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Membrane-Active Polypeptides from Snake Venom: Cardiotoxins and Haemocytotoxins

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1. *Introduction*

A number of basic polypeptides devoid of enzymatic activity have been isolated from venoms belonging to the Elapidae snakes family. One group of basic polypeptides which act on the pre- or post-synaptic sites[1], is known as venom 'neurotoxins'. Another group having a relatively low toxicity to animals, induce a variety of effects which have been separately recognized and studied in various laboratories. As a consequence a confusion in their nomenclature arose and only recently they were found to be closely related if not identical. Thus, strongly basic polypeptides isolated from a specific venom or from venoms of different species or subspecies have been designated as cardiotoxin[2], skeletal muscle depolarizing factor[3], crobramine A and B[4], cytotoxin[5], toxin γ[6], direct lytic factor (DLF)[7], peak 12 B[8] and others.

Following initial suggestions as to their possible identity[9,10] there is accumulating evidence that the hemolytic, cytolytic, transport-inhibiting, depolarizing and cardiotoxic actions are common to most of these basic polypeptides[11–13]. Moreover, their close relationship is supported by their remarquable structural similarity.

Answering the need for a unifying nomenclature of these polypeptides BRISBOIS et al.[14] suggested the designation 'cobramines' which emphasizes their basic character, while LEE[1] employs the term 'cardiotoxins' in view of their effect on the heart differentiating them from the 'neurotoxins'. Objections can be raised to both designations: the term 'cobramines' does not differentiate this group from the equally basic neurotoxins, and the designation 'cardiotoxins' relates to a pathophysiological effect, whereas the primary action of these venom basic polypeptides is exerted at the level of the cell membrane, leading to disturbance of its organization and function. Their generalized action on membranes differs fundamentally from the selective interference with acetylcholine-release from pre-synaptic membranes or with acetylcholine reception at the post-synaptic ones, which characterizes the neurotoxic polypeptides. In view of these considerations, the haemo-cyto-cardiotoxic factors will be designated as 'membrane-active polypeptides' throughout this survey.

Studies on snake venoms and their components, including the membrane-active polypeptides, have been frequently reviewed, with special emphasis on chemistry and parmacology[1,9,15–18]. The present survey deals with these aspects only briefly, whereas its main topic is the interaction of the polypeptides with membranes at a molecular level.

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[13] C. Y. LEE, J. S. LIN and J. W. WEI, in *Toxins of Animal and Plant Origin* (Eds. A. DE VRIES and E. KOCHWA; Gordon and Breach, New York 1971), p. 307.
[14] L. BRISBOIS and J. PEETERS, in *Toxins of Animal and Plant Origin* (Eds. A. DE VRIES and E. KOCHWA; Gordon and Breach, New York 1971), p. 319.
[15] J. M. JIMENEZ-PORRAS, A. Rev. Pharmac. *8*, 293 (1968).
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2. *Chemical composition and structure*

A) *Amino-acid composition and sequence.* The membrane-active polypeptides have molecular weights ranging between 6000 and 7000, and are remarkably heat stable at acidic pH[1]. The amino-acid composition of the polypeptides from *Naja naja*, *Naja naja atra*, *Naja nigricollis* and *Hemachatus haemachatus* venoms is given in Table I. A discussion on the close correspondance within this group and the differences between them and the neurotoxins is given in the review article by LEE[1]. The amino-acid sequences of the cardiotoxin from *Naja naja atra* venom[19], of the cytotoxins I and II from *Naja naja* venom[20,21], and of the hemolytic protein 12B from *Hemachatus haemachatus* venom[8], illustrated in Table II, show a remarkable homology. Although, as pointed out by LEE[1] and TU[17], the amino-acid composition of the membrane-active polypeptides differs substantially from that of the neurotoxins, their primary structures bear a distinct resemblance in both amino-acid sequences and localization of half-cystine residues.

B) *Secondary structure.* TAKECHI and HAYASHI[22] have recently localized the 4 disulfide bridges in the molecule of cytotoxin II from *Naja naja* venom (Figure). The similarity in location of the half cystine residues in the 4 polypeptides listed in Table II, supports the assumption that cytotoxin I, cardiotoxin and protein 12B have the same arrangement of disulfide bridges as demonstrated for cytotoxin II. Moreover, the cross-linking of the half-cystine residues in cytotoxin II is identical to that of the homologous residues in the 61–62 amino-acid neurotoxins[22].

