Biochimica et Biophysica Acta, 555 (1979) 67—78

BBA 78433

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

I. FORMATION OF TRANSMEMBRANE CHANNELS IN LIPID BILAYERS

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(Received December 11th, 1978)

Key words: Toxin; Membrane damage; Membrane channel; (Sea anemone)

Summary

The addition of nanomolar amounts of a toxin preparation derived from the sea anemone *Stoichactis helianthus* to black lipid membranes increases their electrical conductance by one million-fold. In addition, the membranes become permeable predominantly to monovalent cations. The elevated bilayer conductance is voltage-dependent, and the current-voltage curves of these bilayers display rectification as well as a region of negative resistance. The membrane activity of the toxin is proportional to the third power of its concentration, and at very low concentrations the membrane conductance increases in discrete uniform steps. These observations indicate that the mechanism of toxin action involves the formation of transmembrane channels constructed by the aggregation of protein molecules which are inserted in the bilayer. The voltage-dependent membrane conductance arises from two distinct channel characteristics: (1) the unit conductance of individual channels is dependent on the polarity of applied voltage; (2) the number of ion-conducting channels is influenced by the polarity as well as the magnitude of applied potential. It is believed that these effects are due to the influence of an electric field on the insertion of toxin molecules into the bilayer or on their subsequent association with each other to produce channels. Partial chemical characterization of the toxin material has shown that the membrane active factor is a basic protein with a molecular weight of 17 500.

Introduction

A toxin isolated from the sea anemone *Stoichactis helianthus* has been described by Devlin and demonstrated to be a powerful hemolytic agent [1].

As shown by Bernheimer and colleagues, the hemolytic capacity resides in a basic protein which has a p*I* of 9.8 and a molecular weight of 16 000 [2]. The cytolytic action of the toxin has been attributed to a specific interaction with sphingomyelin in the target cell membrane [2,3]. This hypothesis is based on the observations that the toxin's hemolytic function is inhibited by exogenous sphinogmyelin as well as by the pretreatment of target cells with sphingomyelinase or phospholipase [2,3]. Interestingly, the toxin does not exhibit any sphingomyelinase or phospholipase activity. Thus, the exact mechanism for the protein's action is unclear.

In the present experiments, the planar black lipid membrane has been used as a model target system to study the nature of the interaction between the *S. helianthus* toxin and lipid bilayers. The results of the study support the concept that the cytolytic action of the toxin is directed against the lipid bilayer portion of the cell membrane. Specifically, the experiments show that one consequence of the toxin-bilayer reaction is the formation of transmembrane channels which are produced by the aggregation of toxin molecules within the membrane. This action does not require the presence of sphingomyelin in the bilayer. The latter finding is not in accord with the hypothesis expressed by Bernheimer and co-workers that sphingomyelin is a specific membrane receptor for the toxin [2,3]. Therefore, in the second paper of this series a systematic study of the effect of membrane lipid composition is reported, and the question of the lipid requirements for activity of the *S. helianthus* toxin is resolved [4].

Materials and Methods

Chemicals. Partially purified *S. helianthus* toxin was generously provided by Dr. John Devlin. The material was stored as a lyophilized powder at −20°C. For experimental use a stock solution of 0.1 − 1.0 mg/ml in buffer was prepared and kept at 0°C. This solution was stable for a period of 5 − 7 days with no loss or alteration of membrane activity. Lipids and solvents for the planar lipid bilayer studies were obtained in the highest purity grade available: egg lecithin was purchased from Supelco Inc., Bellefonte, PA, 99 + % cholesterol was obtained through Sigma, St. Louis, MO, and *n*-decane (gold label) was from Aldrich, Metuchen, NJ. Double-distilled water with a specific conductance of 1 μS/cm or less was used for preparing all aqueous solutions.

Polyacrylamide disc gel electrophoresis. The toxin material was examined by polyacrylamide gel electrophoresis on 7.5% gels at pH 4.5. The protocol followed the method of Reisfeld et al. [5] except that the electrode buffer was 70 mM β-alanine buffered at pH 4.5 with glacial acetic acid. The toxin (100 μg) in 50 μl of electrode buffer containing 10% sucrose (w/v) and 0.01% Basic Fuchsin (w/v) was layered onto the gels and electrophoresis was performed at 3 mA per gel for 3 h. Several gels were fixed in 12% trichloroacetic acid (w/v) for 30 min, stained with 0.05% Coomassie brilliant blue (w/v) in methanol/acetic acid/water mixture (5 : 1 : 5, v/v/v) for 2 h, and then destained by diffusion in methanol-acetic acid-water (2 : 3 : 36, v/v/v) overnight. The remaining gels were sliced into 1 mm sections and equivalent discs from each gel were pooled. The protein in these samples was extracted by incubation at 37°C for

4 h with 2 volumes of 0.1 M KCl + 5 mM histidine buffered at pH 7.0. Subsequently, the supernatant from each incubation mixture was assayed for toxin activity by testing its effect on a black lipid membrane.

