Biochimica et Biophysica Acta, 555 (1979) 79—88

BBA 78434

MEMBRANE DAMAGE BY A TOXIN FROM THE SEA ANEMONE *STOICHACTIS HELIANTHUS*

II. EFFECT OF MEMBRANE LIPID COMPOSITION IN A LIPOSOME SYSTEM

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(Received December 11th, 1978)

Key words: Liposome; Membrane lipid; Toxin; Detergent; Membrane channel; (Sea anemone)

Summary

In the first paper of this series, it was shown that a toxin from the sea anemone *Stoichactis helianthus* increased the permeability of black lipid membranes due to transmembrane channel formation. In the present study, we have used liposomes to examine the reactivity of the toxin with different phospholipids. Membrane damage was assessed by measuring the release of $^{86}Rb^+$ and ^{14}C-labeled membrane lipid. For the different lipids, the rank order of marker release was: sphingomyelin > C18 : 2 phosphatidylcholine > C18 : 1 phosphatidylcholine > C18 : 0 phosphatidylcholine > C16 : 0 phosphatidylcholine = C14 : 0 phosphatidylcholine. In C14 : 0 and C16 : 0 phosphatidylcholine liposomes there was no ^{14}C-labeled lipid release and only 13 to 16% $^{86}Rb^+$ release which corresponds to the $^{86}Rb^+$ content in the outermost aqueous shell of multilamellar liposomes. This indicates that membrane damage was limited to the outermost bilayer. In liposomes prepared with the other lipids, the extent of release of both markers increased proportionately with the length and the degree of unsaturation of the lipids' acyl side chains. Sphingomyelin liposomes were the most susceptible with 47% of the ^{14}C-labeled lipid marker and 90% of the $^{86}Rb^+$ marker being released. The large extent of ^{14}C-labeled lipid release is attributed to a detergent-like activity of the toxin which presumably is due to the amphipathic nature of the protein. Thus, the toxin can inflict membrane damage in two ways: (1) channel formation, and (2) detergent

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Abbreviations: C14 : 0, C16 : 0, C18 : 0, C18 : 1, C18 : 2 represent dimyristoyl, dipalmitoyl, distearoyl, dioleoyl, and dilinoleoyl phosphatidylcholine, respectively.

action. The importance of one mechanism or the other apparently varies depending on membrane structure and lipid composition.

Introduction

The sea anemone *Stoichactis helianthus* produces a toxin (presumably originating from the nematocyst cells) of molecular weight 16 000 and isoelectric point 9.8 which lyses various mammalian cells including erythrocytes but not bacterial cells [1—3]. The hemolytic activity is inhibited by exogenous sphingomyelin as well as by pre-treatment of target cells with phospholipases or staphylococcal sphingomyelinase [2]. In light of these observations as well as the demonstration that the toxin forms complexes with sphingomyelin, it has been suggested by Bernheimer and co-workers that sphingomyelin serves as a receptor for the toxin and that the lytic activity is due to a specific reaction with this lipid in the membrane [2,3]. Thus, the cytolytic attack process is thought to be directed against the lipid bilayer portion of the membrane. Interestingly, the *S. helianthus* toxin possesses no sphingomyelinase or phospholipase activity. It is, therefore distinct from two other toxins of anemone origin: one is a 4000 dalton peptide derived from *Anemonia sulcata* which inhibits the inactivation system of the sodium channel in nerve [4], the second one, isolated from *Aiptasia pallida*, exhibits phospholipase activity [5].

