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Ion and Nonelectrolyte Permeability Properties of Channels Formed in Planar Lipid Bilayer Membranes by the Cytolytic Toxin from the Sea Anemone, *Stoichactis helianthus*

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Summary. When present at nanomolar concentrations on one side of a lipid bilayer membrane, *helianthus* toxin (a protein of mol wt$\approx$16,000) increases enormously membrane permeability to ions and nonelectrolytes by forming channels in the membrane. Membranes containing sphingomyelin are especially sensitive to toxin, but sphingomyelin is *not* required for toxin action. Conductance is proportional to about the 4th power of toxin concentration. Single channel conductances are approximately 2×10^{-10} mho in 0.1 M KCl. Toxin-treated membranes are more permeable to K^+ and Na^+ than to Cl^- and $SO_4^=$, but the degree of selectivity is pH dependent. Above pH 7 membranes are almost ideally selective for K^+ with respect to $SO_4^=$, whereas below pH 4 they are poorly selective. The channels show classical molecular sieving for urea, glycerol, glucose, and sucrose – implying a channel radius >5 Å. In symmetrical salt solutions above pH 7, the *I–V* characteristic of the channel shows significant rectification; below pH 5 there is very little rectfication. Because of the effects of pH on ion selectivity and channel conductance, and also because of the rectification in symmetrical salt solutions and the effect of pH on this, we conclude that there are titratable negative charge groups in the channel modulating ion permeability and selectivity. Since pH changes on the side containing the toxin are effective whereas pH changes on the opposite side are not, we place these negative charges near the mouth of the channel facing the solution to which toxin was added.

The sea anemone *Stoichactis helianthus* produces a basic protein (isoelectric point pH 9.8; mol wt $\sim$16,000) which acts as a cytolytic toxin (Devlin, 1974; Bernheimer & Avigad, 1976). From studies of the hemolytic effect of the toxin on red cells and of the release of trapped marker from toxin-treated liposomes, Bernheimer and his colleagues concluded that the toxin acts directly on the plasma membrane and that sphingomyelin is a necessary component in the membrane for its action (Bernheimer & Avigad, 1976; Linder, Bernheimer & Kim, 1977).

Stimulated by these findings, we have investigated the action of this toxin on planar lipid bilayer membranes. A related study has recently been described by Michaels (1979). We find that several toxin molecules combine to form channels permeable to both ions and nonelectrolytes and that sphyngomyelin affects the sensitivity of the membrane to toxin but is *not* an essential component. Negative charge groups at one end of the channel modulate ion permeability and selectivity, in accordance with classical fixed-charge theory.

Materials and Methods

Membranes were formed by the brush technique of Mueller et al. (1963) at 25 ± 2 °C across a 0.03 to 0.9 mm^2 hole in a 125 μm thick Teflon partition separating two Lucite compartments which could be stirred by magnetic fleas. Each compartment contained 3.0 ml of an appropriate salt solution (*see* Results). After the membrane formed, toxin was added to one side, from a concentrated stock solution, to a concentration of from 1 ng/ml to 2 μg/ml. Prior to toxin addition, membrane conductance was approximately 10^{-8} mho/cm^2.

The membrane-forming solutions were primarily 2% egg phosphatidylcholine (PC)+ 2% cholesterol in *n*-decane, with the sphingomyelin concentration varied between 0 and 2%. In a few experiments the solutions were egg PC, egg PC + bovine phosphatidylserine (PS), dioleoyl PC, or bacterial phosphatidylethanolamine (PE) in *n*-decane.

Egg PC was obtained from Sylvana Chemical Company (Orange, N.J.) and from Avanti Biochemicals Inc. (Birmingham, Ala.); the former contained a small percentage of sphingomyelin

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and hence was not used in the experiments designed to test the role of sphingomyelin in toxin sensitivity. Bovine PS and bacterial PE were from Avanti; dioleoyl PC was from Supelco, Inc. (Bellefonte, Pa.); bovine sphingomyelin was from Analabs, Inc. (North Haven, Conn.); cholesterol was from Eastman Kodak Company (Rochester, N.Y.) and twice recrystallized from ethanol; *n*-decane (99.9%) was from Chemical Samples Company (Columbus, Ohio). The *S. helianthus* toxin was a generous gift from Dr. Alan Bernheimer of New York University Medical School. Two different samples were provided: one was the original, partially purified material sent to him by Dr. John Devlin; the other was a further purified fraction (by Bernheimer) of this material. Essentially the same results were obtained with both samples. Stock solutions of toxin (1 mg/ml) in 145 mM NaCl+10 mM Tris (pH 7.2) were stored in small vials at −20 °C. For a given day's experiments, the contents of a vial were thawed and, along with subsequent dilutions, stored on ice.

Steady-state current-voltage (*I–V*) characteristics were generally obtained by passing known currents across the membrane and recording the resulting steady-state voltage response with the same pair of electrodes; either calomel electrodes coupled to the solutions with appropriate salt bridges or Ag/AgCl electrodes in direct contact with the solutions were used. In those experiments in which very high conductances were attained and in all nonelectrolyte-permeability measurements, two pairs of Ag/AgCl electrodes were used, one pair for passing current and the other for recording voltage. The recording electrodes were connected to a high input impedance amplifier and the output displayed on either an oscilloscope screen or a chart recorder. Ion selectivity measurements were performed by establishing salt gradients across the membrane and recording the resulting transmembrane potential differences (at $I=0$) through calomel electrodes coupled to the solutions with saturated KCl junctions.

