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Pore Formation by *S. aureus* α-toxin in Liposomes and Planar Lipid Bilayers: Effects of Nonelectrolytes

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Abstract. Nonelectrolytes such as polyethylene glycols (PEG) and dextrans (i) promote the association of *S. aureus* α-toxin with liposomes (shown by Coomassie staining) and (ii) enhance the rate and extent of calcein leakage from calcein-loaded liposomes; such leakage is inhibited by H^+, Zn^{2+} and Ca^{2+} to the same extent as that of nonPEG-treated liposomes. Incubation of liposomes treated with α-toxin in the presence of PEG with the hydrophobic photo-affinity probe 3-(trifluoromethyl)-3-*m*-[^{125}I]iodophenyl)diazirine(^{125}I-TID) labels monomeric and—predominantly—hexameric forms of liposome-associated α-toxin; in the absence of PEG little labeling is apparent. At high concentrations of H^+ and Zn^{2+} but not of Ca^{2+}—all of which inhibit calcein leakage—the distribution of label between hexamer and monomer is perturbed in favor of the latter.

In α-toxin-treated planar lipid bilayers from which excess toxin has been washed away, PEGs and dextrans strongly promote the appearance of ion-conducting pores. The properties of such pores are similar in most regards to pores induced in the absence of nonelectrolytes; they differ only in being more sensitive to ''closure'' by voltage (as are pores induced in cells).

In both systems, the stimulation by nonelectrolytes increases with concentration and with molecular mass up to a maximum around 2,000 Da. We conclude (i) that most of the α toxin that becomes associated with liposome or planar lipid bilayers does not form active pores and (ii) that the properties of α-toxin-induced pores in lipid bilayers can be modulated to resemble those in cells.

Key words: α-toxin — Protons — Divalent cations —Polyethyleneglycol — 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine — (^{125}I-TID) — Lipid membranes

Introduction

The α-toxin of *S. aureus* is a hemolytic, cytotoxic, dermonecrotic exotoxin, which is considered to be a major virulence factor of *S. aureus* infections (Cassidy, Six, & Harshman, 1974; Thelestam & Blomqvist, 1988). It is secreted as a water-soluble monomer which interacts with target membranes to form oligomeric structures which have a porelike appearance by electron microscopy (Bhakdi, & Tranum-Jensen, 1991). Susceptible cells treated with low amounts of α-toxin leak ions, intermediates of metabolism such as phosphocholine but not proteins (Bashford et al., 1986). Calcein (molecular weight 622), but not fluorescein-dextran (molecular weight 20,000) leaks from α-toxin treated liposomes (Menestrina, Bashford & Pasternak, 1990). In planar lipid bilayers α-toxin induces ion channels which exhibit partial rectification (Menestrina, 1986) and voltage-dependent closure at low pH or in the presence of divalent cations such as Ca^{2+} or Zn^{2+} (Bashford et al., 1988).

The availability and simplicity of action of α-toxin have made it an excellent model for studying the functional assembly of a membrane pore. Its primary struc-

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ture has been determined at the genomic level (Gray & Kehoe, 1984) and site-directed mutants with altered properties (Walker et al., 1992; Palmer et al., 1993), have been prepared. Low resolution electron microscope images of membrane-associated toxin are consistent with the idea that a hexameric structure forms the active pore (Olofsson et al., 1990). However, studies with photoactivatable lipid probes added to rabbit erythrocyte membranes (Thelestam, Jolivet-Reynaud & Alouf, 1983) or to asolectin liposomes (Harshman et al., 1989) show almost exclusive labeling of monomer only. The latter authors ascribed the lack of hexamer labeling to failure of their probes (phosphatidyl choline derivatives) to bind to the relatively hydrophobic surface of the hexamer. We have therefore used the highly hydrophobic probe 3-(trifluoromethyl)-3-*m*-[^{125}I]iodophenyl)diazirine (^{125}I-TID) (Brunner & Semenza, 1981) to investigate this problem. Our initial studies, using ^{125}I-TID to label α-toxin-treated liposomes, revealed no labeling at all, nor could we detect liposome-associated α-toxin by Coomassie Blue staining under conditions where there was significant leakage of liposome-entrapped calcein. To improve binding of α-toxin to liposomes we tried adding nonelectrolytes such as polyethyleneglycol (PEG) or dextran since these are known to favor amphipathic interactions such as membrane fusion (Blow et al., 1978) even though they protect cells against α-toxin induced hemolysis (e.g., Bhakdi, Muhly & Füssle, 1984). The result was startling: binding of α-toxin and calcein leakage were dramatically increased, as was ^{125}I-TID labeling of monomeric *and* hexameric form of α-toxin. When PEG or dextran was added to planar lipid bilayers that had been exposed to α-toxin, a 1,000-fold increase in conductance was observed. These findings, which have been reported in brief (Bashford et al., 1993; Kovacs et al., 1993), are presented below.

