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Membrane damage by *Cerebratulus lacteus* cytolysin A-III. Effects of monovalent and divalent cations on A-III hemolytic activity

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The effects of monovalent and divalent cations on the hemolytic activity of *Cerebratulus lacteus* toxin A-III were studied. The activity of cytolysin A-III is remarkably increased in isotonic, low ionic strength buffer, the HC_{50} (the toxin concentration yielding 50% lysis of a 1% suspension of erythrocytes after 45 min at 37 °C) being shifted from 2 μg per ml in Tris or phosphate-buffered saline to 20–30 ng per ml in sucrose or mannitol buffered with Hepes, corresponding to a 50–100-fold increase in potency. On the contrary, hemolytic activity decreases progressively as the monovalent cation concentration in the medium increases for Na^+, K^+, or choline salts. The divalent cations Ca^{2+} and Zn^{2+} likewise inhibit the cytolysin A-III activity, but more strongly than do the monovalent cations specified above. Zn^{2+} at a concentration of 0.3 mM totally abolishes both toxin A-III-dependent hemolysis of human erythrocytes and toxin-induced leakage from liposomes. The observation of similar effects in both natural membranes and artificial bilayers suggests an effect of Zn^{2+} on the toxin A-III-induced membrane lesion, especially since Zn^{2+} does not alter binding of the cytolysin. The dose-response curve for toxin A-III exhibits positive cooperativity, with a Hill coefficient of 2 to 3. However, analysis of toxin molecular weight by analytical ultracentrifugation reveals no tendency to aggregate at protein concentrations up to 2 mg per ml. These data are consistent with a post-binding aggregational step which may be affected by the ionic strength of the medium.

Introduction

Cerebratulus lacteus cytolysin A-III is a highly basic and cross-linked protein with a molecular weight of 9810, whose complete covalent structure is known [1,2]. A 33 residue amphipathic helix predicted at the C-terminal end of the protein has been shown to be important for cytolytic activity [3]. Furthermore, amino terminal sequences of the polypeptide have the ability to penetrate preformed phospholipid bilayers [4], although the question of whether this penetration is a required step in the cytolytic pathway remains unresolved.

It has been shown that low concentrations of A-III are capable of causing membrane disruption in a variety of cells and in liposomes [1,2]. Human erythrocytes are 50% lysed at an A-III concentration of 3 μg per ml. The interaction of A-III with liposomes causes release of markers trapped within; the A-III concentration dependence of this effect varies with the phospholipid composition of

Abbreviations: HC_{50}, the toxin concentration yielding 50% lysis of a 1% suspension of erythrocytes after 45 min at 37 °C; IC_{50}, concentration of divalent cation causing 50% inhibition of lysis.

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the liposome from 1.6 μg/ml for dilinolenyl PC to 100 μg/ml for distearyl PC [5]. Toxin A-III has also been shown to be devoid of phospholipase A activity [6] in a pH-stat assay.

We have been interested in the mechanism of A-III induced lysis in the hope that the toxin could provide a well-characterized and easily manipulated model for the interaction of polypeptides with membranes. Since the interaction of many cytolysins with membranes is affected by ionic strength and divalent cations in the extracellular medium [7], we anticipated that A-III induced hemolysis might also depend on the extracellular ionic milieu. Thus, manipulation of the external concentrations of mono- and divalent cations could provide us with new insights into the mechanism of toxin-induced hemolysis.

The studies presented here demonstrate that A-III activity is dramatically influenced by ionic strength. Four important observations relating to A-III-dependent hemolytic activity have been made. First. the activity is increased by lowering the ionic strength at constant osmotic pressure and decreased by increasing salt concentration external to the target cells. Second, this activity change is probably not related to a protein conformational change and is likely a post-binding step. Third, divalent cations inhibit A-III activity. Finally, A-III activity displays positive cooperativity, with Hill coefficients of 2 to 3, and is synergistic with Triton X-100. These results suggest that the observed effects of ionic strength on cytolytic activity may be related to the conformation of the polypeptide following its binding to target membranes and that membrane surface potential plays an important role in A-III induced cell lysis.