Samples of the toxin were also characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 7.5% gels following the method of Weber and Osborn [6]. Protein samples (10—100 μg) in 50 μl of buffer were dispersed in an equal volume of buffer containing 2% SDS (w/v) and 2% 2-mercaptoethanol (v/v) by heating at 90°C for 2 min. Electrophoresis was carried out at 4 mA per gel for 3 h at room temperature; the gels were fixed, stained, and destained as described above.

Membranes and electrical measurements. Lipid bilayer membranes were made from a mixture of egg lecithin and cholesterol (20 mg/ml, each lipid) dissolved in *n*-decane. Bilayers were formed by depositing 1—2 μl of lipid solution from a disposable glass microliter pipette (Biotip, Schwarz/Mann Inc., Orangeburg, NY) across a 1 mm aperature in the side of a Kel-F cup which was placed in a small lucite boat. Membrane formation was monitored by visual appearance and electrical capacitance criteria. The unmodified lipid bilayers displayed electrical conductances from $1 \cdot 10^{-9}$ S/cm^2 to $5 \cdot 10^{-8}$ S/cm^2 and electrical capacitances between 0.4 to 0.6 μF/cm^2. Samples of the sea anemone toxin were added to one side of the membrane and the additions were accompanied by vigorous stirring.

Membrane conductance was measured under voltage clamp conditions using a conventional two electrode configuration as described by Jain [7]. Positive membrane current was defined to correspond with cation flow away from the aqueous compartment to which the toxin was added. Electrical contact to each side of the bilayer was made with a calomel electrode via a salt bridge (saturated KCl in 3% agar). The one exception to this protocol was in experiments on ion-selectivity. In these studies, the slow leakage of KCl from the salt bridge into each of the compartments, which were of unequal volume, produced an asymmetry potential which added to the diffusion potential created by the established salt gradient. Therefore, in these experiments two Ag/AgCl electrodes were used and immersed directly into each chamber. Accordingly, the cation transference numbers for the membrane were calculated from the observed cell potentials by the relation:

$$E_{\text{cell}} = 2t^{+} \frac{RT}{F} \ln \frac{[\text{Cl}]_1}{[\text{Cl}]_2},$$

and the liquid-junction diffusion potentials were determined by the equation:

$$E_{\text{D}} = E_{\text{cell}} \frac{(2t^{+} - 1)}{2t^{+}}.$$

Results

Steady-state electrical characteristics of toxin-treated bilayers

The addition of *S. helianthus* toxin to membranes prepared from a mixture of egg lecithin and cholesterol greatly increased membrane permeability and conferred to the bilayer a voltage dependent electrical conductance. One

example of these phenomena is displayed in Fig. 1 which shows data that was recorded after a one-sided addition of toxin to a membrane. Panel A of the figure shows the steady-state current-voltage (I—V) curve which displays rectification as well as a region of negative resistance that occurs with negative voltages in the range from —80 mV to —140 mV. Panel B displays the corresponding conductance-voltage (G—V) curve which was derived from the I—V curve by the relation: $G = I/V$. As shown in Fig. 1B, the toxin-treated membrane has two ohmic conductance levels which are connected by a voltage-dependent conductance curve. The first ohmic conductance level represents a low permeability state which occurs for voltages more negative than —140 mV. The second ohmic conductance level corresponds to a high permeability state which is achieved at positive voltages above +100 mV. The ratio of conductance between the high and low permeability states is six for the experiment shown in Fig. 1. The average ratio of conductance for over one hundred membranes was five.

The capacity of the toxin to increase membrane permeability was not restricted to lecithin bilayers. Fig. 2 shows the G—V curves of toxin-treated bilayers which were prepared from glycerol monoolein, or oxidized cholesterol, or soybean phosphatides (asolectin). In the cases of monoolein and oxidized cholesterol the data are qualitatively similar to that seen in Fig. 1B. These results indicate that the activity of the toxin does not depend on any specific phospholipid or cholesterol requirement. Surprisingly, however, asolectin bilayers did not respond to the toxin even at protein concentrations of 1—5 μg/