Transient current responses (along with the subsequent steady-state responses) and single-channel behavior were studied by applying known voltage steps across the membrane. A single pair of calomel electrodes, coupled to the solutions through appropriate salt bridges, were employed in a simple feed-back circuit for these voltage-clamp experiments (*see*, e.g., Schein, Colombini & Finkelstein, 1976). The compartment *not* containing toxin was virtual ground.

Permeability coefficients (P_d's) of nonelectrolytes at given toxin-induced membrane conductances were determined by the procedure of Holz and Finkelstein (1970). After the conductance attained the desired level, isotopically labeled solute was added to one compartment and samples were taken periodically for counting from the other; unstirred layer corrections were negligible. Some of these experiments were done at conductances at which the access resistance (the resistance in the absence of a membrane) was significant compared to the observed resistance; in those instances, membrane conductance was calculated after subtracting the former from the latter. Urea-^{14}C (5 mCi/mmol), glycerol-^{14}C (8.0 mCi/mmol), glucose-^{14}C (300 mCi/mmol); and sucrose-^{14}C (5 mCi/mmol) were obtained from New England Nuclear Corp.

Results

A. Conductance[1] *Increases Produced by Toxin*

Addition of toxin to one compartment produces a continuous, more or less linear, increase of membrane conductance with time (Fig. 1). No discernable steady state is reached, implying an irreversible interaction of toxin with membrane. Upon removal of toxin from the aqueous compartment (by perfusion with toxin-free solution), the conductance, although ceasing to rise further, does not decline significantly, thus confirming the irreversibility of the interaction. To estimate the functional dependence of conductance on toxin concentration, we chose an arbitrary time interval (e.g., 30 min) between additions of toxin and recorded the conductance at the end of each interval, prior to a new addition of toxin. Such experiments indicate an approximately 4th power dependence of conductance on toxin concentration (Fig. 2). Membranes remain stable for hours, even at conductances as high as 0.1 mho/cm^2 (i.e., at conductances 10^7-fold larger than that of the unmodified membrane).

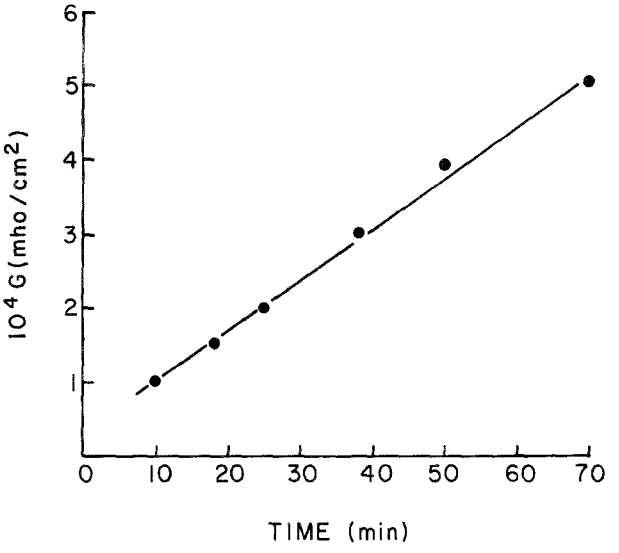

Fig. 1. Membrane conductance as a function of time, following addition of *helianthus* toxin. A membrane formed from 2% PC+2% cholesterol +0.1% sphingomyelin in *n*-decane separated symmetrical salt solutions (100 mM KCl, 5 mM dimethylglutaric acid, pH 7.0); membrane conductance $\approx 10^{-8}$ mho/cm^2. At $t=0$, toxin was added to the solution on one side of the membrane to a concentration of 33 ng/ml and the small-signal conductance recorded over the next 70 min.

At large applied potentials ($|V| \geqq 80$ mV) the conductance is time variant; immediately following the voltage pulse, the current rises to a peak and then decays exponentially to the steady-state value (Fig. 3). At 200 mV, the maximum stimulus we applied, the peak current is about 20% larger than at steady state. Although there is considerable variability in the data, the time constant (τ) of the decay clearly varies with voltage. At ± 100 mV, τ is ~ 10 sec; at 150 mV, it is ~ 3 sec.

[1] Unless otherwise specified, "conductance" means small-signal conductance, which, in symmetrical solutions, is defined as I/V, where $|V| \leqq 20$ mV.