Experimental Procedures

Materials. Toxin A-III was isolated from live *Cerebratulus lacteus* and purified as described elsewhere [6]. Bee venom melittin, phosphatidylcholine, phosphatidylserine, and Triton X-100 were purchased from Sigma, and $Na^{125}I$ from Amersham. *Stoichactis helianthus* venom was the generous gift of Dr. Alan Bernheimer, New York University School of Medicine.

Hemolysis assays. For hemolysis assays, most procedures were done according to Ref. 6 with minor modifications. Human blood, obtained by venipuncture was immediately diluted in cold phosphate-buffered saline (pH 7.4), and then washed four times by centrifugation. Toxin solutions were prepared with either standard phosphate-buffered saline or Hepes-sucrose buffer containing 20 mM Hepes and 260 mM sucrose (pH 7.4 with NaOH). In the experiments to test the effect of ionic strength, sucrose is replaced by different concentrations of the desired salt in Hepes-sucrose buffer of identical total osmolarity. Erythrocytes were at a concentration of 1% in the final assay, giving an absorbance of 1.0 at 540 nm upon complete hemolysis. Each assay was performed in triplicate.

Release of liposomal markers. This procedure was performed essentially as described in Ref. 5. Small unilamellar vesicles were prepared by drying a mixture of phosphatidylcholine and phosphatidylserine (1 : 1 molar ratio) under nitrogen and rehydrating in Hepes-buffered saline (5 mm Hepes, 130 mm NaCl, 5 mM KCl (pH 7.3) at a phospholipid concentration of 4 mg per ml. Under nitrogen, the suspension was briefly vortexed and then sonicated for 5 min. At this point, [^{3}H]sucrose was added, and the suspension frozen, thawed, and sonicated for 45 s. Liposomes were desalted by gel filtration on Sephadex G-50 equilibrated with Hepes-buffered saline. In this procedure, about 1% of the added marker is trapped.

Release of trapped markers was assessed by subjecting 100 μl aliquots of treated or control liposomes to gel filtration on columns of Sephadex G-50 packed in pasteur pipets. The excluded and included volumes, representing liposomal and free marker respectively, were collected and counted.

Binding assays. Binding of iodinated cytolysin A-III to human erythrocyte membranes was done as described by Blumenthal [8].

Results

Hemolytic activity of cytolysin A-III is influenced by ionic strength

The basis of membrane damage in erythrocytes is a breach of the plasma membrane permeability barrier, allowing ions whose intracellular and extracellular concentrations differ to leak into or out of cells leading to colloid-osmotic swelling and

lysis in most cases. It would therefore be expected that changing the osmotic pressure of the external solution should have an effect on the hemolytic activity of A-III. This study is illustrated in Fig. 1.

In phosphate-buffered saline, A-III activity is strongly inhibited by increasing the osmotic pressure with NaCl but, surprisingly, not with sucrose. Different salts, whether permeant or impermeant, show qualitatively similar inhibitory effects, with a rank potency of Na_2SO_4 > NaSCN > KCl > NaCl > choline chloride (Table I). Since increasing ionic strength inhibits A-III activity, it might be anticipated that its diminution would cause a corresponding increase. Indeed, this is the case. In Hepes-sucrose and Hepes-mannitol buffers, the hemolytic activity of A-III is remarkably increased, the HC_{50} being shifted from 2 μg per ml in phosphate-buffered saline to 20–30 ng per ml in Hepes-sucrose. When sucrose is progressively replaced by different concentrations of salts, the hemolytic curves are shifted to higher cytolysin concentrations in parallel (Fig. 2). This experiment clearly indicates that the osmotic pressure alone does not affect A-III activity; rather, the ionic strength has an effect which may be related to membrane surface potential (vide infra).

The fact that hemolytic activity is increased by lowering ionic strength seems to be unique to A-III. Two other polypeptide cytolysins which were tested, honeybee melittin and *Stoichactis helianthus* toxin, (curve not shown) showed no increase in activity comparing phosphate-buffered saline and Hepes-sucrose buffers (Fig. 3). Actually, the hemolytic activity of melittin in Hepes-sucrose buffer is slightly lower than in phosphate-buffered saline, consistent with a previous report by Bashford et al. [7].