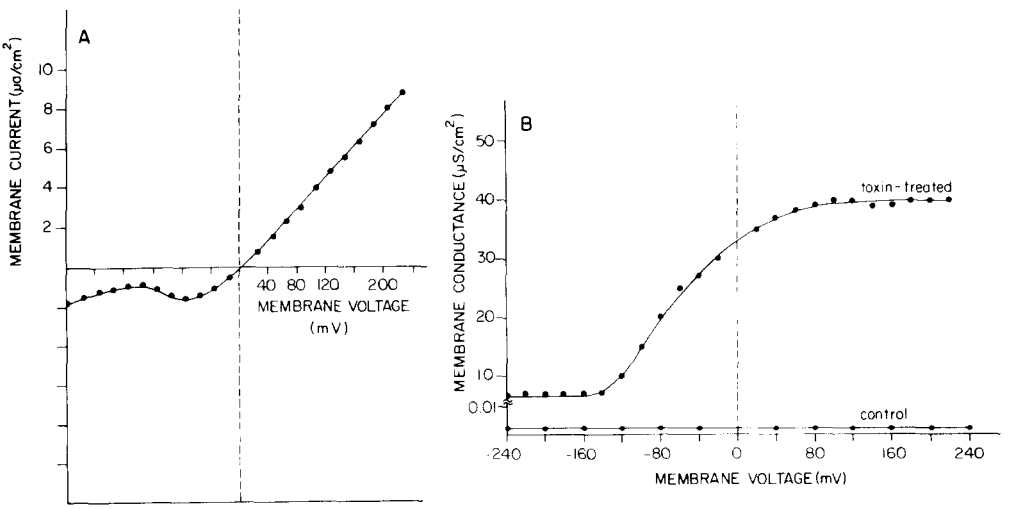

Fig. 1. (A) Steady-state current-voltage curve of a toxin-treated phosphatidylcholine bilayer. The bilayer was formed from egg phosphatidylcholine plus cholesterol (1 : 2 molar ratio) in n-decane. The aqueous phase contained 0.1 M KCl + 5 mM histidine buffered at pH 7.0; toxin (120 ng/ml) was added to one side of the bilayer. The absissa is membrane voltage (mV); the ordinate is the specific membrane current (μA/cm²). (B) Steady-state conductance-voltage curve of a toxin-treated phosphatidylcholine bilayer. The upper conductance curve for a toxin-treated membrane was computed from the data displayed in (A). The lower curve represents data from the same membrane prior to the addition of toxin. See text for details.

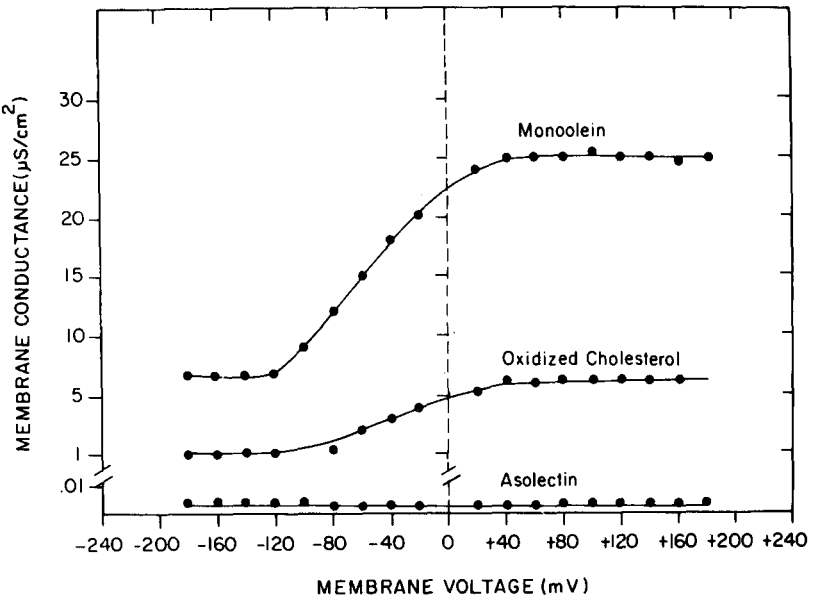

Fig. 2. Steady-state conductance-voltage curves of toxin-treated bilayers prepared from different lipids. Membranes were prepared from monoolein (in *n*-decane), or oxidized cholesterol (in *n*-octane), or asolectin (in *n*-decane). The aqueous phase contained 0.1 M KCl plus 5 mM histidine, buffered at pH 7.0; the concentration of toxin was 120 ng/ml for the monoolein and oxidized cholesterol bilayers, and 5 μg/ml in the case of the asolectin bilayer. The applied membrane voltage is given on the abscissa. The specific membrane conductance is shown on the ordinate which is expanded near the origin in order to record the asolectin data which equals the conductance level of an unmodified bilayer.

ml. For the experiment shown in Fig. 2, the conductance of the unmodified asolectin bilayer was $3 \cdot 10^{-9}$ S/cm^2 and it did not change during a 30 min period following the addition of toxin (5 μg/ml). Thus, the composition of lipid bilayers does modulate their susceptibility to the sea anemone toxin, but the chemical factors which determine this influence are not known.