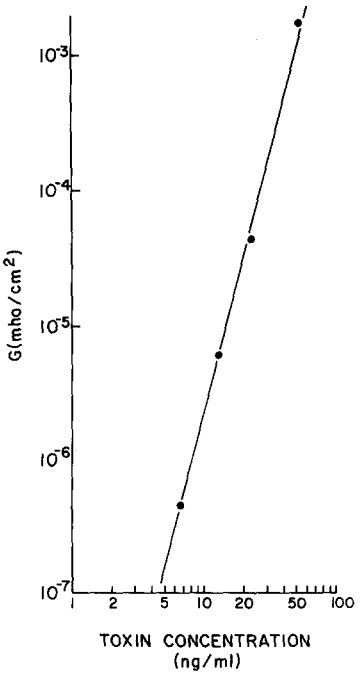

Fig. 2. Double logarithmic plot of membrane conductance *vs. helianthus* toxin concentration. A membrane formed from 2% PC +2% cholesterol +2% sphingomyelin in *n*-decane separated symmetrical salt solutions (100 mM KCl, 5 mM Tris, pH 7.0); membrane conductance $\approx 10^{-8}$ mho/cm^2. Sucessive additions of toxin were made to the solution on one side of the membrane, and the small signal conductance was recorded 30 min after each addition. The slope of the line is 3.8

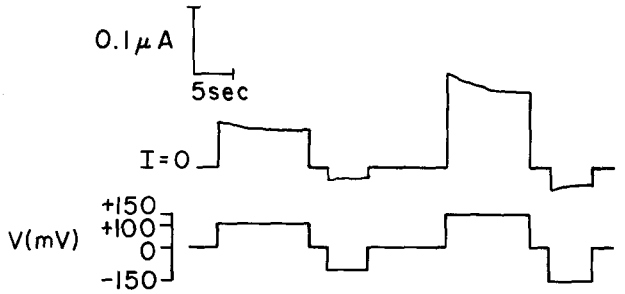

Fig. 3. Current transients in response to large voltage steps for a membrane treated with *helianthus* toxin (33 ng/ml) on one side. Membrane-forming solution: 2% PC+2% cholesterol +0.1% sphingomyelin in *n*-decane; aqueous solution on both sides = 100 mM K_2SO_4, 5 mM Tris (pH 7.5). The potential of the side *not* containing toxin is defined as zero

B. Lipid Dependence of Toxin-Induced Conductance

The 4th power dependence of conductance on toxin concentration is *not* significantly lipid dependent, but the conductance reached with a given toxin concentration (i.e., the sensitivity of the membrane) *is*. Table 1 shows the effect of sphingomyelin on membrane sensitivity to toxin. We have focussed particularly on this lipid because of its reported requirement for toxin action (Bernheimer & Avigad, 1976).

Both PC and PC : cholesterol membranes in the absence of sphingomyelin are sensitive to toxin; sphingomyelin increases that sensitivity (Table 1). Whether the effect of sphingomyelin is considered large or small depends on one's point of view. For a given amount of toxin, the conductance of a PC: sphingomyelin membrane is about 10^4 times that of a PC membrane. Considering the 4th power dependence of conductance on toxin concentration, however, only about 10 times as much toxin is required to achieve the same conductance in a PC membrane as in a PC: sphingomyelin membrane.

C. Toxin Acts by Making Channels

At low toxin concentrations, single channel activity is clearly observed (Fig. 4). The conductance and lifetime of these channels is variable, particularly in sphingomyelin-containing membranes. Single-channel conductances are large, being about 2×10^{-10} mho in 0.1 M KCl (at +50 mV); they increase linearly with KCl concentration between 0.1 and 1.0 M. Generally a channel tends to remain "on" once it appears. The macroscopically observed conductances (Fig. 1) undoubtedly arise from the parallel addition of these channels.

D. Ion Selectivity

Figure 5*A* shows plots of membrane potential vs. the logarithm of $(a_{KCl})_1/(a_{KCl})_2$, where $(a_{KCl})_1$ and $(a_{KCl})_2$ are the activities of KCl in compartments 1 and 2, respectively; essentially the same results are obtained with NaCl. The toxin-treated membrane is

Table 1. Effect of sphingomyelin on membrane sensitivity to *Stoichactus helianthus* toxin

% Sphingomyelin in membrane-forming solution	0%	0.01%	0.05%	0.1%	1%	2%
Conductance (mho/cm^2)	3.0×10^{-8}	7.7×10^{-7}	1.3×10^{-6}	3.4×10^{-4}	4.1×10^{-4}	5.0×10^{-4}

The membrane-forming solutions consisted of 2% egg phosphatidylcholine +2% cholesterol +% of sphingomyelin shown in table, all dissoved in *n*-decane. Each entry is the average of at least 5 experiments. In those experiments, membranes separated symmetrical salt solutions containing 100 mM KCl, 5 mM Tris (pH 7.0). Toxin was added to one side to a concentration of 66 ng/ml, and the conductance was recorded 20 min later.

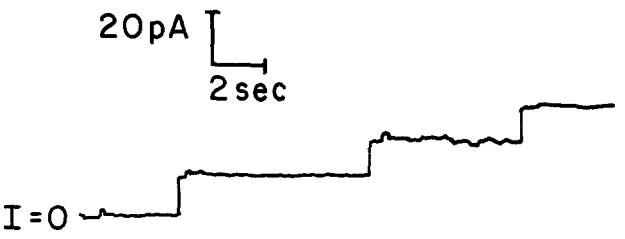

Fig. 4. Single-channel events in a membrane treated with a small amount of toxin (1.6 ng/ml) on one side. The record was obtained with the membrane potential clamped at 50 mV (toxin-containing side positive). Membrane-forming solution: 2% PC+2% cholesterol; aqueous solution on both sides = 100 mM KCl, 5 mM Tris (pH 7.0)

more permeable to univalent cations than to anions[2], but it is difficult to assign a relative selectivity because the plot is not a straight line. The bending of the curve is more pronounced at higher absolute values of salt concentrations (*compare* the curve for which the dilute side is 0.01 M KCl with that for which it is 0.1 M KCl).