Materials and Methods

Chemicals

S. aureus α-toxin was a purified sample donated by Dr. K.D. Hungerer, Behringwerke, Marburg, W. Germany. Palmitoyloleoyl phosphatidylcholine (POPC) and diphytanoyl phosphatidylcholine (DPhPC) were obtained from Avanti Polar Lipids. Cholesterol was from Sigma. 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (^{125}I-TID) was from Amersham International.

Bilayer Experiments

The apparatus consisted of two Teflon chambers (capacity 0.12 ml each) connected by an aperture (10–20 μm diameter) across a 10 μm thick Teflon film. Ag/AgCl electrodes were used; the electrode connected to virtual ground was in the chamber to which α-toxin was added (*cis*). Ion current across such bilayers was measured at different applied voltages. Voltage signs refer to the *cis* compartment; at positive potential cations flow from *cis* to *trans.* One hundred mM KCl buffered to pH 7.4 with 5 mM HEPES was used throughout, except where indicated otherwise. The solutions in either chamber could be removed with a syringe through ports in the teflon housing; complete exchange of solutions, without damaging the bilayer, was achieved by repeated removal and replacement such that the level of the air/solution interface never fell below the orifice connecting the chambers and supporting the lipid bilayer. All the experiments were performed at room temperature.

Planar bilayers were formed from DPhPC or Lettré cell plasma membrane (Schindler, 1980) as described by Korchev et al. (1995). In one experiment, POPC/cholesterol (1/1 w/w) liposomes that had been used for a calcein leakage experiment in the presence of PEG 2,000 and α-toxin were pelleted and bilayers formed by the technique used for Lettré cell plasma membrane. Various amounts of a solution of α-toxin (1 mg protein/ml in 0.16 M NaCl) were added to one chamber (*cis*) as stated in each figure legend. For all experiments, α-toxin was removed after 5 min by perfusion with toxin-free solutions.

Liposome Experiments

Calcein-containing liposomes of POPC/cholesterol (1/1 w/w) were prepared by sonicating 12.5 mg lipid/ml in 80 mM calcein, 50 mM NaCl, pH 7 (NaOH) as described previously (Menestrina, Bashford & Pasternak, 1990; Alder et al., 1991). Nonentrapped calcein was removed either by gel filtration on Sephadex G-50 or by centrifugation at 60,000 × *g* in a Beckman TL-100 ultracentrifuge for 60 min. Fractionated liposomes were resuspended in 160 mM NaCl, 10 mM Hepes, pH 7 (NaOH). Calcein fluorescence was excited at 490 nm (10 nm slitwidth) and monitored at 520 nm (10 nm slitwidth) with a Perkin-Elmer MPF-44A spectrofluorimeter; maximal leakage at the end of each experiment was assessed by lysis with 2% Triton-X-100 (final concentration) (Menestrina, Bashford & Pasternak, 1990; Alder et al., 1991). All experiments were performed at room temperature.

Liposomes were labeled with [^{125}I]TID as follows: prior to addition of triton X-100 a sample (0.8–0.9 ml) of the liposome suspension was removed and the liposomes pelleted at 100,000 rpm for 30 min in a Beckman TL-100 ultracentrifuge, the supernatants were recovered to estimate free calcein and the pellets were resuspended in 0.05 ml of 0.16 M NaCl, 0.01 M Hepes pH 7.0 (buffered saline) and transferred to wells in a 96 place hemolysis plate; 1 μl of [^{125}I]TID was added directly to each pellet and samples were photolyzed under a long wave length at illuminator (Mineralight, Model UVSL-58, Ultra-Violet Products, San Gabriel, CA) for a minimum of 15 min; samples and two 50 μl washings of each well were transferred to 0.9 ml buffered saline in TL-100 tubes and the labeled liposomes were pelleted at 100,000 rpm for 30 min.

