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Release of Liposomal Markers by Cerebratulus Toxin A-III

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Marker release from liposomes induced by the cytolytic protein Cerebratulus lacteus toxin A-III was studied. No phospholipid specificity was apparent, but the sensitivity of liposomes to A-III varied with the membrane fluidity. With dioleylphosphatidylcholine liposomes, complete release occurred at 10-20 μg toxin per ml, depending on marker size. Kinetic experiments showed that release was rapid and exhibited no lag phase. The diameter of the A-III produced membrane lesion must exceed 90 Å, as tetrameric Concanavalin A is quantitatively released from A-III treated liposomes.

The marine heteronemertine Cerebratulus lacteus secretes a number of polypeptides and proteins which are active on cell membranes, among them a 10,000 molecular weight, highly basic cytolysin designated toxin A-III (1). This protein has been shown to cause lysis of human erythrocytes and Ehrlich ascites cells and to induce depolarization of cardiac Purkinje fibers in a tetrodotoxin-insensitive process (2). The covalent structure of toxin A-III is known (3, 4). Examination of this structure suggests the presence of an extended N-terminal region and a hydrophobic C-terminal sequence of approximately 30 residues which is likely to exist as an amphipathic helix. We have recently shown that at least the N-terminal 13 residues of A-III are capable of inserting across liposomal membranes (5) and have provided evidence suggesting that an intact C-terminal helical region is also required for cytolytic activity (6).

Studies of A-III binding to human erythrocyte membranes has provided data consistent with participation of both membrane proteins and phospholipids in the binding step (7). In this communication, we show that A-III does indeed interact directly with membrane phospholipids, that this interaction displays essentially no specificity with respect to phospholipid, and that interaction of A-III with liposomes causes release of markers trapped therein; included among these markers is iodinated Concanavalin A[1], having a monomer molecular weight of 25,000.

[1]The abbreviations are: Con A, Concanavalin A; DOPC, dioleylphosphatidylcholine; PC, phosphatidylcholine.

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Methods

Toxin A-III was purified as described elsewhere (1). Phospholipids, cholesterol, and dicetylphosphate were purchased from Sigma and stored at -20° in chloroform or chloroform-methanol. Purified Con A, obtained from Dr. W. D. Behnke, was iodinated using lactoperoxidase and hydrogen peroxide and desalted on BioGel P-10.

Small unilamellar vesicles were prepared by drying a mixture of phospholipid, cholesterol, and dicetylphosphate (4.1:3:0.4, molar ratio) under N_2 and rehydrating in 10 mM tris-HCl, pH 7.4, containing 145 mM NaCl and 2 mM $CaCl_2$ (Buffer A) at a phospholipid concentration of 4.0 mg per ml. Under N_2, the suspension was briefly vortexed, then sonicated for 4-5 min. At this point, the desired marker was added, and the suspension frozen, thawed, and sonicated 20 sec. Dependent on the trapped marker, liposomes were desalted by gel filtration on either BioGel A-0.5m or Sephadex G-50 equilibrated with Buffer A. Release of trapped markers was assessed by subjecting 100 µl aliquots of treated liposomes to gel filtration on columns of A-0.5m or G-50 packed in Pasteur pipets. Elution was accomplished with Buffer A containing either 100 mM sucrose or 100 µg per ml BSA as carrier; the excluded and included volumes, representing liposomal and free marker, respectively, were collected and counted.

Encapsulation of ^{125}I Con A was probed by protease accessibility. Desalted Con A liposomes were treated with protease K for 60 min, in some cases after a 30 min treatment with 10 µg per ml A-III. After digestion, undegraded Con A was precipitated in 10% TCA, washed, and counted. Nonencapsulated Con A is 40% solubilized by protease K under these conditions.