C) *Tertiary structure.* TAKECHI et al.[21] reported on the remarkable similarity in the CD spectrum of *Naja naja* cytotoxins I and II on one hand, and that of cobratoxin, a neurotoxin isolated from the same venom, on the other. Cobratoxin presents CD and ORD spectra quite different from those of ordinary proteins and suggestive of a large amount of random coil together with small amounts of α-helix and β-structure[23]. However, recent studies by YU et al.[24] on the laser Raman scattering of cobramine B, a membrane-active polypeptide from *Naja naja* venom, suggest that the protein contains a large fraction of antiparallel β-structure coexisting with some random coil and α-helix. The scattering intensity of the tyrosyl ring vibrations indicate that the 3 tyrosines in this protein are 'buried' in the interior of the molecule. Neurotoxin α from *Naja haje haje* venom resembles cobramine B

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Table I. Amino-acid composition of membrane-active polypeptides from snake venoms

	N. naja atra Cardiotoxin	*N. naja* Cobramine B	*N. naja*		*N. naja* F8	*N. nigricollis* F14 (toxin γ)	*H. haemachatus*	
			CM-XI	CM-XII			DLF	F12B
Lysine	9	8	8–9	9	8	9	10	12
Histidine	0	0	0	0	0	0	1	1
Arginine	2	2	2	2	2	2	1	1
Aspartic acid	6	5	7	7–8	8	6	6	6
Threonine	3	3	3	3	3	3	3	3
Serine	2	2	2	2	3	2	3	3
Glutamic acid	0	0	1	0	0	1	1	1
Proline	5	4	5	5	4	6	5	5
Glycine	2	2	2	2	2	2	2	2
Alanine	2	2	2	2	2	2	1	1
Half-cystine	8	6	8	8	8	8	8	8
Valine	7	6	6	7	4	3	4	4
Methionine	2	2	2	2	3	4	2	3
Isoleucine	1	1	2	1	4	3	2	2
Leucine	6	5	6	6	6	5	6	7
Tyrosine	3	3	5	4	2	2	1	1
Phenylalanine	2	1	0	1	1	1	1	1
Tryptophan	0	0	0	0	0	1	0	0
Total	60	52	61–62	61–62	60	60	57	61
N-terminal	Leu		Leu	Leu	Leu	Leu	Leu	Leu
C-terminal	Asn				Asn	Asn	Ser	Asn
Reference	19	4	55		a	a	56	8

[a] EAKER and FRYKLUND, unpublished. Reproduced after C. Y. LEE[1].

Table II. Amino-acid sequences of membrane-active polypeptides from snake venoms

	Ref.	Residues
		1 ... 10
Cardiotoxin	19	H_2N-Leu-Lys-Cys——Asn-Lys-Leu-Val-Pro-Leu-Phe-Tyr-Lys-Thr-Cys-Pro-
Cytotoxin I	20	H_2N-Leu-Lys-Cys——Asn-Lys-Leu-Ile-Pro-Leu-Ala-Tyr-Lys-Thr-Cys-Pro-
Cytotoxin II	21	H_2N-Leu-Lys-Cys——Asn-Lys-Leu-Val-Pro-Leu-Phe-Tyr-Lys-Thr-Cys-Pro-
Protein 12 B	8	H_2N-Leu-Lys-Cys-His-Asn-Lys-Leu-Val-Pro-Phe-Leu-Ser-Lys-Thr-Cys-Pro-
		11
		20 ... 30
Cardiotoxin		Ala-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Phe-Met-Val-Ala-Thr-Pro-
Cytotoxin I		Ala-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Tyr-Met-Val-Ser-Asn-Lys-
Cytotoxin II		Ala-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Tyr-Met-Val-Ala-Thr-Pro-
Protein 12 B		Glu-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Thr-Met-Leu-Lys-Met-Pro-
		21 ... 31
		40
Cardiotoxin		Lys-Val-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Ser-
Cytotoxin I		Thr-Val-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Asn-
Cytotoxin II		Lys-Val-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Ser-
Protein 12 B		Lys-Ile-Pro-Ile-Lys-Arg-Gly-Cys-Thr-Asp-Ala-Cys-Pro-Lys-Ser-
		41
		50 ... 60
Cardiotoxin		Ser-Leu-Val-Leu-Lys-Tyr-Val-Cys-Cys-Asn-Thr-Asp-Arg-Cys-Asn-OH
Cytotoxin I		Ser-Leu-Val-Leu-Lys-Tyr-Glu-Cys-Cys-Asn-Thr-Asp-Arg-Cys-Asn-OH
Cytotoxin II		Ser-Leu-Val-Leu-Lys-Tyr-Val-Cys-Cys-Asn-Thr-Asp-Arg-Cys-Asn-OH
Protein 12 B		Ser-Leu-Leu-Val-Lys-Val-Val-Cys-Cys-Asn-Lys-Asp-Lys-Cys-Asn-OH
		51 ... 61

in both the 'buried' position of the tyrosine residue and in the large amount of antiparallel β-structure[24]. Large amount of β-structure and relative abundance of disulfide bridges in these proteins are considered responsible for their remarkable heat stability at acidic pH[24].