A more clear-cut result is obtained with K_2SO_4. A plot of membrane potential *vs.* $\log (a_{K_2SO_4})_1/(a_{K_2SO_4})_2$ yields a straight line with a slope of 57 mV per 10-fold ratio of salt activity (Fig. 5*B*), indicating almost ideal selectivity for K^+ compared to $SO_4^=$.

E. Current-Voltage (I–V) Characteristics

Figure 6*A* shows the steady-state *I–V* characteristic[3] of a toxin-treated membrane in symmetrical 0.1 M K_2SO_4 solutions. Significant rectification is apparent, with conductance higher for positive voltages (toxin-treated side positive); the ratio of the limiting slope conductances is about 10. The same *I–V* characteristic is obtained for a membrane containing only one channel (Fig. 6*B*). Thus, the rectification in a many-channel membrane reflects the properties of an individual channel and does *not* result from a voltage dependence of channel opening and closing. Much less rectification occurs in 0.1 M KCl than in 0.1 M K_2SO_4 solutions; the ratio of the limiting slope conductances is about 4 instead of 10.

F. Effects of pH

The ion-selectivity and *I–V* characteristics described in the two preceding sections pertain to toxin-treated membranes at pH 7.0–7.8. They, along with the absolute values of the conductance, are significantly affected by pH. As pH is lowered from 7.8 to 2.8,

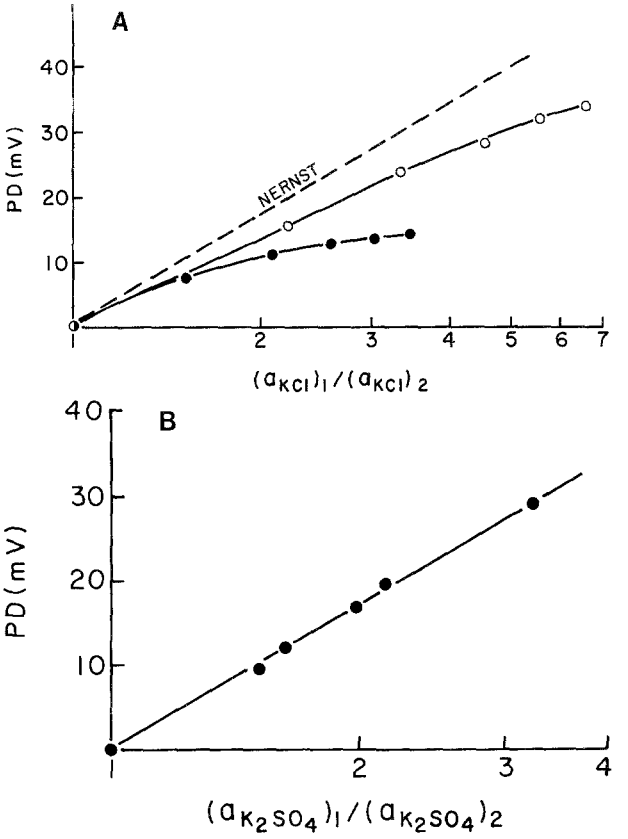

Fig. 5. (*A*): Membrane potential *vs.* logarithm of the ratio of KCl activities on the two sides of a *helianthus* toxin-treated membrane. Each point is the mean of at least 5 experiments. The concentration of KCl on side 2 was either 10 mM (o) or 100 mM (•); the solutions on both sides of the membrane also contained 5 mM Tris (pH 7.0). The concentration of KCl on side 1 was increased to the desired level by additions of small volumes of concentrated KCl solutions. Membrane-forming solution: 2% PC+2% cholesterol. Toxin was present on one side of the membrane at a concentration of 333 ng/ml; membrane conductances were approximately 10^{-4} mho/cm^2. (As the toxin-treated membrane is undoubtedly permeable to $Tris^+$, the cation selectivity for the case of $[KCl]_2 = 10$ mM is probably much greater than implied by the plot of potential *vs.* $(a_{KCl})_1/(a_{KCl})_2$. (*B*): Membrane potential *vs.* logarithm of the ratio of K_2SO_4 activities on the two sides of a *helianthus* toxin-treated membrane. Each point is the mean of at least 5 experiments. Membranes were formed in symmetrical solutions of K_2SO_4+5 mM dimethylglutaric acid (pH 7.6), where $[K_2SO_4]$ was either 100, 200, or 300 mM. After the conductance had been increased (to about 10^{-3} mho/cm^2) by addition of toxin to one side (to a concentration of 70 ng/ml), the concentration of K_2SO_4 on side 1 was increased by the complete exchange of solution with one containing K_2SO_4 (plus 5 mM dimethylglutaric acid) at the desired concentration. The slope of the drawn line is 57 mV per decade. Membrane forming solution: 2% PC+2% cholesterol+0.1% sphingomyelin in *n*-decane

the cation selectivity is reduced (Fig. 7), rectification becomes less significant and is virtually absent at pH 5 (Fig. 6*A*), and small signal conductance (in 0.1 M K_2SO_4) is reduced about fourfold (Fig. 8). Figures 7 and 8 have the appearance of typical titration curves, with the pK of the titratable group around

[2] It is more permeable, however, to Cl^- than to Ca^{++}.