The observation that the lytic activity of A-III is increased by decreasing ionic strength is also seen in rabbit erythrocytes and in mouse 3T3 cells. In rabbit erythrocytes, the HC_{50} of A-III is shifted from 2.5 μg per ml in phosphate-buffered saline to 0.75 μg per ml in Hepes-sucrose buffer. Lysis of mouse 3T3 cells by A-III in phosphate-buffered saline and in Hepes-Sucrose was examined by

TABLE I

INHIBITION OF A-III HEMOLYTIC ACTIVITY BY SALTS

$1 \cdot 10^9$ erythrocytes/ml in phosphate-buffered saline plus 0.25 M salt were treated with 2 μg/ml A-III, and the percent inhibition of hemolysis determined by comparison with activity in phosphate-buffered saline lacking added salts.

Medium	Inhibition(%)
0.25 M Na_2SO_4	95
0.25 M NaSCN	87.7
0.25 M KCl	60
0.25 M NaCl	55.5
0.25 M choline chloride	30.2

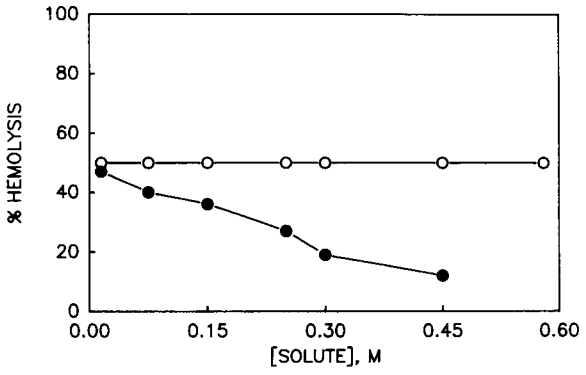

Fig. 1. A-III activity is inhibited by increasing salt concentration. The hemolysis assay was done as described in Experimental Procedures. Each sample contained $1 \cdot 10^9$ cells and an A-III concentration of 2 μg/ml. Hemolysis was measured in phosphate-buffered saline plus different concentrations of NaCl (●) or sucrose (○).

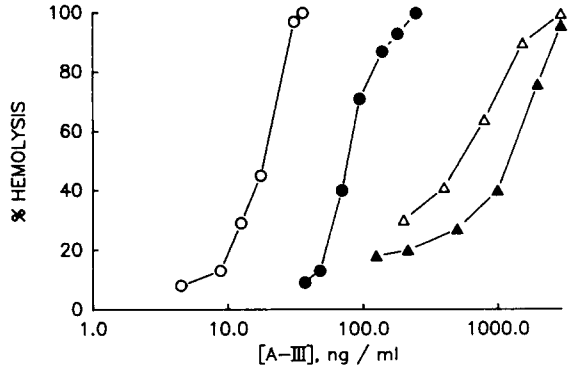

Fig. 2. Hemolytic activity of A-III in isotonic Hepes-sucrose buffer (20 mM Hepes 260 mM sucrose (pH 7.4 by NaOH)) at differing ionic strengths. In the various experiments depicted, sucrose is replaced by NaCl in order to maintain the same osmotic pressure. The NaCl concentrations used are 0 (○), 10 mM (●), 100 mM (△), and 140 mM (▲).

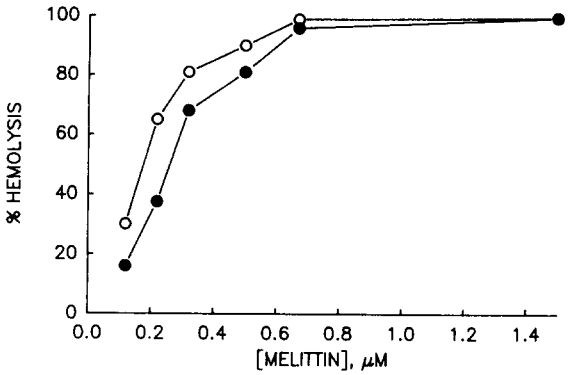

Fig. 3. Hemolysis of erythrocytes by melittin in phosphate-buffered saline and in Hepes-sucrose buffer. Melittin at the indicated concentrations was added to $1 \cdot 10^9$ cells, and the suspension incubated at 37 °C for 45 min. The cells were then removed by centrifugation for 3 min, and the absorbance at 540 nm measured (○) in cells in phosphate-buffered saline; (●) cells in Hepes-sucrose.