Labeled pellets were prepared for polyacrylamide gel electrophoresis by adding: 24 μl distilled deionized water (ddH_2O), 6 μl ''5 × running stain'' (2% SDS, 10% glycerol, 0.0001% bromophenol blue according to Laemmli (1970), and 3 μl of dithiothreitol (>0.1 M). Samples were electrophoresed immediately or stored at −20°C until required. Eight percent or ten percent polyacrylamide separating gels with 4.5% stacking gels were run in an LKB 2050 midgit electrophoresis unit at 200V (120 mA, 30 W) until the front reached the tank buffer (*ca* 1.5 hr). Gels were: (i) fixed for at least 30 min in 10% w/v trichloroacetic acid, 10% v/v glacial acetic acid, 30% methanol and 50% dd H_2O; (ii) washed for 5 min in destain solution (41% ethanol, 7% glacial acetic acid, 52% ddH_2O); (iii) stained with Coomassie brilliant blue (0.125 g/l in 41% ethanol, 7% glacial acetic acid and 52% dd H_2O); (iv) destained by washing with destain solution until the gel was transparent. Gels were vacuum dried onto filter paper using an Edwards Modulyo freeze drier and a Savant gel drier.

Gels were autoradiographed at –70°C for 20–40 hr using Fuji RX 100 medical X-ray film and were developed conventionally by the hospital radiography department.

Red Cell Experiments

Whole rabbit blood was collected into (in mM): 150 NaCl, 5 KCl, 5 Hepes, 1 $MgSO_4$, pH 7.4 (NaOH; Hepes-buffered saline, HBS) containing heparin (*ca* 10 units/ml). Red cells were pelleted at $1,000 \times g$ in an MSE Chilspin for 5 min and washed three times with 5 volumes of HBS before resuspension at 1% v/v in HBS. Hemolysis was assessed in round-bottomed, 96-well tissue culture plates. 0.05 ml HBS without or with appropriate dextrans (10 mM, final concentration) or PEGs (10% v/v final concentration) was added to all wells; then 0.05 ml toxin-containing medium was added to lane 1 and successive two-fold dilutions were made by removing 0.05 ml from lane 1 into lane 2, mixing and removing 0.05 ml from lane 2 into lane 3 etc.; the last 0.05 ml was discarded. 0.05 ml 1% red cells were added to each well and the plate incubated at 37°C for 60 min. Hemolysis was assessed by measuring absorbency of the supernatant and pellets at 543 nm.

Results

Nonelectrolytes Promote Formation of α-toxin Pores in Liposomes

Leakage of calcein from liposomes exposed to α-toxin is greatly enhanced by the inclusion of polyethyleneglycol (or dextran) in the medium. Figure 1*A* shows typical records of the increase in calcein fluorescence observed when α-toxin is added to liposomes in the presence of 0, 5, 10 or 25% PEG 600. Both the rate and the extent of leakage are increased by PEG 600 in a concentration dependent fashion. PEGs of other molecular weights are also effective (Fig. 1*B*). PEG potency increases with PEG size up to a value of 2,000, and may tail off slightly for larger PEGs. Doubling the concentration of PEG (Fig. 1*B*) stimulates leakage further and shifts the activity-response curve towards lower molecular weight (for PEG). If PEGs are added to liposomes pretreated with α-toxin at 4°C and subsequently washed free of excess toxin, no additional calcein leakage ensues. Toxin-induced calcein leakage in the presence of PEG is inhibited by protons and divalent cations to the same extent as that in the absence of PEG (Fig. 1*C*), at concentrations similar to those found previously (Menestrina et al., 1990).

Labeling of Liposome-associated α-toxin with [^{125}I]TID

The potentiation of α-toxin induced calcein leakage from liposomes by PEGs is accompanied by an increased association of toxin with liposomes. Polyacrylamide gel electrophoresis (PAGE) of toxin-treated liposome pellets prepared without or with PEG reveals substantially more toxin in the latter case on the basis of staining with Coomassie Blue (Fig. 2): compare lane 1 of panel *A* and lane 6 of panel *C* with lane 1 of panel *B* and lane 6 of panel *D;* note that the concentration of α-toxin had to be increased to 1 mg/ml to just visualize α-toxin in the absence of PEG. In these experiments, the liposome pellets had been treated with [^{125}I]TID; the autoradiographs of the gels show that the level of ^{125}I-labeled toxin is greatly increased in the PEG-treated liposomes, with label in both low (monomer) and especially in high molecular weight (hexamer) species of the toxin: compare lane 1 of panel *A* and lane 6 of panel *C* with lane 1 of panel *B* and lane 6 of panel *D.*

In conditions where protons, Zn^{2+} or Ca^{2+} inhibit calcein leakage from toxin-treated liposomes, Zn^{2+} (panels *A* and *B*) increases the proportion of toxin in pellets (both by Coomassie staining and by labeling with ^{125}I-TID) found as monomer and other low molecular weight forms. Protons (panels *C* and *D*) have a similar effect, whereas Ca^{2+} (lanes 5 and 6 of panel *B*) does not. Note that under the conditions used for these experiments (higher concentration of liposomes and α-toxin than for the experiments illustrated in Fig. 1), the effect of PEG on calcein leakage is less pronounced.