[3] Essentially the same curve is obtained if the instantaneous *I–V* characteristic is plotted, since the maximum difference between the instantaneous and steady-state *I* is no more than 20%.

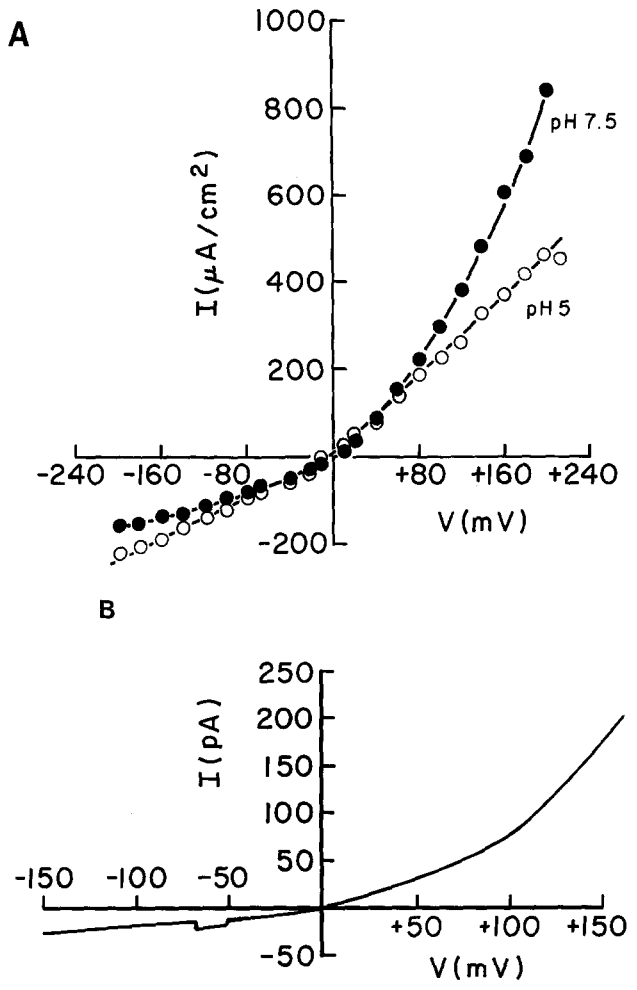

Fig. 6. Steady-state current-voltage (*I–V*) characteristics of a *helianthus* toxin-treated membrane. Membrane-forming solution: 2% PC+0.1% sphingomyelin. Membrane was formed in symmetrical salt solutions consisting of 100 mM K_2SO_4, 5 mM dimethylglutaric acid (pH 7.5). Toxin was added to one side of the membrane; the potential of the opposite side is defined as zero. (*A*): Macroscopic *I–V* characteristic for a membrane treated with toxin at a concentration of 33 ng/ml. Solid dots are for membrane at pH 7.5; open circles are for same membrane after pH was lowered to 5.0 by addition of H_2SO_4. (*B*): *I–V* characteristic of a single channel. Toxin was added to one side to a concentration of 1.6 ng/ml. After the appearance of a single channel the *I–V* characteristic was determined by applying a voltage ramp between −150 and +150 mV; this voltage range was covered in a thousand seconds. (A second channel popped on at −70 and off at −50 mV)

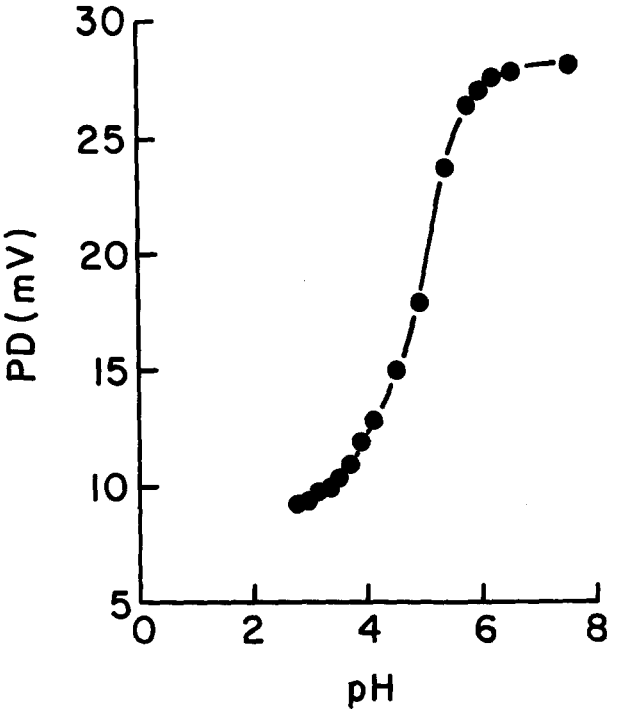

Fig. 7. Effect of pH on the ion selectivity of a *helianthus toxin*-treated membrane. The membrane separated 100 mM K_2SO_4 from 570 mM K_2SO_4; both solutions also contained 5 mM dimethylglutaric acid. The experiment was begun at pH 7.8 and the pH lowered in steps by additions of H_2SO_4 to both sides. Membrane-forming solution: 2%PC+2%cholesterol + 0.1% sphingomyelin; toxin concentration =70 ng/ml (on the 100 mM K_2SO_4 side); membrane conductance $\approx 10^{-4}$ mho/cm²

5.0. Essentially identical results are obtained for pH changes on the toxin-treated side alone as for symmetrical pH changes; little effect is obtained for pH changes on the toxin-free side alone. Apparently, the titratable group is accessible only from the toxin-treated side of the membrane.