In the preceding paper of this series, it was shown that the *S. helianthus* toxin can increase the permeability of planar black lipid bilayers by six orders of magnitude [6]. The mechanism of this action involves the formation of small transmembrane channels (approx. 6 Å diameter). The capacity of the toxin to increase membrane permeability was observed in a wide variety of lipid bilayers including egg phosphatidylcholine, glycerol monoolein and oxidized cholesterol. The susceptibility of such a variety of membranes to the sea anemone toxin casts doubt on the proposal that the toxin reacts specifically with sphingomyelin. Therefore, it was of interest to explore systematically the reactivity of the toxin with different phospholipids. Specifically, we have examined the susceptibility of phosphatidylcholines differing with respect to acyl chain length as well as acyl chain unsaturation. For the purpose of this study, liposomes were used because of the ease with which the lipid composition can be changed. The extent of liposomal membrane damage by toxin has been assessed by two criteria. First, the release of internally trapped $^{86}Rb^{+}$ was monitored as a measure of membrane permeability. Second, the release of ^{14}C-labeled phospholipid was measured in order to determine whether the toxin causes a gross destruction of the lipid bilayers. We have previously shown that the latter phenomenon occurs in the process of liposomal membrane damage by the terminal complement components [7]. Thus, we were curious to determine whether this phenomenon is shared by other amphipathic proteins.

Materials and Methods

Toxin

Sea anemone toxin was the generous gift of Dr. John Devlin. Its properties were described previously [1—3,6].

Radioisotopes

$^{86}Rb^+$ as RbCl and [^{14}C]phosphatidylcholine were purchased from New England Nuclear, Boston, MA. The [^{14}C]phosphatidylcholine was of algal origin with a specific activity of 1.7 Ci/mM. The specific activity of $^{86}Rb^+$ ranged from 1 to 3 Ci/g RbCl.

Lipids

Dimyristoyl phosphatidylcholine (C14 : 0), dipalmitoyl phosphatidylcholine (C16 : 0) and dicetyl phosphate were purchased from P-L Biochemicals Inc., Milwaukee, WS; C18 : 0. C18 : 1, C18 : 2 lecithins and bovine sphingomyelin were obtained from Supelco, Bellefonte, PA.

Buffer

A 5 times concentrated stock solution of veronal-buffered saline was prepared by dissolving 10.19 g sodium veronal and 83.0 g NaCl in 1500 ml H_2O, adjusting to pH 7.4 with 1 N HCl and making up to 2000 ml with H_2O. For experimental use, the 5X stock solution was diluted 5-fold with H_2O (veronal-buffered saline, ionic strength = 0.15), and gelatin was added to a final concentration of 0.02%.

Preparation of liposomes

Liposomes were prepared by the method of Haxby et al. [8], except for minor modifications. In brief, a chloroform solution of the desired phospholipid was mixed in a 25 ml conical flask with stock solutions of cholesterol, dicetyl phosphate and [^{14}C]phosphatidylcholine. The last component was added as a trace marker. The molar ratios of phospholipid/cholesterol/dicetyl phosphate were 1 : 1 : 0.3. The organic solvent was evaporated and the dry lipid film was dispersed in 300 μl of veronal-buffered saline containing $^{86}Rb^+$ by agitation on a Vortex mixer at full speed for 1.5 min in the presence of a few glass beads. In the case of unsaturated lipids, the procedure was performed under nitrogen. In order to remove untrapped $^{86}Rb^+$, the liposome suspension was passed through an agarose 0.5 m gel (Bio-Rad Laboratories) column (30 X 0.8 cm). The liposome preparation, which eluted in the void volume of the column, was stored at 4°C and was stable for 5 to 10 days as judged by the degree of spontaneous leakage of $^{86}Rb^+$.

Measurements of marker release from liposomes treated with toxin

A mixture of 20 μl liposome suspension and 230 μl veronal-buffered saline was placed in an ice-water bath and 100 μl of toxin solution at various concentrations was added. Following incubation for 30 min at 37°C, the reaction mixtures were quantitatively transferred to an agarose 50 m (Bio-Rad Laboratories) column (30 X 0.8 cm) followed by elution with veronal-buffered saline to separate the released markers from the liposomes. In order to reduce the loss of lipid through absorption on the agarose gel, the columns were pretreated with 200 μl of unlabelled liposome suspension containing 0.5 μmol phospholipid. The recovery of radioisotopes from the column was between 93 and 100%. 1 ml fractions were collected from the column and the β-emissions for ^{14}C and $^{86}Rb^+$ were counted in a Beckman LS-233 liquid scintillation

counter. The specific toxin dependent marker release was calculated by subtracting the values for spontaneous marker release obtained in parallel control experiments.