Nonelectrolytes Promote Formation of α-toxin Pores in Planar Lipid Bilayers

Addition of PEG or dextran to phospholipid bilayers containing α-toxin channels from which excess α-toxin has been removed by washing, induces substantial, additional ionic current that remains stable when the PEG is washed away (Fig. 3*A*). (We use the word ''channel'' in the context of bilayer experiments where ion current is being measured, and the word ''pore'' in relation to liposomes and cells, without implying particular structures in either case). This shows that PEG induces the formation of channels from prebound toxin. PEG has to be added to the same side of the bilayer that was exposed to α-toxin (*cis*); if added *trans,* no effect is seen. At pH 7.4 and a transmembrane potential of +30 mV, current remains constant at 0.9 nA (conductance of 3 nS); at +60 mV, current (of approx 1.7 nA; conductance of 2.8 nS) decreases to around 0.85 nA, (conductance of 1.4 nS). In other words, increased voltage favors the ''closed'' state of α-toxin channels. This is true also of pores formed in the absence of PEG, except that in that case much higher voltages are necessary to induce the low-conducting state (Menestrina, 1986). A plot of the ratio of residual current/initial current (I/I_{max}) against voltage gives the curve indicated by the filled circles in Fig. 3*B;* comparison with data from nonPEG treated bilayers (open circles) shows marked difference in voltage sensitivity, indicative of a different type of pore; rectification at pH 7.4 (Menestrina, 1986) is also increased in