Dose-response characteristics

The dose-response curve of the toxin in a lecithin/cholesterol membrane is shown in Fig. 3 which records the logarithm of membrane conductance on the ordinate versus the logarithm of toxin concentration along the abscissa. The slope of the line is 3.0, and the range of bilayer conductance extends over six orders of magnitude which reflects a 100-fold variation in toxin concentration. A simple interpretation of this experiment is that the conductance element produced by the toxin is an aggregate of three toxin molecules. More accurately, the active element is a trimer of the toxin species that is present in aqueous solution which may or may not be a protein monomer.

Membrane channels

The enhanced membrane permeability produced by aggregates of the *S. helianthus* toxin is due to the formation of transient transmembrane channels. This fact is demonstrated in panels A and B of Fig. 4. The two panels are

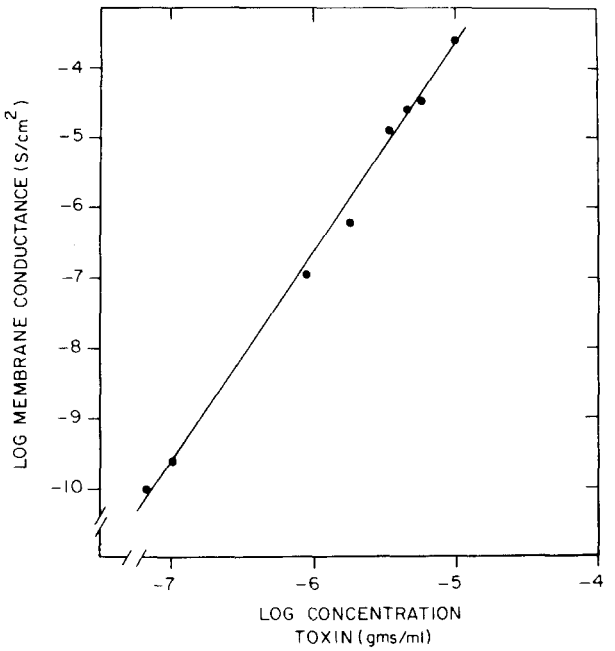

Fig. 3. Dose-response curve for *S. helianthus* toxin in bilayers. Experimental conditions were the same as in Fig. 1A. Aliquots of a stock solution of toxin were added to one side of the membrane with vigorous stirring. Membrane conductance was monitored continuously by clamping the bilayer to +10 mV. The final steady-state conductance was assumed when no further current increments occurred over a 3—5 min observation period. The abscissa displays the logarithm of toxin concentration; the ordinate represents the logarithm of the actual membrane conductance (uncorrected for membrane area).

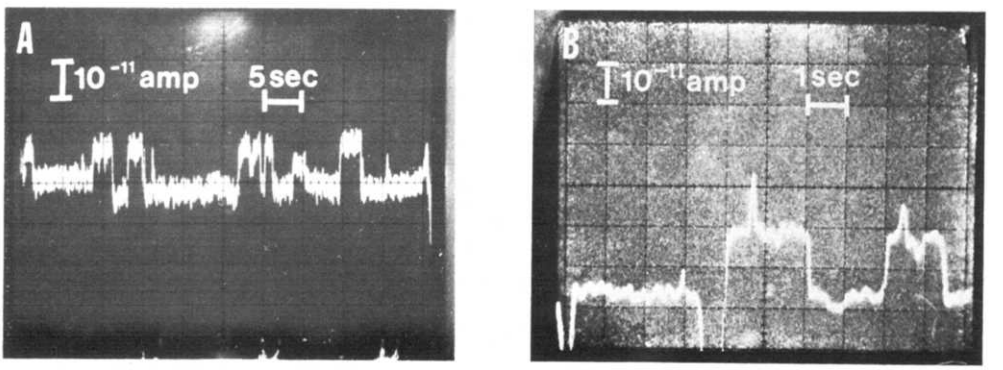

Fig. 4. Single channel current fluctuations in toxin-treated membranes. The data records show the fluctuation of membrane current with time which was observed in toxin-treated lecithin/cholesterol bilayers. Toxin (10 ng/ml) was added to one side of the membrane; the aqueous phase contained 0.5 M KCl + 5 mM histidine, buffered at pH 7.0. Panel A. The record was taken at a clamp potential of —100 mV and shows uniform discrete current fluctuations above the baseline level of the unmodified bilayer. The membrane contained only one channel which had a conductance of $1 \cdot 10^{-10}$ S and an average lifetime of 2 s. Panel B. The record was taken at a potential of +100 mV and was recorded 2 min after the record in panel A. During this interval a second channel appeared in the membrane and was open during most of the time period covered by the second oscillogram. Hence, the baseline current of the untreated bilayer was below the oscilloscope screen. The conductance of both channels was $1.8 \cdot 10^{-10}$ S and the lifetime for the channel which was opening and closing is around 2 s.