Results

Quantative analysis of marker release from liposomes

In order to quantitate the extent of liposomal membrane damage by toxin, it was necessary to separate released markers from trapped markers after the liposomes were treated with toxin. This was accomplished by agarose gel column chromatography. Fig. 1 shows the elution profiles for liposomes before and after treatment with the sea anemone toxin. The elution profiles contain two peaks; the first peak corresponds to liposomes eluted in the void volume, and the second peak reflects the released markers which eluted in the internal volume of the column. The released markers are well separated from the liposome peak. Hence, the concentration of free and trapped labels can be measured by summing the radioactivity associated with individual peaks. In control experiments, the degree of spontaneous $^{86}Rb^{+}$ release varied between 2 and 8% and the release of [^{14}C]phosphatidylcholine ranged between 0.1 and 1.5%.

Kinetics of marker release

Fig. 2 shows a kinetic experiment which was performed with C14 : 0 lecithin liposomes in order to determine the minimum time required to achieve an end-

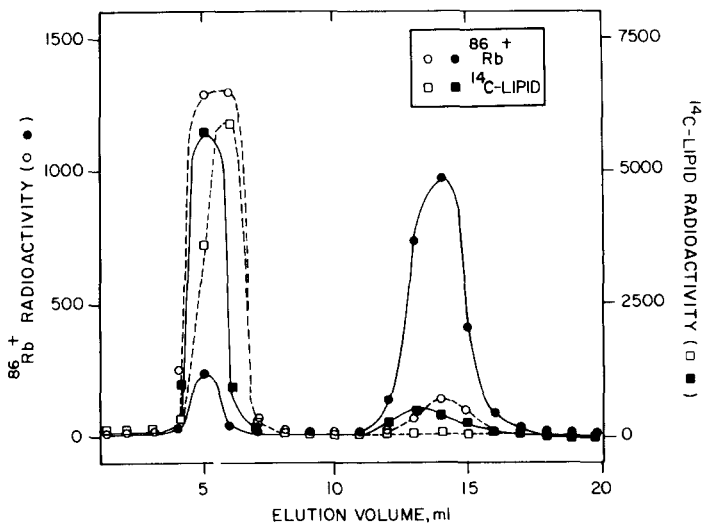

Fig. 1. Quantitative determination of marker release from liposomes by agarose 50 m gel column chromatography. Liposomes contained trapped $^{86}Rb^{+}$ (circles) as a water soluble marker and [^{14}C]phosphatidylcholine (squares) as a membrane marker. The first set of four peaks represents the liposomes which eluted in the void volume of the column. The second set of four peaks represents the released markers which eluted within the internal volume of the column. The dotted lines trace the release profile (open symbols) of markers in a control experiment with only buffer. The solid lines represent the release profile (solid symbols) of markers from a C18 : 1 phosphatidylcholine liposome after treatment with sea anemone toxin (33 μg/ml).