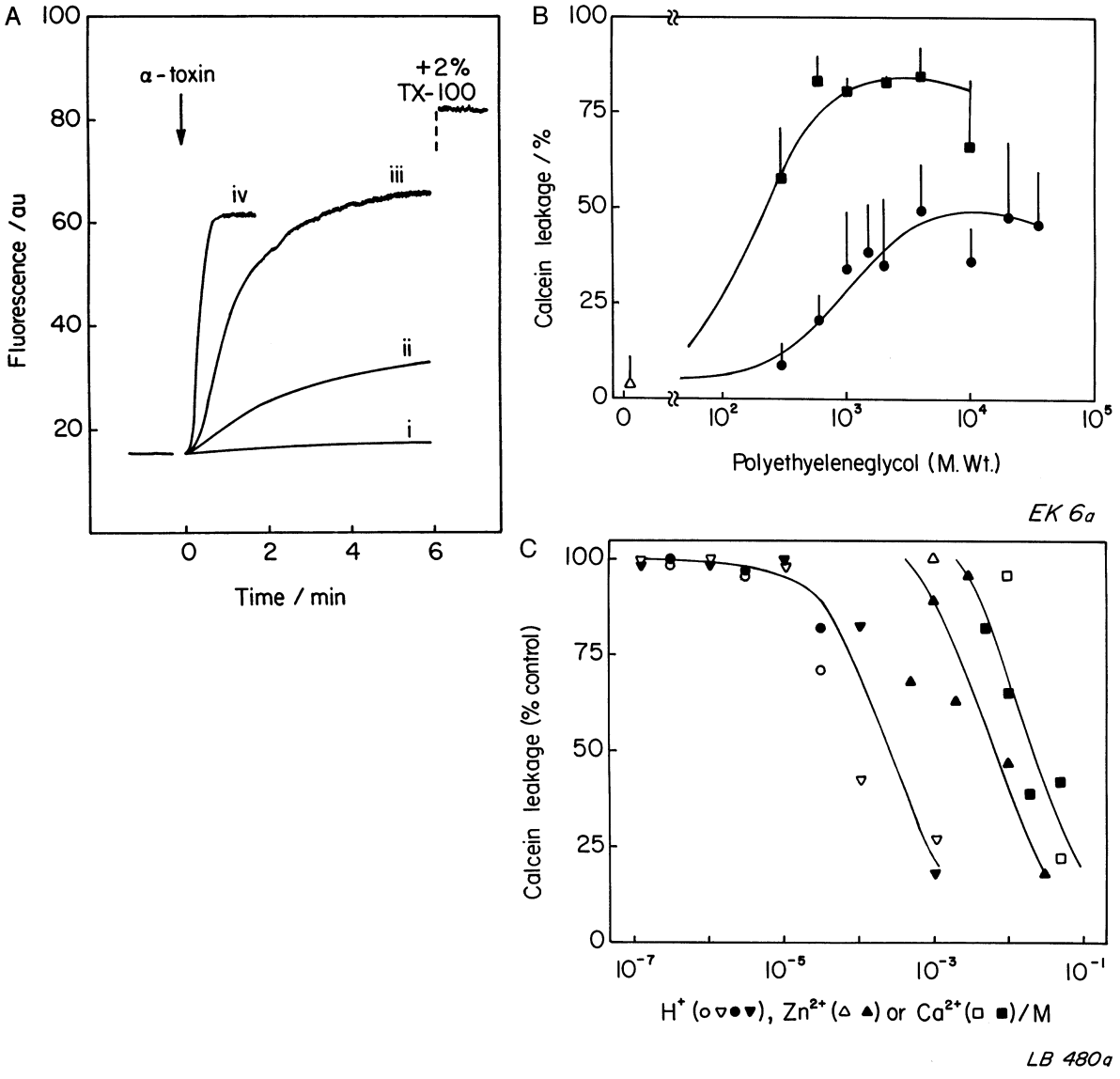


Fig. 1. Effect of PEG on α-toxin induced calcein leakage from liposomes. POPC/cholesterol liposomes containing entrapped calcein were prepared as described in Materials and Methods and incubated at room temperature. (*A*) Time course of calcein leakage. Liposomes (10 μg/ml) without (trace i) or with 5% (trace ii), 10% (trace iii) or 25% (trace iv) PEG 600 (all w/v) were treated with α-toxin (2 μg/ml) at the time indicated by the arrow. Fluorescence (expressed in arbitrary units) was monitored continuously. At the end of each run triton X-100 was added to determine the maximal extent of leakage. A typical experiment is shown. (*B*) Dependence on concentration and molecular weight of PEG. Liposomes (10 μg/ml) without (△) or with 5% (●) or 10% (■) PEG (w/v) were treated with α-toxin (2 μg/ml) and increase of fluorescence monitored as in *A;* when fluorescence had reached a steady value (>10 min), triton X-100 was added to determine maximum leakage. The ordinate shows the mean % of maximum leakage for several experiments (error bars indicate SEM of 3 (■), 4 (●) or 7 (△) experiments). (*C*) Effect of protons and divalent cations. The pH of the liposome suspension (10 μg/ml) was either kept at 7 and $ZnSO_4$ (upward triangles) or $CaCl_2$ (squares) added to give the final concentrations indicated, or was reduced with HCl (circles) to give the final H^+ concentration indicated. Five percent PEG 4,000 (final; w/v) was added (filled symbols) or not (open symbols), followed by α-toxin (2 μg/ml). An additional experiment with liposomes (100 μg/ml) in 0.16 M NaCl, phosphate/citrate buffers, diluted tenfold to give the final H^+ concentration indicated (downward triangles) is also shown. Ten percent PEG 1500 (final; w/v) was added (filled symbols) or not (open symbols), followed by α-toxin (10 μg/ml). The increase in fluorescence was monitored as in *A;* percentage of maximum leakage was calculated as in *B* and assigned a value of 100 for leakage (+/–PEG) at pH 7 without divalent cations: Percentage of maximum leakage under conditions of altered pH or added Zn^{2+} or Ca^{2+} is expressed as a % of that control value (+/–-PEG). Symbols refer to individual experiments (each with its pH 7, no divalent cations, control).