3. *Structure-function relationship*

The elucidation of the chemical composition and structure of a number of membrane-active polypeptides promoted attempts to relate these data with their biochemical and pharmacological properties. The active conformation of the polypeptides seems to depend on intact disulfide bridges, since the toxicity of both cytotoxin I and II disappears on reduction and either carboxymethylation or aminoethylation[20]. Similarily, reduction of *Naja naja* direct lytic factor with mercaptoethanol abolishes both its direct hemolytic effect and the potentiated hemolysis induced in combination with phospholiphase A[25].

A comparison between the membrane-active polypeptides and the closely related neurotoxins allows speculations as to the chemical or structural differences which might be responsible for their specific effects. Thus, while there are no significant differences between their secondary or tertiary structures, their amino acid composition is grossly dissimilar, and possibly significant differences in primary structure have been pointed out. The membrane-active polypeptides are characterized by a high content of lysine residues distributed evenly throughout the molecule and by a predominance of hydrophobic amino acids in the N-terminal half (Figure), while in a typical neurotoxin the residues at the N-terminal half are predominantly hydrophilic[1]. FRYKLUND and EAKER[8] point to the absence, in the lytic protein and its homologues, of the invariant sequence Lys-X-Trp-X-Asp-X-Arg-Gly, found so far in all neurotoxins. In their view, the lytic activity of these membrane-active polypeptides could result from the high charge density combined with the high content of nonpolar amino-acids, giving the molecule a tensid character[8]. TAKECHI et al.[21] point to the replacement of lysine residues 2, 18, 35 and 50

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Table III. Synergistic action of DLF and phospholipase A on cellular and subcellular membranes

Substrate	*Vipera palestinae* Phospholipase	*Vipera palestinae* Phospholipase + DLF	Ringhals Phospholipase	Ringhals Phospholipase + DLF
Erythrocyte[7]	—	Hydrolysis + Hemolysis	—	Hydrolysis + Hemolysis
Erythrocyte osmotic ghost[7]	—	Hydrolysis	Hydrolysis	Hydrolysis
Platelet[57]	—	Hydrolysis + No clot retraction	Hydrolysis + No clot retraction	Hydrolysis + No clot retraction
Liver Mitochondria[58]	—	Hydrolysis + Swelling	Hydrolysis + Swelling	Hydrolysis + Swelling

Hydrolysis: phospholipid splitting

in cytotoxin and cardiotoxin by glutamic acid residues, as a possible source of the pharmacological differences between the membrane active polypeptides and the neurotoxins. A similar consideration applies to the protein 12 B[8].

4. *Effects on cell membranes*

A) *Direct and potentiated hemolysis.* The membrane-active polypeptides are characterized by a direct lytic effect on washed red blood cells suspended in isotonic media, thus being designated as 'direct lytic factors' (DLF)[7] or 'hemolytic proteins'[8]. The degree of hemolysis is dependent on the type of red cell. When erythrocytes from various animal species are subjected to the action of DLF, a range of sensitivities is evident, guinea-pig and dog erythrocytes being the most susceptible, human and rabbit having moderate susceptibility while camel and sheep erythrocytes are entirely resistant[26]. The low susceptibility of human erythrocytes to the lytic action of the membrane-active polypeptides might explain the report on toxin γ as being nonlytic[27]. Indeed, in experiments with Ringhals (*Hemachatus haemachatus*) venom DLF, using concentrations up to 200 µg/ml, hemolysis of human erythrocytes did not exceed 10%[28,29]. JACOBI et al.[30] and OLDINGS et al.[31] added to the findings of MAMMON et al.[26] by showing that rat erythrocytes are completely resistant to amounts of *Naja naja* DLF which induced 26% hemolysis of guinea-pig erythrocytes. In order to obtain a similar degree of hemolysis, 50 times more DLF is needed. The range of sensitivities to the lytic action of *Naja naja altra* cardiotoxin for erythrocytes from 9 species, reported by LEE et al.[13], is in full agreement with the previous data.

Studies with labelled DLF showed that the polypeptide becomes bound to the erythrocytes from various species in amounts which correlate with their specific sensitivity towards DLF-induced hemolysis[32].