Results and Discussion

In order to assess the phospholipid specificity of toxin A-III, the release of ^{22}Na from liposomes incubated under iso-osmotic conditions was studied. Liposomes contained either sphingomyelin or a phosphatidylcholine of defined fatty acid composition, as well as cholesterol and dicetylphosphte (molar ratio = 4.1:3:0.4). The data shown in Table 1 indicate that A-III recognizes liposomes containing any of the phospholipids tested, as measured by marker release. The extent of release shows a positive correlation with membrane fluidity, especially within the C18 phosphatidylcholine series. Similar results have been obtained by Duncan and Buckingham in studies of streptolysin S (8). It is therefore unlikely that A-III exhibits a specificity for a unique phospholipid, as has been

Table 1

Effect of Different Phospholipids on $^{22}Na^+$ Release

Phospholipid	EC_{50}, µg per ml	Minimal (AIII) giving 100% release
Sphingomyelin	90	n.d.
Dimyristylphosphatidylcholine	>100	n.d.
Dipalmityl PC	>100	n.d.
Distearyl PC	~100	n.d.
Dioleyl PC	2.5	10
Dilinoleyl PC	2.1	10
Dilinolenyl PC	1.6	7.5

Release of $^{22}Na^+$ from liposomes containing the indicated phospholipid, cholesterol, and dicetylphosphate was measured after 60 min at 37° as described in Methods. EC_{50} is the toxin concentration causing 50% release of the marker. n.d. = not determined.

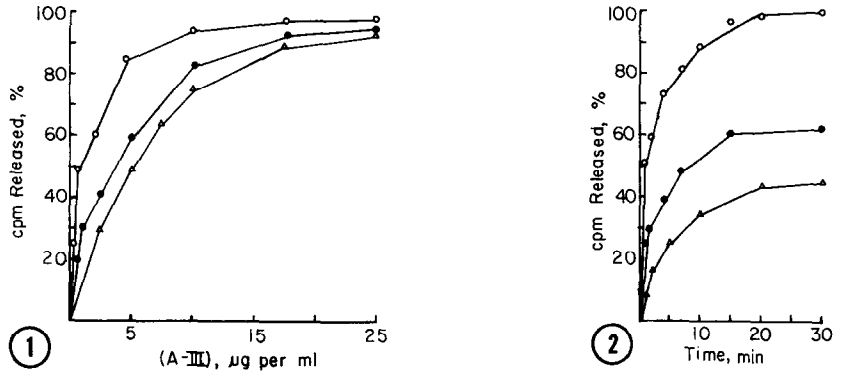

Figure 1. A-III concentration dependence for release of $^{22}Na^+$ (o), ^3H-sucrose (•), or ^{125}I-Con A (△) from liposomes containing dioleyl phosphatidylcholine. All samples were incubated for 60 min at 37° and then separated into liposomal and free fractions by gel filtration on 2.1-ml columns of Bio Gel A-0.5 m or G-50 as described in methods. Efflux in the absence of toxin has been subtracted from the data shown. The % efflux is calculated as $[(\text{cpm out})_t - (\text{cpm out})_0/\text{cpm added} - (\text{cpm out})_0]$; the total recovery of counts ranged from 89-97% in all experiments.

Figure 2. Time course of A-III dependent efflux of $^{22}Na^+$ (o), ^3H-sucrose (•), or ^{125}I-Con A (△) from DOPC liposomes. Liposomes containing the indicated marker were incubated at 37° with 5 µg per ml A-III and aliquots removed and separated by gel filtration of the indicated times. Efflux in the absence of A-III has been subtracted at each time point, and % release calculated as described in Figure 1.

suggested for Stoichactis toxin and sphingomyelin (8). All subsequent studies used dioleylphosphatidylcholine as phospholipid.