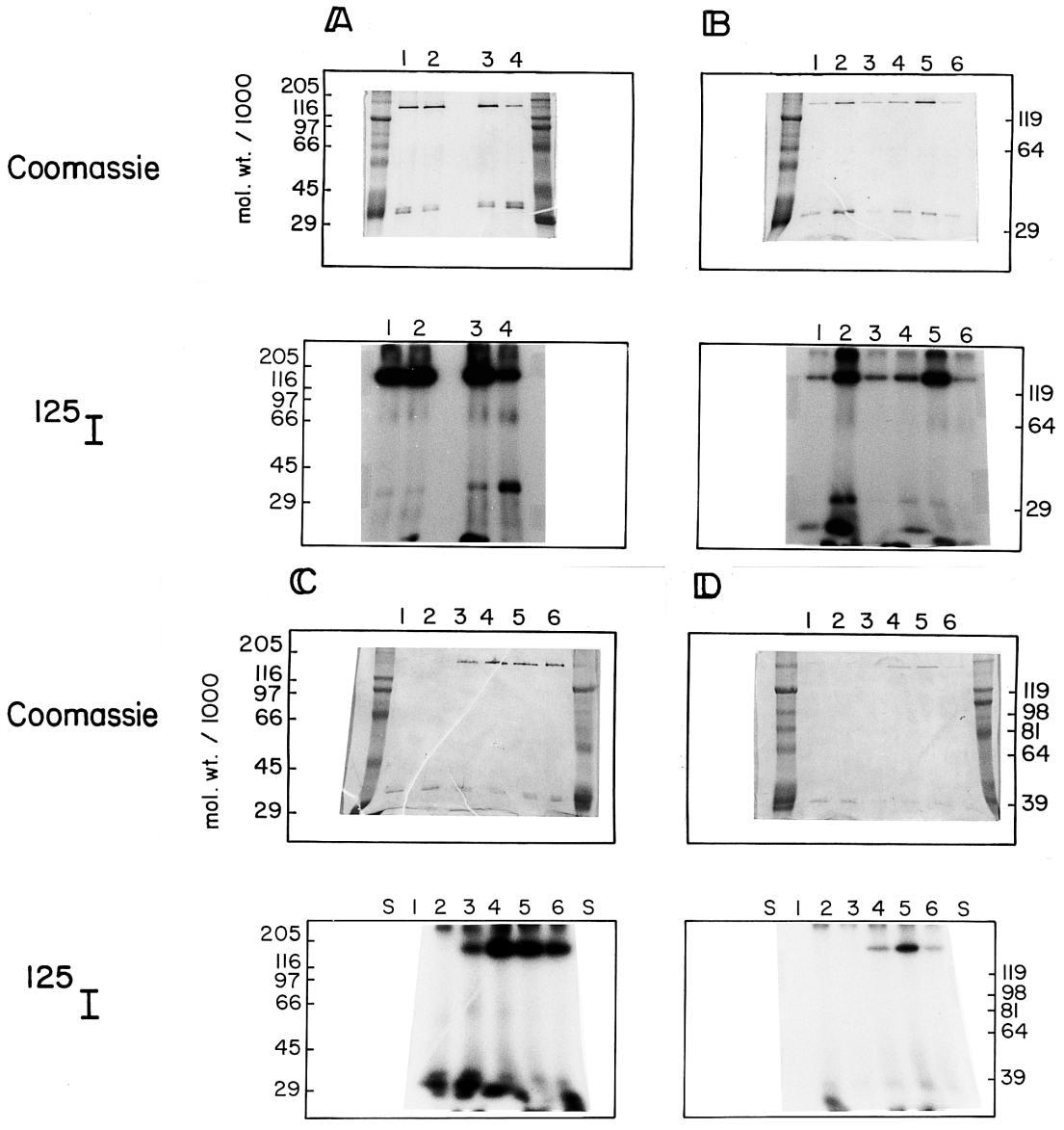
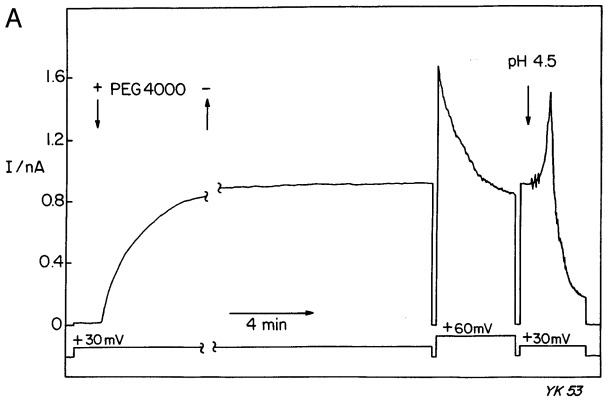
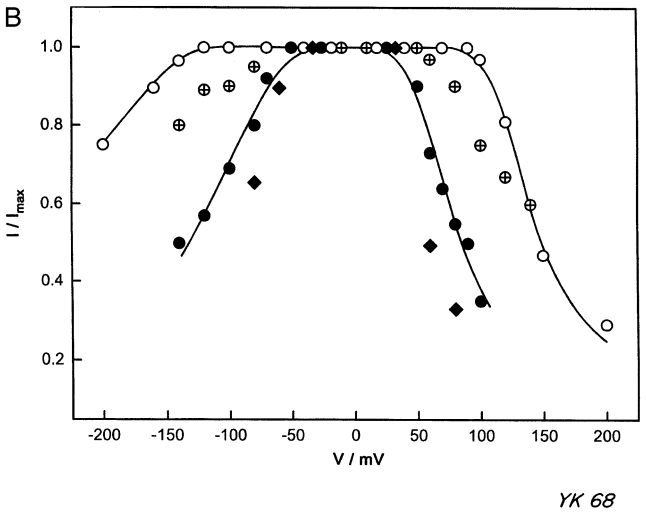
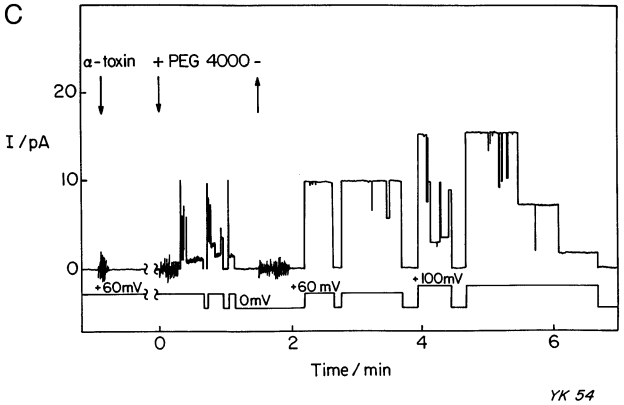