[26] E. CONDREA, Z. MAMMON, S. ALOOF and A. DE VRIES, Biochim. biophys. Acta *84*, 365 (1964).
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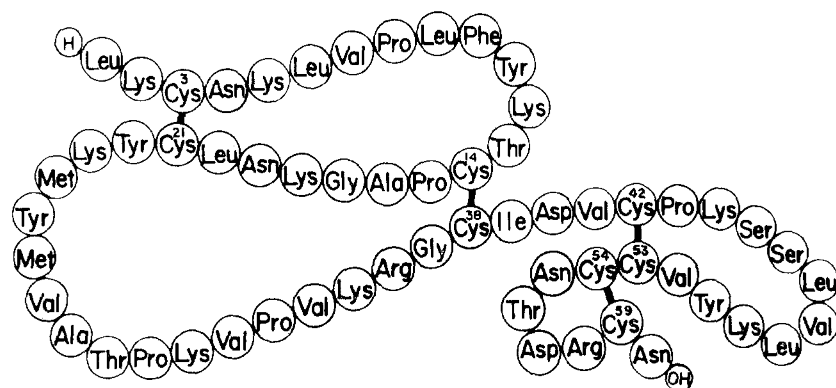
[28] C. KLIBANSKY, Y. LONDON, A. FRENKEL and A. DE VRIES, Biochim. biophys. Acta *150*, 15 (1968).
[29] E. CONDREA, M. BARZILAY and J. MAGER, Biochim. biophys. Acta *210*, 65 (1970).
[30] C. JACOBI, P. G. LANKISCH, K. SCHONER and W. VOGT, Naunyn Schmiedeberg's Arch. Pharmac. *274*, 81 (1972).
[31] H. D. OLDINGS, L. LEGE and P. G. LANKISCH, Naunyn Schmiedeberg's Arch. Pharmac. *268*, 27 (1971).
[32] E. CONDREA, I. KENDZERSKY and A. DE VRIES, Experientia *21*, 461 (1965).



The amino acid sequence of cytotoxin II showing the positions of the disulfide bridges. Reproduced after M. TAKECHI and K. HAYASHI[22].

The lysis induced by the membrane-active polypeptides in erythrocytes of the 'sensitive' species is greatly potentiated by addition of another venom factor, the phospholipase A_2, which in itself is nonlytic[7]. Thus, the presence of both phospholipase and membrane-active polypeptides in whole cobra venoms explains their known ability to lyse rapidly and completely red blood cells from a number of animal species. Erythrocytes not susceptible to direct hemolysis by DLF, are also resistant to the potentiated hemolysis by the joint action of DLF and phospholipase or by whole cobra venom[26].

B) *Synergistic action with phospholipase A_2 in promoting membrane phospholipid splitting.* The observation by CONDREA et al.[7] that hemolysis induced by the combined action of DLF and phospholipase A is associated with splitting of membranal phospholipids, helped clarify the mechanism of potentiated hemolysis. While, as mentioned above, the membrane-active polypeptides are only moderately hemolytic, snake venom phospholipase A_2 is neither lytic nor able to hydrolyze significantly the phospholipid substrates in intact erythrocyte membranes[7]. A recent report by GULL and SMITH[33] indicates that *Naja naja* phospholipase hydrolyzes to some extent the lecithin in intact human erythrocytes without causing hemolysis. It is therefore reasonable to assume that in intact erythrocytes the glycerophospholipids are not freely exposed to the exterior and thus are not available to the attack by the venom phospholipase A_2. Hydrolysis, however, becomes possible when membrane structural changes are induced by hypotonic media or by a number of membrane-active agents including the snake venom polypeptides. The hydrolysis products evolving in situ, lysolecithin and fatty acids, are themselves lytic, and hemolysis is therefore promoted in an autocatalytic fashion.

Cobra venom phospholipase A_2 hydrolyzes freely the glycerophospholipids in osmotically prepared red cell ghosts. The phospholipase A_2 from *Vipera palestinae* venom is unable to do so unless supplemented with DLF. It is noteworthy that in the presence of DLF *Vipera palestinae* phospholipase is able to split ghost phospholipids but only of the 'sensitive' erythrocytes, while in the presence of detergents like saponin, phospholipids are split in the ghosts from all erythrocytes species, indiscriminately. On this basis it was concluded that the different red cell sensitivities to whole cobra venom reflects primarily a different susceptibility to the action of the membrane-active polypeptides present in the venom, and not to the phospholipase[26]. OLDINGS et al.[31] support a different view. Comparing the potentiated hemolysis of sensitive guinea-pig erythrocytes with that of resistant rat erythrocytes, they showed that similar hemolysis times can be obtained by using, for the resistant cells, 50-fold more DLF and 100-fold more phospholipase and concluded that specific sensitivity derives from susceptibility not only to DLF, but also to the phospholipase.

The synergistic action of the membrane-active polypeptides with phospholipases A in promoting splitting of membrane phospholipids has been demonstrated also on membranes other than the red cell, as illustrated in Table III. As seen in the table splitting of phospholipids results in red cell hemolysis, inhibition of platelet clot-retracting activity and mitochondrial swelling.