oscilloscopic records of membrane current fluctuations which were observed after the addition of toxin (10 ng/ml) to an egg phosphatidylcholine/cholesterol membrane. The fluctuations reflect discrete changes of membrane conductance which correspond to the formation and breakdown of individual channels. The data trace in panel A was recorded at an applied potential of −100 mV, and the record in panel B was taken at a potential of +100 mV. The current scale (vertical axis) is the same for both records; however, the time scale (horizontal axis) for panel B is expanded five times in relation to the scale in panel A. There are two parameters contained in the data records which are important for describing the channels. First, the average lifetime of a single channel is around two seconds and is independent of the polarity of applied voltage. Second, the average conductance of a single channel at −100 mV is $1 \cdot 10^{-10}$ S (panel A), and at +100 mV it is $1.8 \cdot 10^{-10}$ S (panel B). Thus, the conductance of a channel is not equal for voltages of +100 mV and −100 mV. It should be noted that the dependence of single channel conductance on voltage polarity is in the same direction as the rectification effect which is observed in the steady-state I—V curve of a 'many channel' membrane (Fig. 1A).

Time-dependent conductance changes

The difference between channel conductance for voltages of opposite polarity partially accounts for the asymmetry in the electrical characteristics of toxin-treated bilayers. The remaining portion arises from a time-dependent conductance change which occurs at negative potentials. This characteristic is shown in Fig. 5. At the start of this experiment (left hand portion of the figure), the membrane was clamped at 0 mV for one minute (complete period not shown). At point *a* the membrane potential was stepped to +100 mV, and the resulting current response was a change from 0 to $4.8 \cdot 10^{-8}$ A. The potential was returned to 0 mV at *b* and next changed to −100 mV at *c*; this time the current response was a step change from 0 to only $-2.3 \cdot 10^{-8}$ A. Moreover,

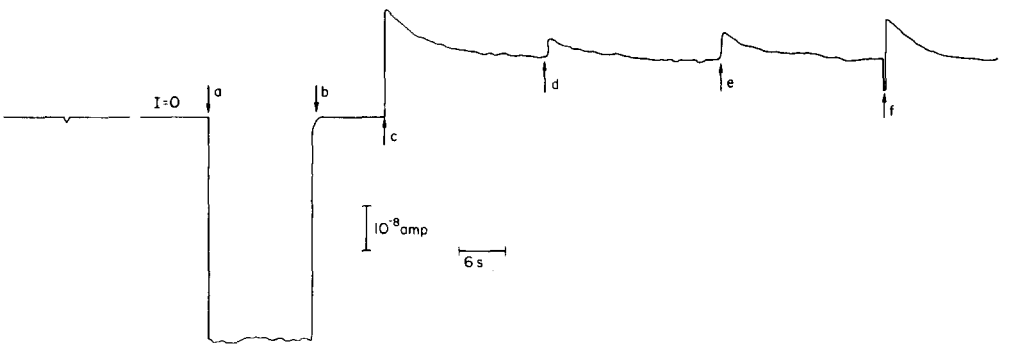

Fig. 5. Time-dependent changes of membrane conductance. Experimental conditions were as described in Fig. 1A. At the start of the experiment, the membrane potential was clamped at 0 mV (horizontal line in the center of the record at the lefthand side corresponds to $I = 0$). The arrows mark the times at which the applied membrane potential (V) was changed. The vertical axis indicates membrane current, the horizontal axis corresponds to increasing time. Experimental protocol proceeds from left to right: a, V = +100 mV; b, $V = 0$ mV; c, $V = -100$ mV; d, $V = 0$ mV pulse for 2 ms and return to −100 mV; e, V = 0 mV pulse for 10 ms and return to −100 mV; f, $V = 0$ mV pulse for 100 ms and return to −100 mV. See text for details.

this initial response was followed by a decay of membrane current over the course of 21 seconds to a final steady-state value of $-1.2 \cdot 10^{-8}$ A, i.e., the bilayer conductance undergoes inactivation at negative voltage. For the experiment shown in Fig. 5, the time course of the inactivation process is given by a single exponential function with a time constant (τ) of three seconds (analysis not shown).