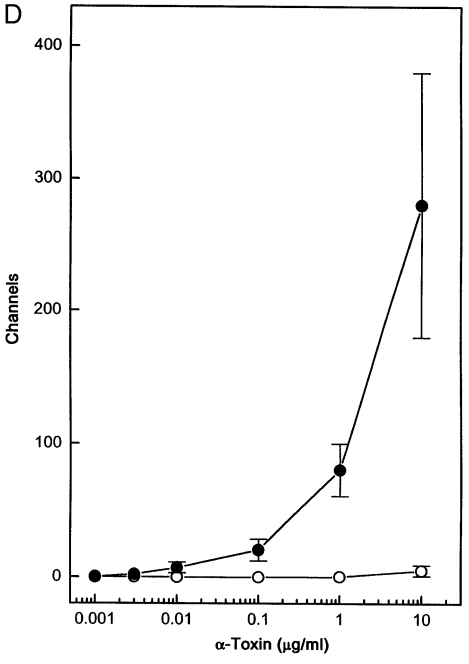
Fig. 2. Effect of PEG on binding and oligomerization of α-toxin by liposomes. Liposomes (100 μg/ml) were incubated at room temperature without or with PEG 1500 (10% w/v final) in 160 mM NaCl buffered with 10 mM Hepes (pH 7.4) for the experiments shown in panels *A* and *B* or with 10 mM phosphate/citrate buffers for the experiments shown in panels *C* and *D*. α-toxin (10 μg/ml) was added and calcein leakage was monitored as shown in Fig. 1, panel *A*. After 5 min a portion of the suspension was removed, the liposomes pelleted and the amount of calcein in the supernatant assessed and the pellets labeled with [^{125}I]TID as described in Materials and Methods. The % calcein leakage quoted refers to a value of 100% after the addition of triton (Fig. 1). Each panel shows Coomassie Blue staining (upper) and autoradiography for ^{125}I (lower) of the same polyacrylamide gel. Molecular weight markers (upper panels) are shown to the sides of the experimental lanes. *Panel A:* Effect of Zn^{2+} in the presence of PEG. Lanes 1, 2, 3 and 4 correspond to the addition of 0, 3, 10 and 30 mM Zn^{2+} respectively. The % calcein leakage was 65, 60, 30 and 15% respectively. *Panel B:* Effect of Zn^{2+} in the absence of PEG (lanes 1–4) and of Ca^{2+} with or without PEG. Lanes 1, 2, 3 and 4 correspond to the addition of 0, 30, 10 and 3 mM Zn^{2+} respectively. The % calcein leakage was 55, 15, 35 and 40% respectively. Lanes 5 and 6 correspond to the addition of 50 mM Ca^{2+} with (lane 5) or without (lane 6) PEG. The % calcein leakage was 25 and 15% respectively. *Panel C:* Effect of pH in the presence of PEG. Lanes 2, 3, 4, 5 and 6 correspond to pH 3, 4, 5, 6 and 7 respectively. The % calcein leakage was 15, 60, 75, 75 and 75% respectively. A standard (unlabeled) sample of α-toxin was run in lane 1. *Panel D:* Effect of pH in the absence of PEG. Lanes 2, 3, 4, 5 and 6 correspond to pH 3, 4, 5, 6 and 7 respectively. The % calcein leakage was 20, 30, 65, 65 and 65% respectively. A standard (unlabeled) sample of α-toxin was run in lane 1.