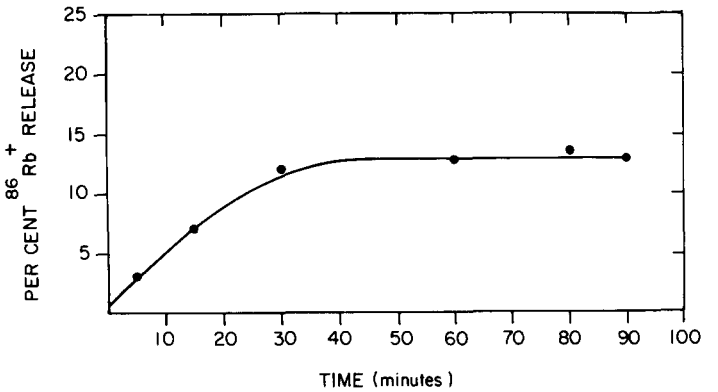

Fig. 2. Kinetics of $^{86}Rb^{+}$ release from C14 : 0 phosphatidylcholine liposomes. Liposomes were incubated at 37°C together with toxin (50 μg/ml). Samples were taken at various times and applied to an agarose column in order to separate released marker from liposomes. The graph records the percentage of $^{86}Rb^{+}$ released (after subtraction of the control sample containing only buffer) versus the time of sampling.

point in the toxin-liposome reaction. The graph displays the specific percentage of $^{86}Rb^{+}$ release (after subtraction of the buffer control) versus time. As shown in the figure, the 30 min time point corresponded to a 97% reaction completion level and was selected as a standard condition for all subsequent experiments.

Effect of lipid composition on the release of $^{86}Rb^{+}$

Fig. 3 displays a family of $^{86}Rb^{+}$ release curves as a function of toxin concentration for a series of liposome samples prepared from different phosphatidylcholine as well as bovine sphingomyelin *. It is evident that the toxin caused the release of marker from all of the preparations. However, there are marked quantitative differences between samples which correlate with the length and the degree of unsaturation of the acyl chains of the lipids. The rank order for $^{86}Rb^{+}$ release from the different preparations is in the sequence: sphingomyelin > C18 : 2 phosphatidylcholine = C18 : 1 phosphatidylcholine > C18 : 0 phosphatidylcholine > C16 : 0 phosphatidylcholine = C14 : 0 phosphatidylcholine. Thus, the extent of the toxin-liposome interactions was promoted by lipids which contain long and unsaturated acyl side chains. It should be noted that all liposome samples were prepared with 50 mol% cholesterol in order to minimize the differences of membrane fluidity due to varying lipid acyl chains.

Effect of lipid composition on the release of [^{14}C]phosphatidylcholine

The results shown in Fig. 4 demonstrate that the *S. helianthus* toxin caused the release of substantially different amounts of [^{14}C]phosphatidylcholine from liposomes of different bulk lipid composition. It is of interest that the various lipids generally displayed a qualitative similarity between [^{14}C]phos-

* The fatty acid composition of the bovine sphingomyelin samples was provided by Supelco, Inc.; the identity of the acyl chains and their proportion (mol%) was: C14 : 0 (0), C16 : 0 (3), C18 : 0 (21), C18 : 1 (1), C20 : 0 (1), C22 : 0 (4), C24 : 0 (6), C24 : 1 (52), other (12).

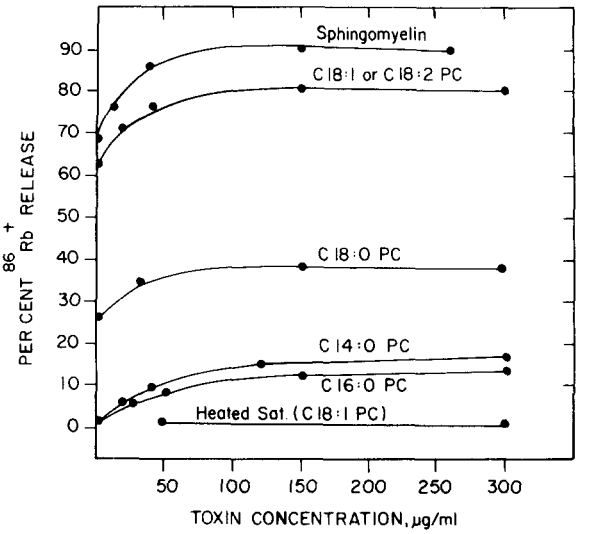

Fig. 3. Effect of lipid composition on the release of $^{86}Rb^{+}$ from toxin-treated liposomes. Liposomes were prepared with dimyristoyl (C14 : 0), dipalmitoyl (C16 : 0), distearoyl (C18 : 0), dioleoyl (C18 : 1) or dilinoleoyl (C18 : 2) lecithin, or bovine sphingomyelin. All of the preparations contained 50 mol% cholesterol to minimize changes in membrane fluidity. Samples were incubated for 30 min at 37°C with various amounts of toxin. The released $^{86}Rb^{+}$ marker was separated from liposomes by column chromatography on agarose 50 m.