C) *Effects on membrane permeability and transport.* Cobramine B, a membrane-active polypeptide from *Naja naja* venom, inhibits accumulative transport across cell membranes[34]. The inhibition seems to be of a general nature since it affects the transport of various classes of compounds such as small anions I^-, ReO_4^-), amino-acids, 3-*O*-methyl glucose and the organic ion *p*-aminohippurate, in a variety of tissues such as the thyroid, parotid, choroid plexus, small intestine and kidney, and in all animal species examined so far[34]. The question was raised as to whether membrane-active polypeptides produce this effect by an interaction with specific carriers, by interference with metabolically activated pump systems or by inducing a membrane change leading to leakiness. Studing the inhibition of iodine accumulation in thyroid slices treated with cobramine B, WOLFF et al.[11] conclude that the major portion of the inhibition is due to an increased I^- efflux resulting from leakiness of the membrane which becomes permeable to iodide as well as to potassium ions. Data obtained with red blood cells are also in favor of membrane leakiness. Thus, JACOBI et al.[30] showed that the moderate hemolysis and increased osmotic fragility of guinea-pig cells induced by a *Naja naja* membrane-active polypeptide are accompanied by an increased passive permeability to Na^+ ions. At the same time, the activity of the pump ATP-ase in human and guinea-pig erythrocytes is little or not affected[35]. Furthermore, efflux rates of Na^+ from preloaded human erythrocytes remains normal[34], suggesting that inhibition of active transport is not involved in the hemolytic mechanism. However, this may not be the case for all tissues since membrane-active polypeptides from cobra venom inhibit the Mg^{2+}-dependent, Na^+, K^+-activated ATP-ase of ox brain[35] and the Na^+ pump of toad bladder[34].

Data on the transport inhibition in KB cells under the action of *Naja nigricollis* γ-toxin[36] correlate well with the findings on red cells and thyroid slices. Finding that the toxin abolishes the accumulation

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[36] P. BOQUET, C.r. Acad. Sci. Paris *271*, 2422 (1970).

of a number of amino acids, BOQUET[36] first considered the possibility of the toxin affecting primarily the energy-generating processes. A lowering of cellular ATP level and a reduction in oxygen consumption were in favor of such a mechanism. However, the more likely mode of action seems to be an increased secondary efflux from the cells, while the primary phase of accumulation is less affected.

It can therefore be reasonably concluded that the membrane-active polypeptides induce initially a perturbation of permeability with outward leakage of accumulating compounds, before an impairment of the active transport becomes evident, if at all.

D) *Effects on membranal enzymes.* The membrane-activity of the venom polypeptides is further exemplified by their effect on membrane-bound enzymes. It has been shown that treatment of red cell osmotic ghosts with Ringhals venom DLF results in a marked increase in glyceraldehyde 3-phosphate dehydrogenase, adenylate kinase, 3-phosphoglycerate kinase and aldolase activities[37]. The activating effect was attributed to a disorganization of the membrane structure possibly resulting in uncovering of previously unavailable sites of the enzyme. In higher concentrations, DLF further loosens the membrane structure up to a point where the enzyme elutes from the membrane. Other basic proteins or polypeptides such as melittin, protamine and histone mimic, to some extent, the effects of DLF[37].

Similar to the glycolytic enzymes, the Mg^{++}-dependent, (Na^+-K^+)-ATP-ase is a membranal enzyme, however distinct from them in depending on the structural integrity of the membrane for normal functioning. Inhibition of ATP-ase activity by the membrane-active polypeptide documents the latters ability to disrupt the membrane structure. As shown in the previous chapter, this inhibition occurs in some tissues[34,35] while not in others[35], again reflecting different susceptibilities of the cell membranes to the polypeptide action.

The membrane-active polypeptide from *Naja naja atra* venom, identified with the 'cardiotoxin' by LEE et al.[13], inactivates the acetylcholinesterase in media devoid of mono- and bivalent cations and of polyanions[1]. The anti-cholinesterase activity, however, bears no relationship to the previously described effects since, resulting from a direct binding of the basic polypeptide to the anionic sites of the enzyme molecule, it is not a membrane phenomenon and can not be demonstrated in biological systems.

E) *Effects on excitable membranes: nerve and muscle.* A characteristic effect of the membrane-active polypeptides is deplorization of the membrane in excitable cells. Conduction is blocked in lobster and squid peripheral axons[38,39]. Treatment with Ringhals venom DLF, however ,failed to significantly increase the penetration of acetylcholine into the axons[39]. The high concentrations of DLF needed to produce effects on excitable membranes are not surprizing, in view of the fact that they are not directly exposed to the action of DLF, being covered by a protective layer.

The membrane-active polypeptides depolarize skeletal muscle and induce paralysis. Their action on the heart, systolic arrest in isolated heart preparations and death by ventricular fibrillation when administered to animals, led to their designation as 'cardiotoxins'.

The pharmacological effects of the polypeptides on nerve and muscle have been exhaustively reviewed by LEE[1] to whom I refer for further details. Noteworthy is the liberation of calcium from muscle fibers by the action of *Naja nigricollis* γ-toxin[36,40], which indicates a change in permeability as the primary effect of the polypeptide. Calcium administration supresses the ventricular fibrillation induced by the toxin[40].