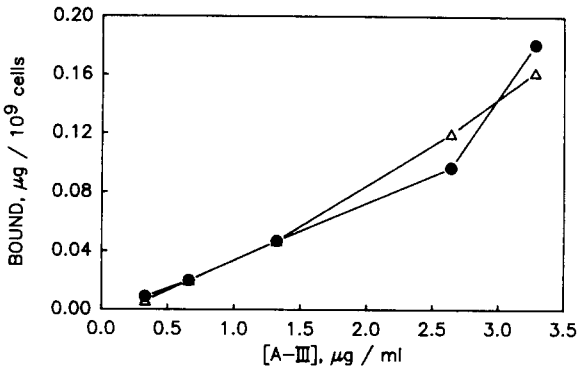

Fig. 4. Specific binding of ^{125}I-labeled A-III to proteinase inhibitor treated erythrocyte ghosts in either phosphate-buffered saline (●) or Hepes-sucrose buffer (△) was measured by rapid filtration after 20 min incubation in the indicated buffer. Specific binding was determined as the difference between total binding and that in the presence of 500 μg/ml of nonlabeled A-III.

microscopy. A-III at a concentration of 20 μg per ml in phosphate-buffered saline causes no significant lysis of a confluent monolayer of 3T3 cells. In contrast, 3T3 cells are completely lysed in Hepes-sucrose buffer at an A-III concentration of 8 μg per ml. Assessment of cytolysis by measurement of solubilized protein is consistent with this observation.

The activity change is a post-binding step

One simple explanation of the data described above is that binding of A-III to erythrocyte membranes is enhanced under conditions of low ionic strength. However, as shown in Fig. 4, this is not the case. Binding of iodinated A-III to human erythrocyte ghosts is identical in both phosphate-buffered saline and Hepes-sucrose.

A second potential basis for the altered activity is that the conformation of the cytolysin is sensitive to ionic strength, adopting a more active structure upon decreasing the salt concentration. However, analysis of the physical properties of A-III by analytical ultracentrifugation (data not shown) or intrinsic fluorescence emission spectroscopy (Fig. 5) both fail to reveal any significant buffer-dependent changes in conformation or aggregation state. These findings imply that the observed change in activity is a post-binding step which is unrelated to the solution conformation of the protein.

Inhibition by divalent cations

Kem and Posner [9] have reported that A-III activity is inhibited at 50 mM Ca^{2+}, a result which we confirm here. Furthermore, we find that lysis of erythrocytes by A-III is inhibited by other divalent cations in the rank order $Zn^{2+} > Co^{2+} >$

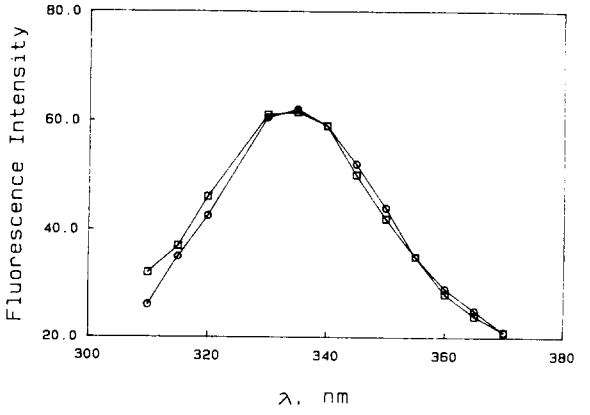

Fig. 5. The fluorescence emission spectrum of A-III (45 μg per ml) in phosphate-buffered saline (□) or in Hepes-sucrose (○) was determined at 37 °C on a Perkin-Elmer MPF-44A recording spectrofluorometer with excitation at 295 nm. The spectra shown represent an average of a minimum of three sweeps.