Continuing in the same experiment, at point *d* the membrane voltage was changed to 0 mV for 2 ms and then returned to −100 mV (the current transient is not seen in the record because its duration was much shorter than the recorder's response time). The initial current value (and hence, membrane conductance) immediately after the 0 mV pulse is greater than the previous steady-state level. This observation indicates that a significant activation or 'turn-on' of conductance was achieved during the 2 ms period that the membrane was clamped at 0 mV. Next, at points *e* and *f* the voltage applied to the bilayer was returned to 0 mV for 10 and 100 ms respectively. In each case the initial current after returning to −100 mV was successively larger. After the 100 ms pulse the initial current upon returning to −100 mV is equal to that seen at point *c*. The kinetics of the 'turn-on' conductance mechanism have not been analyzed in detail. However, the data shown in Fig. 5 conform to a first-order rate process with a time constant of 2 ms. Thus, the activation and inactivation processes have drastically different kinetics, the inactivation reaction being 1000 times slower.

Ion-selectivity of toxin channels

The ion selectivity of toxin-treated membranes is shown in Table I which records the cation transference numbers of the alkali metal and alkaline earth

TABLE I

ION-SELECTIVITY OF TOXIN-TREATED MEMBRANES FOR MONOVALENT AND DIVALENT METAL CHLORIDES

Membranes were prepared from egg phosphatidylcholine/cholesterol (1 : 2) in *n*-decane. Initially the composition of the two aqueous compartments was set at the low salt concentration level, toxin (100—200 ng/ml) was added to the inside chamber with stirring, and 30—40 min was allowed for the membrane conductance to stabilize. Subsequently, the inside chamber was adjusted to the high salt concentration level by addition of an aliquot of a concentrated stock solution (1—4 M), an equivalent volume of buffer at the low salt concentration was added to the outside chamber to equalize hydrostatic pressure. The membrane diffusion potential (E_D) developed within 1—2 min and was stable for at least 5 min. For each of the different salts, two to five experiments (n) were conducted on different membranes. The calculations for E_D and t^+ are described in Methods.

Salt	Inside chamber (mM)	Outside chamber (mM)	n	E_D (mV)	t^+
LiCl	100	10	2	−26 ± 4	0.75
NaCl	100	10	3	−32 ± 3	0.80
KCl	100	10	5	−40 ± 2	0.85
RbCl	100	10	4	−41 ± 3	0.87
CsCl	100	10	2	−36 ± 3	0.22
$MgCl_2$	50	5	2	+31 ± 5	0.32
$CaCl_2$	50	5	4	+18 ± 2	0.47
$SrCl_2$	50	5	3	+40 ± 4	0.14
$BaCl_2$	50	5	2	+51 ± 2	0.05

chlorides. For the Group 1A chlorides the channel was cation selective, and the selectivity ratios (t^+/t^-) varied from 3 to 9. However, the intercation selectivity was low based on the Nernst potentials (E_D), a conclusion which was confirmed by bi-ionic cell potentials of only 5—10 mV (data not shown). When the alkali metal salts were replaced with Group IIA chlorides, the membranes became anion selective. The selectivity ratios varied from 0.9 for $CaCl_2$ to 0.05 in the case of $BaCl_2$.

Analysis of toxin by polyacrylamide gel electrophoresis

In order to characterize the chemical nature of the sea anemone toxin, the preparation was analyzed by polyacrylamide gel electrophoresis. The results of this study are summarized in Fig. 6. Panel A of the figure shows the electrophoretic pattern of three gels. Gel No. 1 is a SDS gel of the stock toxin material, and six bands are clearly discernible. Gel No. 2 is a polyacrylamide gel pattern obtained at pH 4.5 of the same material. Under these conditions, the toxin sample showed only two equally stained bands with R_F values of 0.90 and 0.82. The protein corresponding to these bands was recovered by extraction from sliced gels as described in Methods. When the extracts from individual samples were tested on a black lipid membrane only the R_F 0.90 band showed activity (indicated in Gel No. 2 by +), and its behavior was identical to the original preparation. The R_F 0.90 band was also analyzed on SDS-gels. The