F) *Cytotoxic effects.* BRAGANCA et al.[5] separated from *Naja naja* venom a basic fraction migrating as a single band on agarose gel electrophoresis. This fraction had cytotoxic effects on a number of cells, the most sensitive being the Yoshida sarcoma (YS) cells, while human leukocytes, rat lymphocytes and rat bone marrow cells were of moderate sensitivity. Human and rat erythrocytes showed resistance to the cytotoxic action[5]. Furthermore, PATEL et al.[41] demonstrated that cobra cytotoxin is able to differentiate between closely related cell types such as different strains of YS cells. The finding that phosphatidylethanolamine and phosphatidylserine inhibit to a considerable extent the lysis of YS cells by *Naja naja* cytotoxin, led PATEL et al.[41] to suggest that these phospholipids compete with receptor sites for cytotoxin on the cell membrane. Phospholipids and sialic acids were considered as possible candidates for receptor sites, and the specific architecture of individual cell membranes, resulting in different exposure of these sites, might acount for differences in toxin binding. It is noteworthy that removal of sialic acids from human erythrocytes with sialidase does not prevent DLF from inducing hemolysis and promoting phospholipid splitting[42]. A direct demonstration of polypeptide binding to YS cells and chicken erythrocytes was obtained by means of dansylated cytotoxin[41]. With chicken erythrocytes, which

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have a moderate susceptibility to cytotoxin, a progressive penetration could be observed. Thus, the dansylated toxin becomes attached to the membrane, enters the cytoplasm and reaches the nucleus. Swelling of the YS cells exposed to the toxin mark the early permeability changes, followed by extensive membrane damage as revealed by progressive release of RNA and protein. While K^+ and Mg^{++} stimulated lysis, Ca^{2+} had no effect in concentrations up to 10^{-3} *M* and, at higher concentrations, became inhibitory[41].

The cytotoxicity of another membrane-active polypeptide, the toxin γ from *Naja nigricollis* venom, was demonstrated on KB cells in culture (BOQUET[36]). Toxicity was revealed by a decreased susceptibility to neutral-red stain and by a diminution in amino-acid transport, nucleotide triphosphate contents and oxygen consumption[36]. A purified basic polypeptide from *Naja naja atra* venom produced complete inhibition of anaerobic glycolysis in Ehrlich ascites tumor cells[43]. Further study of this venom fraction, designated F_{12} or cytotoxic factor, led the authors to the conclusion that inhibition of glycolysis is a consequence of the toxin's membrane-activity[14]. In the hands of LEE et al.[44], fractionation of *Naja naja atra* venom produced 3 fractions all being cardiotoxic, hemolytic and having cytopathic effects on both HeLa and KB tumor cell cultures. Each of these fractions was able to disrupt cell monolayers[13].

5. *Characteristics of the polypeptide interaction with membranes*

A) *Time course.* The few data available on the time-dependence of polypeptide interaction with membrane suggest a fast process. Thus, the effect of cobramine B on I^- accumulation by thyroid slices was found to be nearly independent on the duration of preincubation of the tissue with the polypeptide; maximal effect was obtained in 5 to 15 min[11]. Similarly, the binding of $^{131}I^-$ labelled DLF to erythrocyte ghosts from various species was nearly maximal after 10 min incubation[32]. In contradistinction, intact red blood cells of the 'sensitive' species bind DLF progressively with increasing incubation time and hemolysis proceeds slowly[32,45]. It seems that in the osmotic ghost the binding sites for DLF are readily available while in the intact erythrocyte new binding sites are revealed stimultaneously with the progression of hemolysis.

B) *Temperature dependence.* The direct lytic effect of *Naja naja* DLF on guinea-pig erythrocytes is promoted by raising the temperature of incubation[25]. A similar temperature-dependence has been shown in protamine-induced hemolysis[46]. Also lysis of YS cells by *Naja naja* cytotoxin[41] is more pronounced at 37 °C than at 27 °C. Conversely, the inhibitory action of cobramine B on I^- accumulation by thyroid slices is markedly decreased by lowering the preincubation temperature from 37 °C to 4 °C[11].

C) *Prevention and reversal.* A number of polyanions were found able to combine with the basic membrane-active polypeptides, forming soluble or insoluble complexes, and preventing their effects. Thus heparin, dextran sulfate, RNA, gangliosides and suramine prevent the inhibition of iodide accumulation by cobramine B[11] and the synergistic action of Ringhals DLF with phospholipase A on red cells[7]. WOLFF et al.[11] noticed that once thyroid inhibition by cobramine B is established, polyanions fail to reverse it. This does not seem to hold for the hemolytic and phospholipid-promoting actions of DLF combined with phospholipase A. Addition of heparin following incubation of red cells with DLF did reverse the phospholipid splitting and potentiated hemolysis by phospholipase A[7]. Antisera to *Naja nigricollis* venom are able to prevent the action of toxin γ on abdominal muscle and isolated diaphragm preparations, but cannot reverse its effects when added after the toxin[40]. These data suggest that once the polypeptides have acted on membranes, the change is irreversible. In the specific case of the synergistic action with phospholipase A however, prior modification of the membrane by the polypeptide is insufficient and simultaneous presence of both DLF and phospholipase A are necessary for the phospholipid splitting to occur[7].