Ca^{++} has a similar effect to that of H^+ on ion selectivity and probably through a similar mechanism (*see* section *E*-1 in the Discussion). A toxin-treated membrane at pH 9 separating 10 mM KCl from 100 mM KCl shows a potential difference of 42 mV (10 mM KCl side positive). When $CaCl_2$ is added to both sides to a concentration of 5 mM, the potential drops to 13 mV.

G. Nonelectrolyte Permeability

Permeability coefficients (P_d's) for urea, glycerol, glucose, and sucrose increase linearly with membrane conductance (Fig. 9). The linear relation strongly implies that the P_d's reflect the nonelectrolyte permeability of the ion-conducting channels, for it strains credulity to believe that the toxin creates separate ion and nonelectrolyte permeation pathways and that the number of both of these pathways is proportional to the 4th power of the toxin concentration. It is convenient, for calculating relative nonelectrolyte permeabilities, to normalize the values to some arbitrary membrane conductance (Table 2). Toxin-treated membranes show a graded permeability to nonelec-

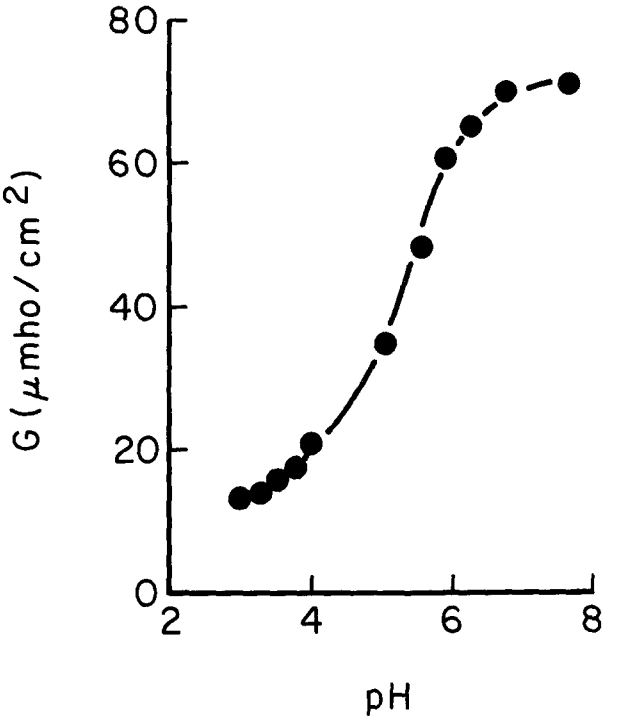

Fig. 8. Effect of pH on the conductance of a *helianthus* toxin-treated membrane. The membrane separated symmetrical solutions containing 100 mM K_2SO_4+5 mM dimethylglutaric acid. The experiment was begun at pH 7.8, and the pH was lowered in steps by additions of H_2SO_4 to both sides. Conductance was determined from the current response to a 30 mV stimulus (toxin-containing side negative). Membrane-forming solution: 2% PC +2% cholesterol +0.1% sphingomyelin in *n*-decane; toxin concentration= 33 ng/ml on one side

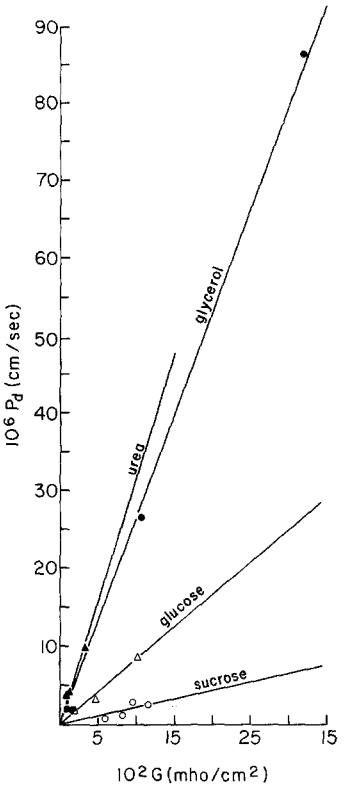

Fig. 9. Permeability coefficients (P_d's) for several nonelectrolytes as a function of membrane conductance in *helianthus* toxin-treated membranes. Each point was obtained on a different membrane. All experiments were done with membranes formed from 2% PC+2% cholesterol in *n*-decane and separating symmetrical solutions containing 100 mM KCl, 5 mM dimethylglutaric acid (pH 7.6)

trolytes; i.e., P_d values decrease with increasing molecular size (Fig. 9 and Table 2). P_d(urea):P_d(glycerol):P_d(glucose):P_d(sucrose)=1:0.8:0.3:0.07. Thus, the toxin channels manifest classical molecular "sieving" properties.