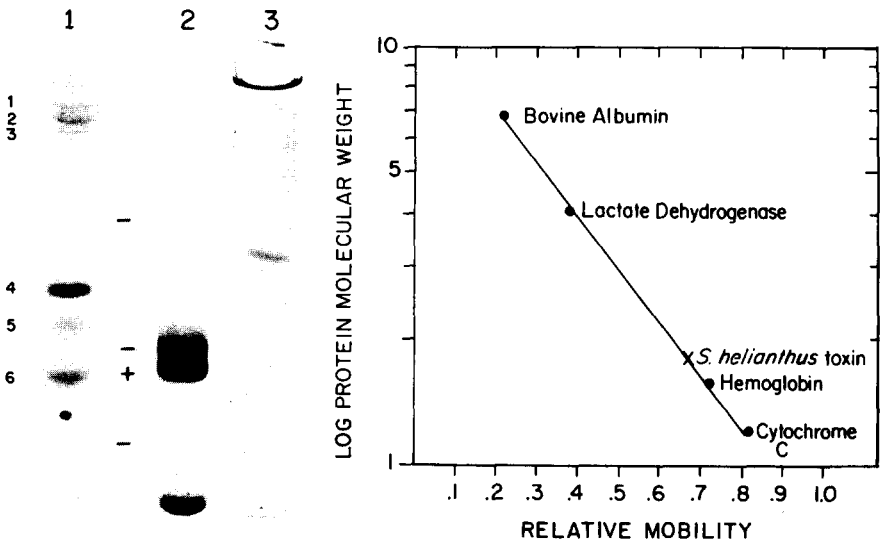

Fig. 6. Analysis of toxin by polyacrylamide gel electrophoresis. Panel A. The electrophoretic pattern of three gels are shown which were prepared as described in Methods: (1) SDS gel of the crude toxin sample. (2) Polyacrylamide gel at pH 4.5 of the same material. After electrophoresis, the latter gels were cut into 1 mm sections, and the protein in pooled equivalent slices was extracted by incubation with excess buffer. Subsequently, the extracts of individual samples were tested for activity on a black lipid bilayer. A single membrane-active fraction was found which corresponded to the front-running protein band seen in Gel No. 2 (position indicated by '+'). All other extracts were membrane inactive (representative samples are indicated by '—'. (3) SDS gel analysis which shows a single protein band for the membrane active sample isolated from Gel No. 2. Panel B. Semilogarithmic graph of molecular weight for designated standard proteins versus mobility in SDS gels. The purified membrane-active toxin protein falls on the curve corresponding to a molecular weight of 17 500.

results are given in the electrophoretic pattern of Gel No. 3 which shows a single protein band that corresponds to band 4 in Gel No. 1. Estimation of the molecular weight of the membrane active protein is shown in panel B of Fig. 6 which is a semilogarithmic graph of molecular weight for standard protein samples versus mobility in SDS gels. The bilayer-active protein falls on the curve corresponding to a molecular weight of 17 500.

Discussion

The present experiments have shown that a toxin derived from the sea anemone *S. helianthus* can increase the permeability of black lipid membranes over six orders of magnitude with no compromise in membrane stability. The mechanism of this action involves the formation of transmembrane channels which are constructed by the aggregation of toxin molecules. Analysis by polyacrylamide gel electrophoresis of the membrane active protein (Fig. 6). indicates that it is a basic protein of 17 500 daltons. The fact that the toxin bears a strong positive charge at neutral pH does not contradict the implied hydrophobic character necessary for the insertion of the protein into a lipid membrane. The size of a 17 500 dalton protein greatly exceeds the length required to span a bilayer; actually, a peptide of about 2000 daltons would be sufficient. Thus, only a small portion of the toxin molecule needs to be hydrophobic.

Bilayer lipid membrane activity of the toxin parallels its powerful hemolytic capacity previously reported by Devlin [1] and by Bernheimer and Avigad [2]. The latter workers have purified the hemolysin to homogeneity and shown it to be a 16 000 dalton protein with a p*I* of 9.8. The similarity between this substance and the protein described in the present study as regards membrane reactivity and chemical characteristics suggested that the two proteins were identical. This conclusion was confirmed when the purified hemolysin (the generous gift of Dr. Alan Bernheimer) was tested on black lipid bilayers and its reaction characteristics were identical with our preparation of toxin.

The assignment of a channel mechanism for *S. helianthus* toxin rests on two salient observations. The first observation is the dependence of the macroscopic membrane conductance on $[\text{toxin}]^3$ which is displayed in Fig. 3. The simplest explanation for this dose-response relation is that three molecules of toxin associate with each other within the membrane to form an ion-conducting pathway. The second observation is the occurrence of discrete current fluctuations, as shown in Fig. 4. The magnitude of these fluctuations, 10^{-10} S in 0.5 M KCl, corresponds to an ion flux of about 10^8 ions/s across the membrane at a potential of 100 mV. This rate exceeds that of a possible carrier type mechanism (i.e., valinomycin) by four orders of magnitude. Further, the lifetime of a single fluctuation (2 s in phosphatidylcholine/cholesterol bilayers at 23°C) is larger than could be expected for a transient perturbation or 'tear' in the bilayer. Hence, we are dealing with a stabilized high permeability structure. The increase in membrane conductance by discrete steps as well as the high power dose-response curve are characteristics which have been repeatedly demonstrated with other channel-forming agents such as gramicidin [8], alamethicin [9—11], and monazomycin [12].