D) *Effects of calcium ions.* In earlier studies by VILLE and VOGT[47] and VOGT et al.[25] it was reported that Ca^{++} enhances the direct lytic action of *Naja naja* DLF, while EDTA inhibits it. It became clear later[45] that the apparent Ca^{++}-activation and EDTA inhibition were due to residual phospholipase A in the DLF preparation. Using purified DLF, addition of Ca^{++} to the medium actually decreased its hemolytic effect[45] (and CONDREA, personal observation). The antagonistic effect of Ca^{++} has been observed in many systems in which membrane-active polypeptides from various snake venoms were acted upon a diversity of membranes. Thus, studying the effects of toxin from *Naja nigricollis* venom on KB cells, BOQUET[36] found that addition of 10^{-2} *M* Ca^{++} to the medium prevents the inhibition of amino-acid transport and oxygen consumption as well as the efflux of nucleotide triphosphates induced by the toxin. In vivo administration of $CaCl_2$ to rats antagonizes completely the ventricular fibrillation induced by the toxin[40]. The authors connect this finding with the toxin's ability

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to liberate Ca^{++} from muscular fibres[40]. Furthermore, the lytic effect of *Naja naja* cytotoxin on YS cells in vitro[41] is stimulated by addition of K and Mg ions, while calcium ions have no effect up to 10^{-3} *M*, and become inhibitory from 3×10^{-3} *M* up.

Unlike the polyanions discussed in the previous chapter, the inhibition caused by Ca ions does not seem to result from a direct interaction with the toxin or from competition for membrane binding sites[29], but rather from their known membrane-stabilizing effect[48].

6. *Mode of action*

According to modern concepts membranes are a fluid mosaic of lipid and protein[49]. The phospholipids are organized in a bimolecular layer in which the fatty acid chains stretch inwards and the polar ends are oriented outwards. Intrinsic proteins penetrate the fatty acid region of the bilayer, with which they interact hydrophobically, while extrinsic proteins are bound by mainly ionics bonds on both sides of the lipid polar ends. The structure is stabilized also by lipid-lipid and protein-protein interactions and by Ca^{++}-mediated bonds. This general model allows for specific structural differences between membranes of particular chemical composition and/or function.

The membrane-active polypeptides are able to disturb this normal architecture and impair membrane function. Regarding the mechanism by which this result is achieved, two different approaches have been advanced so far.

One view is concerned both with the direct lytic effect and with the phospholipid splitting-facilitating action of the polypeptides. According to this view, the venom polypeptides become first attached to the membranes owing to their electropositive surface charge, and then penetrate the membrane structure through their lipophilic residues. This mode of action would resemble that of the lytic protein from bee venom, melittin, which disrupts membranes by penetrating their hydrocarbon region with which it interacts hydrophobically[50,51]. Further support for this view derives from the study of a number of synthetic basic polymers and co-polymers as possible DLF substitutes in inducing direct hemolysis and promoting phospholipid splitting by phospholipase A in osmotic ghosts[28]. The common characteristic of the active co-polymers is the presence of both basic and lipophilic groups such as in polyornithine-leucine, polynithine-leucine-alanine and polylysine-leucine. The basicity of the polypeptides is held responsible for their attachment to the membrane whereas the lipophilic side chains are invoked in the facilitation of the approach of phospholipase to phospholipid substrates situated in the membrane. Protamine[7] and the basic polymers polylysine and polyornithine[28] failed to duplicate the synergistic action of DLF with phospholipase A on human red cell membrane, but were moderately lytic and able to inhibit I^- accumulation by thyroid slices, although to a lesser extent than cobramine B[11]. A polylysine with average molecular weight of 14,000 was found optimal for the inhibitory effect. Polyarginine was also inhibitory but the respective monomers were not. Inactive were the natural basic proteins ribonuclease, spermine, lysosyme and histones[11].

However similar, the activity of the analogues is not identical to that of the venom polypeptides and the conclusions drawn by comparison should be cautions. Thus, the basic copolymers which promote splitting of red cell phospholipids by phospholipase A are, unlike DLF, markedly lytic. Moreover, they migrate in lipid solvents on chromatography, while DLF does not[28].