phatidylcholine release and $^{86}Rb^{+}$ release (Fig. 3). However, there are three notable quantitative differences. First, for the C14 : 0 and the C16 : 0 phosphatidylcholine liposomes only 1% of the available [^{14}C]phosphatidylcholine was released, whereas approx. 15% of the trapped $^{86}Rb^{+}$ was released. Second, there is a disparity in the release of ^{14}C-labeled lipid marker from C18 : 1 and C18 : 2 phosphatidylcholine liposomes, although the $^{86}Rb^{+}$ release curves were identical. Third, for all of the C18 phosphatidylcholine samples as well as for

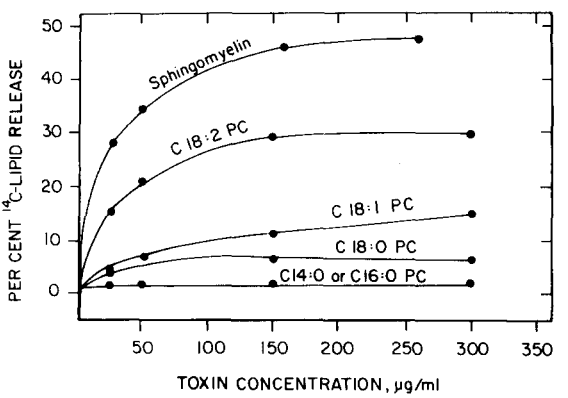

Fig. 4. Effect of lipid composition on the release of [^{14}C]phosphatidylcholine from toxin-treated liposomes. The experimental protocol is described in Fig. 3; [^{14}C]phosphatidylcholine and $^{86}Rb^{+}$ release were determined simultaneously in the same experiment. The graph displays a family of dose-response curves for different liposome preparations and records the percent specific ^{14}C-labeled lipid release versus the concentration of toxin in the reaction mixture.

sphingomyelin liposomes the release of [^{14}C]phosphatidylcholine at low toxin concentration (1 μg/ml) was very much less (1—3%) than the release of $^{86}Rb^+$ (25—67%).

Discussion

In the preceding paper, one of us has shown that the *S. helianthus* toxin can increase the permeability of planar black lipid bilayers prepared from various lipids (e.g., egg phosphatidylcholine, glycerol monoolein, and oxidized cholesterol) by one million-fold [6]. The mechanism of this action involves the insertion of toxin molecules into the lipid bilayer and the assembly of transmembrane channels via an aggregation reaction between three protein molecules. These observations correlate with the potent hemolytic activity of the sea anemone toxin previously reported by others [1—3]. However, the capacity of the toxin to increase the permeability of lipid bilayers which do not contain sphingomyelin is discordant with the previous suggestion that sphingomyelin is a specific receptor for the toxin [2].

The purpose of the present experiments was to examine systematically the effect of membrane lipid composition in a liposome system on the process of membrane damage by the *S. helianthus* toxin. The results on $^{86}Rb^+$ and [^{14}C]-phosphatidylcholine release, shown in Figs. 3 and 4, are of interest in two respects: (1) the rank order of marker release from liposomes with different lipid composition, (2) the large extent of $^{86}Rb^+$ and ^{14}C-labeled lipid release which reached 90% and 47%, respectively, in the case of sphingomyelin liposomes.

