo, Italy.

Nakashima, K., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H. and Ohno, M. (1993) Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A_2 isozymes. *Proc. natn. Acad Sci. U.S.A.* **90,** 5964–5968.

Nikai, T., Komori, Y., Yagihashi, S., Ohara, A., Ohizumi, Y. and Sugihara, H. (1993) Isolation and characterization of phospholipase A_2 from *Agkistrodon bilineatus* (common cantil) venom. *Int. J. Biochem.* **25,** 879–884.

Nishioka, S. A. and Silveira, P. V. P. (1992) A clinical and epidemiologic study of 292 cases of lance-headed viper bite in a Brazilian teaching hospital. *Am. J. trop. Med. Hyg.* **47,**805–810.

Otero, R., Tobon, G. S., Gomez, L. F., Osorio, R., Valderrama, R., Hoyos, D., Urreta, J. E., Molina, S. and Arboleda, J. J. (1992) Accidente ofidico en Antioquia y Chocó. Aspectos clínicos y epidemiológicos (marzo de 1989-febrero de 1990). *Acta Méd. Colombiana* **17,** 229–249.

Ownby, C. L. and Li, Q. (1993) Molecular cloning of the gene encoding a myotoxin from the venom of the broad-banded copperhead (*Agkistrodon contortrix laticinctus*). Abstracts of the XI European Meeting of the International Society on Toxinology, p. 69, Arezzo, Italy.

Ownby, C. L., Fletcher, J. E. and Colberg, T. R. (1993) Cardiotoxin I from cobra (*Naja naja atra*) venom causes necrosis of skeletal muscle *in vivo*. *Toxicon* **31,** 697–709.

Pedersen, J. Z., de Arcuri, B. F., Morero, R. and Rufini, S. (1994) Phospholipase-like myotoxins induce rapid membrane leakage of non-hydrolyzable ether-lipid liposomes. *Biochim. biophys. Acta* **1190,** 177–180.

Pedersen, J. Z,, Lomonte, B., Massoud, R., Gubenšek, F., Gutiérrez, J. M. and Rufini, S . (1995) Autocatalytic acylation of phospholipase-like myotoxins. *Biochemistry* **34,** 4670–4675.

Pestronk, A., Parhad, I. M., Drachman, D. B. and Price, D. L. (1982) Membrane myopathy: morphological similarities to Duchenne muscular dystrophy. *Muscle Nerve* **5,** 209–214.

Pruzanski, W. and Vadas, P. (1991) Phospholipase A_2: a mediator between proximal and distal effectors of inflammation. *Immun. Today* **12,** 143–146.

Queiroz, L. S., Santo Neto, H., Rodrigues-Simioni, L. and Prado-Franceschi, J. (1984) Muscle necrosis and regeneration after envenomation by *Bothrops jararacussu* snake venom. *Toxicon* **22,** 339–346.

Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J. and Sigler, P. B. (1985) A comparison of the crystal structures of phospholipase A_2 from bovine pancreas and *Crotalus atrox* venom. *J. biol. Chem.* **260,** 11,627–11,634.

Renetseder, R., Dijkstra, B. W., Huizinga, K., Kalk, K. H. and Drenth, J. (1988) Crystal structure of bovine pancreatic phospholipase A_2 covalently inhibited by *p*-bromophenacyl-bromide. *J. molec. Biol.* **200,** 181–188.

Roden, L., Ananth, S., Campbell, P., Curenton, T., Ekborg, G., Manzella, S., Pillion, D. and Meezan, E. (1992) Heparin—an introduction. In: *Heparin and Related Polysaccharides*, pp. 1–20 (Lane, D. A., Björk, I. and Lindahl, U., Eds). New York: Plenum Press.

Rosenberg, P. (1990) Phospholipases. In: *Handbook of Toxicology*, pp. 67–277 (Shier, W. T. and Mebs, D., Eds.). New York: Marcel Dekker.

Rosenfeld, G. (1971) Symptomatology, pathology and treatment of snake bites in South America. In: *Venomous Animals and Their Venoms*, Vol. 2, pp. 345–403 (Bucherl, W. and Buckley, E. E., Eds). New York: Academic Press.