One characteristic of the synergistic action of DLF with phospholipase A is the necessity for their simultaneous presence, which suggests that DLF participates not only as a membrane modifier but also as a mediator in binding the enzyme to the membrane substrate[7,29]. The cationic groups of the membrane-active polypeptide might perform this function in a way similar to the mechanism of enzyme activation by Ca ions through formation of an enzyme-Ca-substrate complex[52]. Indeed, DLF and Ca^{++} are interchangeable as activators of phospholipid splitting in erythrocyte ghosts by *Vipera palestinae* phospholipase[29]. However, here again, the similarity is limited since DLF does not replace Ca^{++} in non-membranal systems while Ca^{++} does not replace DLF in mediating hemolysis and phospholipid hydrolysis by phospholipase A in the intact erythrocyte[29,45].

A different view in interpreting the mode of action of the venom polypeptide on red cell membranes has been advanced by the group of VOGT[25]. In their view, the potentiation of phospholipase A hemolysis by DLF is due to an alteration of the red cell membrane structure caused by the interaction of DLF with SH groups in the membrane. Combination of electropositive change with disulfide bonds is considered to be the structural feature enabling DLF and a number of peptide analogues such as an apamine fraction, vasopressin, anaphylatoxin[25] and viscotoxin B[53], to promote hemolysis by phospholipase A. VOGT et al.[25] consider the direct and the potentiated hemolysis with

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phospholipase A as independent on the detergent property of DLF, since, by reduction, the lytic activities are abolished while the detergent effect is not. In addition, synthetic SH-reagent like *p*-chloromercurybenzoate and N-ethylmaleimide, which are devoid of detergent properties, mimick the action of DLF when combined with phospholipase A. However, the basic membrane-active polypeptide from bee venom, melittin, potentiates the phospholipase A while being devoid of S-S bridges. An additional argument in favor of a possible interaction between the disulfide bonds of DLF and the SH groups of membrane proteins derives from the positive correlation between glutathion reductase activity and sensitivity to DLF in erythrocytes of various animal species[54]. The mode by which high enzyme activity would enhance the sensitivity to DLF rather than protect the cells against it is not easily explained. SCHROETER et al.[54] consider the possibility of a dual action: first, cleavage of the S-S bonds of DLF at the membrane site, enabling binding to a membrane constituent by new S-S bridges, and second, reduction of this bond and detachment of DLF.

The interaction of the disulfide bonds in DLF with membrane SH groups is still to be directly tested. Loss of activity by reduction of DLF does not necessarily demonstrate such an interaction; an alternative interpretation might be that reduction results in unfolding of the polypeptide and loss of the proper configuration which might be instrumental for its membrane-activity. Nor do the arguments derived from analogy with other natural or synthetic polypeptides support unequivocally one or the other of the two different approaches as to the mode of action of the venom polypeptides. As VOGT et al.[25] pointed out, there are apparently several possibilities of facilitating hemolysis and phospholipid cleavage by phospholipase A. The particular case of the venom polypeptides is still open to further investigation.

7. *Concluding remarks*

The membrane-active polypeptides from snake venoms are substances able to modify cellular membranes. In addition, they pave the way for the action of venom enzymes on normally unavailable membranal substrates. The remarkable ability to promote the action of phospholipases, while being only moderately lytic in themselves, distinguishes the venom polypeptides from membrane-active substances from other sources. The action of the venom polypeptides and enzymes is synergistic, the toxicity of whole venom exceeding the sum of the toxicities of the individual venom components.

It is noteworthy that specific cell types respond differently to the action of the membrane-active polypeptides: neoplastic cells react more strongly than normal cells, erythrocytes from certain animal species more strongly than from others. Understanding of the particular feature of the membrane which renders it susceptible to the action of the polypeptides calls for an identification of their site of attachment. Meanwhile, the sialic acids having been ruled out, acidic groups of phospholipids or proteins remain as possible candidates.

Résumé. On a isolé à partir du venin de serpents un nombre de polypeptides fortement basiques, à poids moléculaire réduit, qui n'exhibent pas d'activité enzymatique, ne sont pas neurotoxiques et ont un effet léthal modéré. Néanmoins, ces polypeptides sont hémolytiques, cytotoxiques et cardiotoxiques. Il semble que ces polypeptides excercent leur action au niveau de la membrane cellulaire en modifiant sa structure. Ces modifications structurales sont à la base des changements des propriétés membranaires telles que perméabilité, transport, excitabilité et activité enzymatique. De plus, les polypeptides basiques excercent une action synergique avec un enzyme présent dans le venin, la phospholipase A_2. Sous l'action combinée des polypeptides et de la phospholipase, les phospholipides membranaires deviennent susceptibles à l'hydrolyse par l'enzyme.

Deux mécanismes permettant d'expliquer le mode d'action des polypeptides sur les membranes cellulaires ont été proposés: 1. les polypeptides, grâce à leur structure réunissant basicité et résidus lipophyliques, modifient la membrane en la pénétrant; 2. l'altération de la membrane est la conséquence d'une interaction des groupes S-S des polypeptides avec les groupes -SH membranaires.

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