Several additional features of the electrical characteristics of toxin-treated membranes are important, because they relate to the process of protein insertion into the bilayer and the nature of the transmembrane channel. One observation is that a one-sided addition of toxin produces a membrane conductance which is dependent on the polarity as well as the magnitude of the applied voltage, as shown in Fig. 1B. The asymmetry of the G—V curve with respect to voltage polarity demonstrates that the toxin molecules which are available for channel formation are not distributed evenly on both sides of the bilayer. Specifically, it suggests that the toxin inserts in the bilayer and is anchored, presumably by ionic or hydrophilic head-groups, on the side of the membrane to which the protein addition was made. Thus, the toxin does not behave like a simple lipid-soluble substance which would partition between the aqueous phase and the hydrocarbon milieu of the membrane. This conclusion is consistent with the fact that the *S. helianthus* toxin is a positively-charged protein at neutral pH.

The dependence of membrane conductance on the magnitude of applied voltage indicates that either the number of channels or the conductance of individual channels is voltage dependent. The data which are relevant to this issue are shown in Figs. 4 and 5. First, panels A and B of Fig. 4 show that individual channels exhibit rectification with the same voltage polarity dependence that is observed for a 'many channel' bilayer. Such a phenomenon could conceivably take place in two ways; one choice is that the channel undergoes a change in configuration which alters its conductance level when the voltage polarity is reversed. Precedent for this type of mechanism is provided by the action of hemocyanin, another putative channel-forming agent, in lipid bilayers [13]. An alternative explanation is that the change in the direction of the electric field results in a redistribution of ions at the mouth of the channel and alters the concentration of charge carriers which move through the channel. It should be possible to distinguish between these two alternatives in future experiments by studying the effect of applied potential, within the range of approx. ±100 mV, on single channel conductance. For the configuration-state mechanism, the channel should display discrete changes in conductance as a function of voltage, whereas for the ion-relaxation mechanism the conductance should be a smooth and continuous function.

Next, Fig. 5 shows that the membrane conductance inactivates with time when the bilayer is held at a negative voltage. For the specific experiment shown in the figure, the decay of conductance is exponential and has a time constant of three seconds. Interestingly, the membrane conductance can be returned to its initial high state by briefly removing the negative voltage. In the experiment shown, the potential was taken to 0 mV for only a few milliseconds in order to accomplish the reactivation of conductance. Hence, the 'turn-on' kinetics (τ = 2 ms) are very much faster than the 'turn-off' kinetics. It should be emphasized that the activation and inactivation of membrane conductance seen in Fig. 5 is not due to a time-dependent transition of channels between different conductance levels in response to the changes of membane voltage. As shown in the single-channel experiments (Fig. 4), the conductance of a channel throughout its lifetime is constant. Instead, it is believed that the 'turn-on' and 'turn-off' of membrane conductance reflects a change in the number of

channels in the bilayer. This type of mechanism has been clearly demonstrated in lipid bilayer reconstitution experiments with an anion transport protein isolated from paramecium which exhibits a similar phenomenon of activation and inactivation of membrane conductance in response to voltage polarity [14].

The direction of the conductance changes with reversals in voltage polarity in toxin-treated bilayers is consistent with the idea that positively charged toxin molecules are driven into or out of the membrane by the applied electric field. However, it is unlikely that this process involves the transfer of material back and forth between the membrane and the aqueous phase. If this were the case, the application of a large negative potential to the bilayer for a long time (compared to the diffusion time through the unstirred aqueous layer at the bilayer interface) would cause an electrophoresis of the toxin away from the membrane. This effect would manifest itself as a diminution in the bilayer's initial current response to a subsequent positive voltage pulse in comparison to a membrane which had been held at 0 mV. Such an effect was not observed. Tentatively, it is believed that either (or both) the tilting into the bilayer of toxin molecules which are already aligned on the membrane surface or the subsequent aggregation between inserted molecules is voltage dependent. This mechanism has already been described for other channel-formers, notably alamethicin [15] and has been thoroughly discussed by Mueller [16].

Acknowledgement

The author expresses his appreciation to Dr. Manfred M. Mayer for advice, discussion, and financial support. This work was supported in part by Grant PCM77-16002 from the National Science Foundation, Grant RO A102566-18 from the United States Public Health Service and Contract N00014-75-C-0142 from the Office of Naval Research.

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