Rovira, M. E., Carmona, E. and Lomonte, B. (1992) Immunoenzymatic quantitation of antibodies to *Bothrops asper* myotoxins after polyvalent antivenom administration in mice. *Braz. J. Med. Biol. Res.* **25,** 23–33.

Rufini, S., Cesaroni, P., Desideri, A., Farias, R., Gubensek, F., Gutiérrez, J. M., Luly, P., Massoud, R., Morero, R. and Pedersen, J. Z. (1992) Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. *Biochemistry* **31,** 12,424–12,430.

Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H. and Sigler, P. B. (1990) Interfacial catalysis: the mechanism of phospholipase A_2. *Science* **250,** 1541–1546.

Scott, D. L., Achari, A., Vidal, J. C. and Sigler, P. B. (1992) Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. *J. biol. Chem.* **267,** 22,645–22,657.

Selistre, H. S., Queiroz, L. S., Cunha, O. A. B., De Souza, G. E. P. and Giglio, J. R. (1990) Isolation and characterization of hemorrhagic, myonecrotic and edema-inducing toxins from *Bothrops insularis* (jararaca ilhoa) snake venom. *Toxicon* **28,** 261–273.

Sharp, N. J. H., Kornegay, J. N., Bartlett, R. J., Hung, W. Y. and Dykstra, M. J. (1993) Notexin-induced muscle injury in the dog. *J. neurol. Sci.* **116,** 73–81.

Takasaki, C., Yutani, F. and Kajiyashiki, T. (1990) Amino acid sequences of eight phospholipases A_2 from the venom of Australian king brown snake, *Pseudechis australis*. *Toxicon* **28,** 329–339.

Tomasselli, A. G., Hui, J., Fisher, J., Zurcher-Neely, H., Reardon, I. M., Oriaku, E., Kezdy, F. J. and Heinrikson, R. L. (1989) Dimerization and activation of porcine pancreatic phospholipase A_2 via substrate level acylation of lysine 56. *J. biol. Chem.* **264,** 10,041–10,047.

Valiente, C., Moreno, E., Sittenfeld, A., Lomonte, B. and Gutiérrez, J. M. (1992) An electrophoretic study on phospholipase A_2 isoenzymes in the venoms of Central American crotaline snakes. *Toxicon* **30,** 815–823.

Van Den Bergh, C. J., Slotboom, A. J., Verheij, H. M. and De Haas, G. H. (1989) The role of Asp-49 and other conserved amino acids in phospholipases A_2 and their importance for enzymatic activity. *J. cell. Biochem.* **39,** 379–390.

Vishwanath, B. S., Fawzy, A. A. and Franson, R. C. (1988) Edema-inducing activity of phospholipase A_2 purified from human synovial fluid and inhibition by aristolochic acid. *Inflammation* **12,** 549–561.

Vracko, R. and Benditt, E. P. (1972) Basal lamina: the scaffold for orderly cell replacement. Observations on regeneration of injured skeletal muscle fibers and capillaries. *J. Cell Biol.* **55,** 406–419.

Wery, J. P., Schevitz, R. W., Clawson, D. K., Bobbitt, J. L., Dow, E. R., Gamboa, G., Goodson, T., Hermann, R. B., Kramer, R. M., McClure, D. B., Mihelich, E. D., Putnam, J. E., Sharp, J. D., Stark, J. D., Teater, C., Warrick, M. W. and Jones, N. D. (1991) Structure of recombinant human rheumatoid arthritic synovial fluid phospholipase A_2 at 2.2 Å resolution. *Nature* **352,** 79–82.

Westerlund, B., Nordlund, P., Uhlin, U., Eaker, D. and Eklund, H. (1992) The three-dimensional structure of notexin, a presynaptic neurotoxic phospholipase A_2 at 2.0 Å resolution. *FEBS Lett.* **301,** 159–164.

Yoshizumi, K., Liu, S. Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S. and Kihara, H. (1990) Purification and amino acid sequence of basic protein I, a lysine-49 phospholipase A_2 with low activity, from the venom of *Trimeresurus flavoviridis* (habu snake). *Toxicon* **28,** 43–54.