$Ca^{2+} = Mg^{2+}$ (Table II). Zn^{2+} is the most potent inhibitor of cytolysis, yielding 50% inhibition at a concentration of 0.5 mM in phosphate-buffered saline; in Hepes-buffered saline this value is reduced to 0.16 mM. Similar results have been obtained by Bashford et al. [7] in studies of Lettre cell lysis by Sendai virus, melittin, complement, or *Staphylococcus* α-toxin. To assess whether the effect of Zn^{2+} is simply due to inhibition of A-III binding, red blood cells were incubated with A-III (10 μg/ml), and zinc (4 mM) for 45 min at 37°C. This treatment resulted in no detectable hemolysis, although in the absence of Zn^{2+} this cytolysin concentration causes 100% hemolysis. After incubation, the cells were isolated by centrifugation, resuspended in Zn^{2+}-free, toxin-free phosphate-buffered saline, and incubated for an additional 30 min at 37°C. This treatment resulted in 65% lysis, and indicates that Zn^{2+} at a concentration completely inhibiting hemolysis does not prevent toxin binding to the cell membrane.

In order to assess whether the effect of Zn^{2+} was directly on the phospholipid bilayer and not on membrane proteins, release of liposomal markers in the presence and absence of Zn^{2+} was studied (Table III). Marker release was measured as described in 'Experimental Procedures'. After 30 min at 37°C, 60% of the [^{3}H]sucrose trapped counts were released from liposomes at an A-III concentration of 5 μg per ml, in agreement with earlier studies [5]. The presence of 0.2 mM Zn^{2+} totally abolishes this release. The inhibitory effect of Zn^{2+} on A-III dependent release of marker was reversed in the presence of 0.4 mM EDTA, although EDTA at 0.4 mM and Zn^{2+} at 0.2 mM in the absence of the cytolysin have no detectable effects on release of ^{3}H-sucrose from liposomes. This study further confirms the notion that divalent cations have their major effect on membrane phospholipid.

TABLE II

INHIBITION OF HEMOLYTIC ACTIVITY BY DIVALENT CATIONS

$1 \cdot 10^9$ erythrocytes/ml, Hepes-buffered saline plus different concentrations of divalent cations were treated with 10 μg/ml A-III and the IC_{50} values determined by comparison with activity in Hepes-buffered saline lacking divalent cations. In phosphate-buffered saline, the IC_{50} for Zn^{2+} is 0.5 mM.

Cation	IC_{50}
Zn^{2+}	0.16 mM
Co^{2+}	10 mM
Mg^{2+}	20 mM
Ca^{2+}	27 mM

TABLE III

EFFECT OF Zn^{2+} ON [^{3}H]SUCROSE RELEASE FROM LIPOSOMES

100 μl aliquots of [^{3}H]sucrose-containing liposomes (3000–3500 cpm) were treated with 5 μg/ml A-III in the presence or absence of 0.2 mM Zn^{2+} and/or 0.4 mM EDTA for 60 min at 37°C. The treated or control liposomes were subjected to gel filtration on columns of Sephadex G-50.

Medium	%cpm released
1. Buffer alone	0
2. Buffer alone + A-III	60 ± 0.2%
3. Buffer alone + A-III + Zn^{2+}	0
4. Buffer alone + A-III + Zn^{2+} + EDTA	47 ± 3%
5. Buffer alone + Zn^{2+}	0
6. Buffer alone + EDTA	0