The infleunce of membrane composition on the release of [^{14}C]phosphatidylcholine from liposomes treated with toxin is shown in Fig. 4 for a homologous series of phosphatidylcholine and bovine sphingomyelin. In the case of sphingomyelin the composition of the two acyl side chains is different. One chain is part of the sphingosine base and has a constant length of 15 carbon atoms. The second chain is acylated to the amine group of sphingosine and has a variable composition depending on the source, but characteristically is comprised of long unsaturated fatty acids. As shown in Fig. 4, the quantity of ^{14}C-labeled lipid release in our experiments varied from 1% (C14 : 0 and C16 : 0 phosphatidylcholine) to 47% (sphingomyelin). Specifically, the rank order of marker release was: sphingomyelin > C18 : 2 phosphatidylcholine > C18 : 1 phosphatidylcholine > C18 : 0 phosphatidylcholine > C16 : 0 phosphatidylcholine = C14 : 0 phosphatidylcholine. The arrangement of this sequence, which also applies to the release of $^{86}Rb^+$, is obviously in the order of decreasing acyl chain length and unsaturation. Thus, the susceptibility of liposomes to the sea anemone toxin is more dependent on the chemical nature of the acyl chains of the membrane lipids than on class (e.g., sphingolipid or phospholipid). This result is not in accord with the view of Linder et al. that sphingomyelin is a specific and unique membrane receptor for the toxin [3].

However, it must be emphasized that the extent of [^{14}C]phosphatidylcholine removal in our experiments may not be directly related to the binding capacity of the toxin for the bulk membrane lipid. Instead, the ^{14}C-labeled lipid removal is a measure of the extent of interaction between the toxin and the liposomal

membrane. This interaction is dependent on the ability of the toxin to insert into the lipid bilayer which is, presumably, determined by the packing-geometry of the lipids. The possibility that the [^{14}C]phosphatidylcholine introduced reaction centers for the toxin into the bilayer is unlikely, because the extent of marker release did not change when the concentration of the marker was increased ten-fold (Shin, M.L., unpublished observation).

The release of ^{14}C-labeled lipid marker from liposomes cannot be due to the hydrolysis of phospholipid, because the *S. helianthus* toxin does not possess phospholipase activity [2]. It is also unlikely, on a thermodynamic basis, that the released lipid exists in a 'free' form in the aqueous phase. Rather, we are inclined to believe that the release of the marker represents the extraction of lipid from liposomes by toxin and that it exists in a co-micelle state bound to toxin protein. Thus, the toxin acts like a detergent. This appears reasonable since this protein is an amphipathic molecule.

Mechanism of $^{86}Rb^+$ release from liposomes by toxin

In previous experiments with black lipid bilayers it was shown that the toxin produces transmembrane channels [6]. It is reasonable to assume that such channels are also formed in liposomes. Precedent for this assumption is provided by the antibiotic gramicidin which has been shown to produce channels in black lipid membranes [9] as well as liposomes of greatly different lipid composition [10—11]. Thus, we believe that the sea anemone toxin inflicts membrane damage, indicated by the release of $^{86}Rb^+$, in two ways: (1) channel formation, and (2) detergent action.

The fact that a large part of the $^{86}Rb^+$ release is attributable to channels can be seen most readily by considering the extent of $^{86}Rb^+$ release relative to phospholipid release at very low toxin concentration, e.g., 1 μg/ml. At this protein concentration there is so little phospholipid removal (3% from sphingomyelin liposomes and less from lecithin liposomes) that extensive membrane damage would not be expected. Yet, sphingomyelin liposomes released 67% of their $^{86}Rb^+$ at this toxin concentration and C18 : 1 or C18 : 2 phosphatidylcholine liposomes released 62% of the $^{86}Rb^+$.