Table 2. Nonelectrolyte permeability coefficients (P_d's) for membranes treated with *Stoichactis helianthus* toxin

Molecule	P_d (cm/sec)
Urea	3.2×10^{-6}
Glycerol	2.6×10^{-6}
Glucose	8.5×10^{-7}
Sucrose	2.1×10^{-7}

P_d values are normalized to a conductance of 10^{-2} mho/cm^2 in 100 mM KCl, 5 mM Tris (pH 7.5).

Discussion

A. Lipid Dependence of Stoichastis helianthus Toxin

Previous work has suggested that *S. helianthus* toxin specifically requires sphingomyelin for its action on lipid vesicles or mammalian cells (Bernheimer & Avigad, 1976; Linder et al., 1977). Our result with planar lipid bilayer membranes (Table 1) show that although the toxin's ability to increase membrane conductance is enhanced by the presence of sphingomyelin in the membrane, the toxin is still very active on membranes containing no sphingomyelin. These include PC, PC +cholesterol, PC+PS, and PE membranes. Michaels (1979) has also shown that PC+cholesterol, oxidized cholesterol, and glycerol monoolein membranes are sensitive to toxin. Thus, sphingomyelin is not a specific and unique membrane receptor for the toxin, a view also reached by Shin, Michaels and Mayer (1979) from experiments with liposomes. They concluded that toxin sensitivity depends more on acyl chain length and degree of saturation than on lipid class.

B. Channel Formation

S. helianthus toxin increases membrane conductance by forming ion-conducting channels in the bilayer.

This conclusion follows directly from the discrete conductance "jumps" of $\sim 2 \times 10^{-10}$ mho in membranes treated with very small amounts of toxin (Fig. 4). At a membrane potential of 50 mV, this represents a flux of 6×10^7 ions per second, much too large for a carrier-type mechanism (*see* Hladky & Haydon, 1972). Also consistent with channel formation is the dependence of conductance on the 4th power of toxin concentration and the graded permeability of toxin-treated membranes to nonelectrolytes (*see* following section).

The most obvious interpretation of the 4th power dependence of conductance on concentration is that the channel is formed by four toxin molecules. Several other examples of multimeric channel formation in lipid bilayers are known, such as nystatin and amphotericin B (Finkelstein & Holz, 1973), alamethicin (Eisenberg, Hall & Mead, 1973), and monazomycin (Muller & Finkelstein, 1972). In those instances the monomeric units have mol wt $\sim$ 1000–2000; the multimeric *helianthus* toxin channel is composed of protein subunits of mol wt $\sim$ 16,000. In the absence of structural data for the toxin, we can safely assume that the channel is formed by a "barrel stave" arrangement similar to that postulated for the nystatin and amphotericin B channels (Finkelstein & Holz, 1973), the alamethicin channel (Baumann & Mueller, 1974), and the monazomycin channel (Heyer, Muller & Finkelstein, 1976).

Michaels (1979) reports results similar to ours but with some quantitative differences. He obtains a 3rd power dependence of conductance on toxin concentration rather than a 4th power dependence. His single-channel conductance value of 10^{-10} mhos in 0.5 M KCl is smaller than our 2×10^{-10} mhos in 0.1 M KCl. He reports single-channel life times of ~ 2 sec, whereas we find that once a channel appears it tends to remain *on* most of the time. There are also detailed differences in the *I–V* characteristics and the time-variant conductances observed by Michaels and by us. These discrepancies could result from minor variations in toxin preparations.

C. Channel Size

The "sieving" property of toxin-treated membranes (Table 2), i.e., the decrease in nonelectrolyte permeability with increasing molecular size, is in itself strong evidence that the toxin forms channels (pores) in bilayer membranes; it also provides an estimate of channel size. The ratio of P_d (urea) to P_d (glycerol) is approximately the ratio of the diffusion coefficients of urea and glycerol in free solution (1.3). Thus, the toxin channel does not significantly discriminate between those two solutes. On the other hand, P_d(urea)/P_d(glucose) = 4 and P_d(urea)/P_d(sucrose) = 15, demonstrating that there is increasing sieving of glucose and sucrose. We did not attempt to find the "cut-off" size for the channel. Since sucrose is permeable, the channel radius is *at least* 5 Å.

Table 3 compares the permeability properties of the *helianthus* toxin channel with those of the amphotericin B channel (Holz & Finkelstein, 1970). The latter is clearly a smaller channel, having a cut-off for molecules about the size of glucose, whereas the toxin channel is still significantly permeable to sucrose. This size difference is also manifested by the relative permeabilities of permeant molecules. Thus, whereas P_d(urea)/P_d(glycerol) is only 1.2 for the toxin channel, it is 8.3 for the amphotericin B channel; as expected, the smaller polyene channel discriminates much more between urea and glycerol than does the larger, toxin channel.