It has been previously reported that the activity of some cytolysins is inhibited by divalent cations [7,10]. The fact that A-III shows a similar response to external divalent cations supports the notion that the action of divalent cations is at least in part on the structure of the lesions caused by membrane damaging agents, rather than on the mechanism of their formation. It is worthwhile to mention that it has been reported that the apparent inhibitory effect on Zn^{2+} on hemolysis induced by certain cytolysins such as vibriolysin is due to precipitation of hemoglobins by Zn^{2+} in Hepes-NaOH buffer [20,21]. This results in a false inhibition of hemolysis by Zn^{2+} when assayed by measuring absorbance at 540 nm due to released hemoglobins. To avoid this problem, inhibition of hemolytic activity by Zn^{2+} was compared in Hepes buffer and in phosphate-buffered saline, where no such precipitation occurs [20,21]. In Hepes-buffered saline, the IC_{50} of Zn^{2+} is 0.16 mM, slightly lower than the 0.5 mM observed in phosphate-buffered saline. This difference is probably due to precipitation of hemoglobins by Zn^{2+}. It should also be noted (vide supra) that the IC_{50} for inhibition of release of [^{3}H]sucrose from liposomes by Zn^{2+} is < 0.2 mM.

A-III exhibits positive cooperativity and shows synergy with detergents

The extent of hemolysis induced by different concentrations of A-III in phosphate-buffered saline is illustrated in Fig. 6. A simple hyperbolic dependency of leakage on A-III concentration is not observed. In both phosphate-buffered saline and Hepes-sucrose buffers the A-III concentration dependence of lysis is sigmoidal, with Hill coefficients in the range of 2–3, despite the dramatic differences in potency in these buffers. The observation of similar Hill coefficients in different buffers is also consistent with the change in A-III potency being a post-binding step. Furthermore, the observation of positive cooperativity indicates the importance of protein-protein interactions in cytolysis. Recent experiments in this laboratory have demonstrated aggregation of A-III to a tetramer induced by low levels of oleic acid (unpublished data).

Some membrane damaging agents including detergents and cytolysins, show synergy at subcytolytic concentrations [7]. Since A-III itself exhibits positive cooperativity it is possible that when it is added together with another cytolysin or detergent at a concentration at which each is near its threshold value (i.e., little hemolysis), extensive leakage might be induced. Combination of sublytic concentrations of A-III and 0.025% Triton X-100 causes 40% lysis of cells (data not shown).

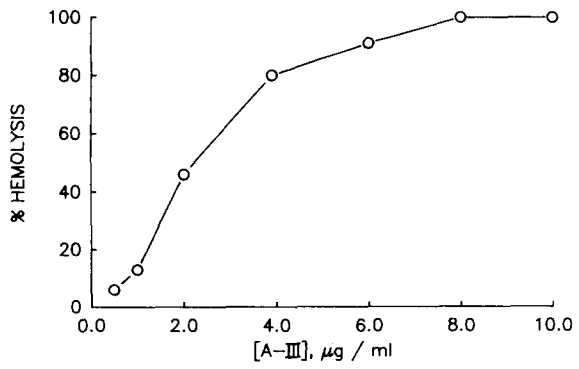

Fig. 6. Dependency of hemolysis on the concentration of A-III in phosphate-buffered saline. Erythrocytes were treated with A-III at the concentration indicated and the hemolysis was measured as described in the Experimental Procedures.

However, no apparent synergy between A-III and either melittin or *Stoichactis* toxin is observed.

Discussion

The fact that the activity of membrane damaging agents is affected by mono- and divalent cations is not restricted to the toxin A-III: erythrocyte system; a number of other reports on this topic have appeared [7,10]. However, the work presented here is to our knowledge, the first to show that cytolysin activity is enhanced by decreasing ionic strength, in contrast to data previously obtained with other cytolysins [7], such as melittin and Sendai virus where activity is decreased under such conditions.

The data reported herein demonstrate that the hemolytic activity of cytolysin A-III exhibits four major properties: (1) activity is inhibited by increasing ionic strength; (2) activity is stimulated by lowering ionic strength; (3) the activity change is not related to changes of protein conformation or aggregation state in solution and (4) divalent cations strongly inhibit cytolysis.