The extent to which $^{86}Rb^+$ release can be attributed to removal of phospholipid by the toxin would be expected to depend on the amount of phospholipid solubilized. This relation should apply particularly when the extent of phospholipid removal becomes large, as in the case of sphingomyelin or C18 phosphatidylcholine liposomes treated with toxin in excess of 30 or 50 μg/ml. In these cases, one would expect damage to the structure of one or more liposomal bilayers and such damage could augment the extent of $^{86}Rb^+$ release. Unfortunately, it is not possible at present to determine the proportion of $^{86}Rb^+$ release which is due to this mode of membrane damage.

Release of $^{86}Rb^+$ from interior aqueous compartments

The amount of $^{86}Rb^+$ release from C18 : 1 or C18 : 2 phosphatidylcholine liposomes (62%) and sphingomyelin liposomes (67%) at low toxin concentration, e.g., 1 μg/ml, where phospholipid release is negligible is very noteworthy. The magnitude of these values is much greater than the 15 to 20% proportion of marker estimated to be in the outermost aqueous space of the multilamellar

liposomes *. These results suggest that the toxin can cause the release of $^{86}Rb^{+}$ from interior aqueous compartments by traversing internal bilayers. However, the mechanism by which toxin transfers between successive membranes poses a difficult problem because the electrical characteristics of toxin-treated black lipid membranes indicate that the toxin does not equilibrate across the membrane following a one-sided addition [6]. Therefore, it will be necessary to search for the mechanism of transit in future experiments.

One line of speculation involves the concept that the toxin channel may collapse inwardly such that hydrophilic or polar head groups are pulled through the aqueous cavity of the channel as it closes. In this fashion, the channel is pulled inside-out like a sock and toxin molecules are deposited on the opposite side of the membrane. Such a hypothesis has recently been proposed for the action of monazomycin in lipid bilayers [13]. An alternative speculation involves the idea that the toxin and membrane lipid may form co-micelles within the bilayer resulting in a structural change of the membrane from a lamellar to a micellar state in the immediate vicinity of the inserted proteins. If this were to occur, toxin molecules would be expected to traverse the bilayer in the co-micelle state.

Mechanisms of membrane damage in lipid bilayers

The observation that the *S. helianthus* toxin can potentially inflict membrane damage by channel formation as well as by detergent action is an interesting and important point. Very similar phenomena were observed in our studies on membrane damage by activated complement proteins [7,14]. It is reasonable to speculate that this feature of dual action may be characteristic of many other membrane-reactive proteins. Such behavior is probably due to the amphipathic nature of these molecules. In addition to hydrophilic regions these proteins possess hydrophobic domains which play a key role in their function [15]. This chemical duality introduces a subtle aspect to the problem of defining molecular mechanisms. Thus, under certain conditions membrane reactivity depends on the proteins' ability to insert hydrophobic peptides into the lipid bilayer so as to form a transmembrane channel by the aggregation of several protein molecules. This mechanism is operative for the sea anemone toxin in black lipid bilayers and, presumably, in liposomes. On the other hand, under different conditions the protein can behave as a detergent due to its amphipathic character. In the case of the toxin reacting with the C18 phosphatidylcholine or sphingomyelin liposomes, the latter mechanism may contribute to the process of membrane damage in view of the large amount of ^{14}C-labeled lipid release. The channel mechanism and the detergent mechanism are not mutally exclusive. However, the relative importance of one or the other can vary depending on membrane structure and lipid composition as well as the nature of the protein and its concentration.

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* The estimate of the proportion of $^{86}Rb^{+}$ trapped in the outermost aqueous compartment was calculated with data taken from electron micrographs of liposomes [12]. Average values used in the computation were: liposome diameter = 160 nm, number of bilayers = 8 and inter-membrane spacing = 2.0 nm. A two to three-fold variation in these values would not alter our interpretation of the data on marker release.

Acknowledgement

This work was supported in part by Grant PCM77-16002 from the National Science Foundation, Grant RO-AI-02566-16 from the United States Public Health Service, and contract N00014-75-C-0142 with the Office of Naval Research.

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