As shown in Figures 1 and 2, toxin A-III also induces release of ^3H-sucrose or ^{125}I-Concanavalin A from DOPC[1]-containing liposomes. Figure 1 shows the toxin concentration dependence for release of either $^{22}Na^+$, ^3H-sucrose, or ^{125}I-Con A, measured after 60 min at 37°. Under these conditions, spontaneous loss of marker represents less than 10% of the total; this background level has been subtracted to give the family of curves shown. Although the concentration dependence is similar for all markers tested, the differences observed are reproducible. While $^{22}Na^+$ is released at a toxin concentration less than that required for hemolysis of human erythrocytes (HC_{50} = 3 µg per ml (1)), both sucrose and Con A release, and hemolysis display rather similar concentration dependences. Essentially complete release is obtained at 10 µg per ml for Na^+, 15 µg per ml for sucrose, and 20 µg per ml for Con A. That the Con A is indeed within the liposomes, rather than simply absorbed to the surface, is shown in Table 2. Liposomal Con A is fully resistant to external protease K, whereas free Con A is partially degraded. Pretreatment of Con A liposomes with A-III (10 µg per ml) renders the lectin partially sensitive to protease K. At 10 µg per ml, Con A is approximately 60% released (Fig. 1). Of this, 40-

Table 2

Effect of protease K on ^{125}I-Con A and ^{125}I-Con A liposomes

Sample	Protease K	cpm TCA-precipitable, %
Con A	-	90.4
Con A	+	49.7
Con A-Lp	-	86.5
Con A-Lp	+	78.2
Con A-Lp + AIII	+	63.2

Samples were incubated 60 min at 37 ° in the presence or absence of 5 μg protease K; for the experiment with Con A-Lp and A-III, the liposomes were pretreated for 45 min with 10 μg per ml A-III. After hydrolysis, samples were mixed with 100 μg BSA and precipitated in 10% TCA. Pellets were washed twice with 10% TCA, solubilized, and counted.

50% should be rendered TCA-soluble by protease K (or 24-30% of the total cpm added). The observed value is consistent with this prediction. Furthermore, iodinated Con A is bound to Sephadex G-50, known to be an affinity ligand for this lectin (10), while liposomal Con A is not (data not shown). Thus, the release observed in Figures 1 and 2 must represent transfer of the protein out of an internal compartment and not merely discharging of surface associated material.

The kinetics of marker efflux were studied at a number of different toxin concentrations; a representative experiment, carried out at 5 μg per ml A-III is shown in Figure 2. Consistent with the concentration dependence data, Na^+ is released most rapidly, followed in order by sucrose and Con A. Similar data are obtained at 2.8 and 10 μg per ml toxin, with the outside:inside ratio at each time point being smaller at the former concentration and larger at the latter. It is noteworthy that no lag in efflux is observed even at the lowest A-III levels tested. This is in contrast to the results obtained by Buckingham and Duncan (11) using Streptolysin S where a lag of 5 to 15 min was observed, but similar to their data for Streptolysin O. It may be noteworthy that the latter peptide is proposed to cause lysis via a noncolloid-osmotic process (12), and to induce membrane lesions of >128 Å diameter.

Two other points regarding our data should be noted. First, under the conditions of our studies, Con A should exist as a tetramer with an approximate diameter of 90 Å (13, 14). Thus whatever the molecular nature of the membrane lesion caused by A-III, it must permit passage of relatively large molecules. Second, doubling the concentration of liposomes, while holding toxin concentration constant, results in a plateau at about 60%

release of any of our trapped markers; kinetics of release are unaltered (data not shown). This suggests that either a large fraction of the liposomes are toxin free, which seems unlikely on statistical grounds, or that a threshold level of A-III molecules per liposome is required for marker release to be observed.

In summary, the conclusions of this study, which suggest that a protein receptor for A-III may not be an absolute requirement for hemolysis, are threefold: (1) that Cerebratulus toxin A-III causes release of liposomally-entrapped markers in a process which displays no phospholipid specificity, (2) that the concentration dependence of release of small molecules from DOPC-containing liposomes is similar to the concentration-dependence of hemolysis, and (3) that the membrane lesion produced by A-III must exceed 90 Å in diameter, as it allows escape of tetrameric Con A from liposomes. Experiments designed to describe the nature of the lesion are in progress.

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