We have also shown that lysis of cultured 3T3 cells by A-III is significantly enhanced in low ionic strength buffers. Although lysis of erythrocytes is more readily quantitated, microscopic examination of 3T3 cultures, following treatment with A-III, reveals complete lysis in Hepes-sucrose at 8 μg per ml cytolysin, as contrasted with a lack of significant lysis in phosphate-buffered saline at a 2.5-fold greater A-III concentration. Measurement of the liberation of cellular contents into the medium also occurs at a lower A-III concentration in Hepes-sucrose buffer than in phosphate-buffered saline. Thus, the two unrelated cell types display qualitatively similar responses.

Bashford et al. [7] have shown that the cytolytic agents Sendai virus, *S. aureus* alpha toxin, melittin, and Triton X-100 have four properties in common. First, for all four agents, permeability changes are induced sequentially, suggestive of a growing lesion or pore. Second, the dose-response curve for agent is sigmoidal, indicating the existence of positive cooperativity. Third, divalent cations inhibit the lytic activity. Fourth, leakage is decreased by lowering the ionic strength of the external medium and can be restored in a Zn^{2+}

and Ca^{2+}-sensitive manner by the addition of salt. In the first three instances, *Cerebratulus* toxin A-III is similar to the other cytolysins tested, i.e., A-III induced hemolysis is sequential, its dose-response curves for hemolysis in different buffers are sigmoidal, indicating positive cooperativity, it displays significant synergy with Triton, and its hemolytic activity is inhibited by certain divalent cations. Surprisingly, however, and in direct contrast to the results of Bashford, et al. A-III dependent hemolysis is dramatically increased in low ionic strength buffers and is inhibited by salts. It is likely that this effect represents contributions of both the membrane surface potential and some unique feature of the structure of cytolysin A-III.

Biological membranes are negatively charged, and both mono- and divalent cations have the capacity to screen these charges. This screening effect is 1000–10000-times stronger for divalent than for monovalent cations [11]. The ability of cations, particularly divalent cations, to inhibit cytolytic activity in our study is consistent with the importance of surface potential in cytolysin-induced membrane damage. Although the mechanism by which the surface potential effect is exerted is presently unknown, our data clearly demonstrate that it is not simply an effect upon lysin binding.

That A-III can be distinguished mechanistically from melittin and/or alpha toxin is not unexpected. *Cerebratulus* cytolysin is a 95 residue polypeptide carrying 15 net positive charges at physiological pH, and analysis of its structure by the method of Kyte and Doolittle [12] reveals the presence of distinct hydrophilic and hydrophobic domains. In this analysis, the amino terminal two-thirds of the molecular is highly hydrophilic, carrying 12 net positive charges, while the C-terminal portion is relatively hydrophobic, carrying only 3. Such a structure leaves A-III without sequence homology to cytolysins such as melittin [13], *Stoichactis helianthus* toxin [14] or *Staphylococcus* alpha toxin [15]. The latter two agents are known to be pore formers [16,17], and all apparently possess the ability to insert into membranes. Based upon the results described herein and on those of others, it appears unlikely that common primary structural motifs exist among cytolytic proteins, even those which are mechanistically related.

Previous studies in this laboratory have implicated the C-terminal hydrophobic regions of A-III as being important for both binding and lysis of erythrocytes [3] and have also shown that the amino terminal portion of the protein has the ability to penetrate preformed liposomal bilayers [4]. Minimally, the N-terminal 13 residues of A-III, which include two charged residues, have been shown in protease encapsulation studies to cross a sphingomyelin-containing bilayer. The driving force for this seemingly unlikely insertional event is unknown, but apparently overall hydrophobicity is not the only factor to be considered in predicting membrane insertion. In this context. Finkelstein and his coworkers [18,19] have recently demonstrated the translocation of large domains of colicin E1, some of them quite polar, across planar lipid bilayers.

Whatever the mechanism of insertion of cytolysin A-III, our data strongly suggest that a charge interaction between the positively charged toxin and the negatively charged cell membrane is involved in membrane damage, and that screening of the surface potential attenuates activity. A study of the relationship between positive charges and toxin activity carried out by chemical modification and/or site-directed mutagenesis should afford a better understanding of the mechanism of action of this unusual protein.

Acknowledgements

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