Finally, since single-channel data are available for both toxin and amphotericin B channels, a comparison can be made of the permeability coefficients per channel (Table 3)[4]. Calling the single-channel permeability coefficients p_d, we find that $[p_d(\text{urea})]_{\text{toxin}}/[p_d(\text{urea})]_{\text{amph B}} = 27$ and $[p_d(\text{glycerol})]_{\text{toxin}}/[p_d(\text{glycerol})]_{\text{amph B}} = 180$. Thus, by three criteria – cut-off size, relative permeability of permeable solutes, and absolute solute permeabilities of the individual channels – the *helianthus* toxin channel is significantly larger than the amphotericin B channel.

D. Time-Variant Response

Although we have observed time-variant conductance behavior at voltages larger than ± 80 mV (Fig. 3), we cannot elaborate further on this phenomenon. Our data are not entirely consistent, and we have not obtained effects with large voltages at the single-channel level that could account for the current transients seen in membranes containing many channels. The transient peak, in any case, is maximally only 20% larger than the steady-state level. Michaels (1979) reports more extensively on this phenomenon but arrives at no firm conclusion about its nature.

E. Channel Properties Controlled by Charge Groups

1. Ion selectivity. The univalent cation selectivity of toxin-treated membranes is readily explained by nega-

[4] The permeability coefficients in Table 3 are not exact. For the toxin channel, this is because of the scatter in the single-channel conductance data. For the amphotericin B channel, uncertainty arises because the nonelectrolyte permeability data and the single-channel conductance data were obtained in different laboratories on membranes of different composition, albeit both membranes were made from brain lipids.

Table 3. Comparison of the permeability properties of *Stoichactus helianthus* toxin channels and amphotericin B channels

	Single channel conductance in 0.1 M salt[c] (mho)	P_d(urea) (cm/sec)	P_d(glycerol) (cm/sec)	p_d(urea) (cm^3/sec)	p_d(glycerol) (cm^3/sec)
Amphotericin B	2.5×10^{-13}[a]	95×10^{-6}[b]	11.5×10^{-6}[b]	2.4×10^{-15}	2.9×10^{-16}
Toxin	2.0×10^{-10}	3.2×10^{-6}	2.6×10^{-6}	6.4×10^{-14}	5.2×10^{-14}

P_d values are normalized to a conductance of 10^{-2} mho/cm^2 in 0.1 M KCl (for the toxin) and 0.1 M NaCl (for amphotericin B).

[a] Extrapolated from the data in Fig. 3 of Ermishkin, Kasumov & Potseluyev (1977).

[b] From Holz and Finkelstein (1970).

[c] KCl for toxin and NaCl for amphotericin B.

tive charges within or at the mouth of the channel formed by the toxin molecules. The most convincing evidence for these negative charges is the effect of pH on the channel's relative selectivity for K^+ and $SO_4^=$ (Fig. 7). Above pH 6.2 the channel is almost ideally K^+ selective, whereas below pH 4 there is very little discrimination between K^+ and $SO_4^=$. This indicates that the degree of protonation of charged groups associated with the channel[5] controls ion selectivity. Titration of either positive or negative charge groups is consistent with the effect of pH on selectivity, but given the cation selectivity of the channel, the latter is more likely. Also consistent with negative charge groups is the decrease of cation selectivity produced by Ca^{++} (*see* section *F* of Results). The effect is consistent with Ca^{++} binding to (or screening) negative charge groups associated with the channel. The titration curves of selectivity *vs.* pH (Fig. 7) and of conductance *vs.* pH (Fig. 8) indicate that the pK of these negative charge groups is around 5, suggesting that they are carboxyl groups.

One other aspect of the ion selectivity can be attributed to charge groups in the channel. The bending of the membrane potential vs. log $[KCl]_1/[KCl]_2$ curves and the greater degree of this bending at higher absolute values of KCl concentration (Fig. 5*A*) is consistent with classical fixed-charge theory (Teorell, 1953). In that theory, this phenomenon results from the effect of salt concentration on the Donnan potentials at the two membrane-solution interfaces of a membrane containing fixed charges. In the present instance, the phenomenon similarly results from the effect of salt concentration on the mobile ion distributions within the channel as modified, through the Poisson-Boltzmann relation, by the fixed charges in the channel.

[5] The membrane lipids, PC and cholesterol, have no significant net charge and do not titrate appreciably in the pH range (4 to 8) of our experiments.

2. Rectification. The *I-V* characteristic of a toxin-treated membrane exhibits rectification even when the membrane separates identical salt solutions (Fig. 6*A*), and this characteristic is a property of individual channels (Fig. 6*B*). It is readily explained by assuming that the negative charge groups, described in the preceding section, are asymmetrically distributed, being closer to the mouth of the channel facing the side to which toxin was added. Consequently, there is a higher concentration of mobile cations at that end of the channel than at the opposite end; an applied voltage shifts the concentration profile of these cations to produce rectification as described by the Goldman equation. From the magnitude of the rectification, we estimate that, in 0.1 M K_2SO_4, the potential at the mouth of the channel facing the side to which toxin was added is about 60 mV more negative than the potential at the opposite mouth of the channel.

Our finding that the charge groups are titratable only from the side to which toxin was added (*see* Results) strongly supports the assumption of an asymmetrical charge distribution. pH changes on the opposite side have no effect on selectivity or conductance. This apparently places the negative charge groups that modulate conductance and ion selectivity, and are also responsible for the rectification seen in symmetric salt solutions, near the mouth of the channel facing the side to which toxin was added.

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