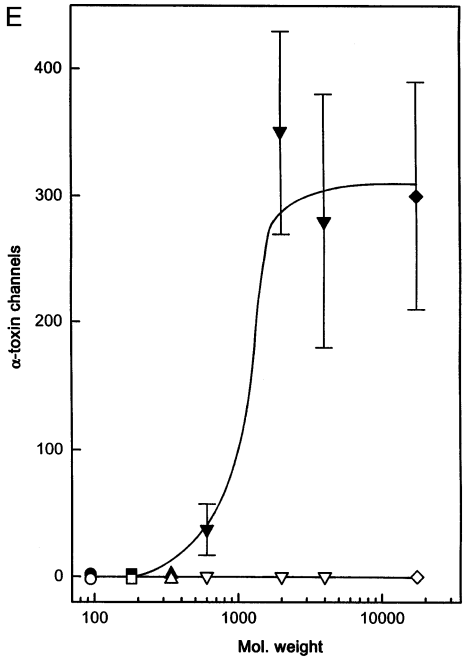
PEG-induced channels relative to nonPEG induced channels (*not shown*). When PEG-induced channels are exposed to pH 4.5, there is an initial increase of current (as with nonPEG pores: Korchev et al., 1995*a*), followed by a rapid decline (Fig. 3*A*). The residual current remains low if the pH is returned to 7.4, and now shows voltage sensitivity approaching that of nonPEG-treated bilayers (crossed circles in Fig. 3*B*).

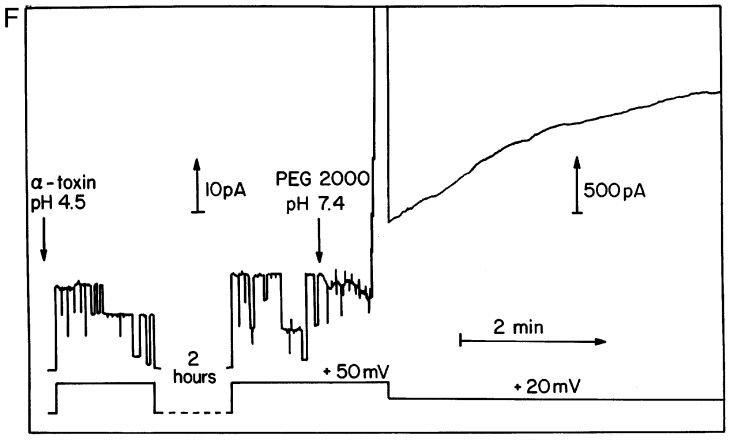

A
+ PEG4000 −
pH 4.5
I/nA
1.6
1.2
0.8
0.4
0
4 min
+30mV
+60mV
+30mV
YK 53


B
I / I_max
1.0
0.8
0.6
0.4
0.2
-200 -150 -100 -50 0 50 100 150 200
V / mV
YK 68


C
α-toxin + PEG 4000 −
I/pA
20
10
0
+60mV
0mV
+60 mV
+100mV
0 2 4 6
Time / min
YK 54


D
Channels
400
300
200
100
0
0.001 0.01 0.1 1 10
α-Toxin (μg/ml)
YK 62


E
α-toxin channels
400
300
200
100
0
100 1000 10000
Mol. weight


F
α-toxin
pH 4.5
10pA
PEG 2000
pH 7.4
500pA
2 min
2
hours
+50mV
+20mV

In other words, PEG-induced channels are unstable at low pH [induction of pores at pH 4.5 by PEG also gives a reduced current relative to that induced at pH 7.4 (*not shown*)]; this may be contrasted with the stability of channels induced in the absence of PEG: loss of current at pH 4.5 is fully reversible simply by switching voltage to 0 (as is loss of current by divalent cations: Menestrina, 1986). By exposing planar bilayers to PEG after treatment with extremely low amounts of α-toxin—that on its own induces no channels—''single-channel'' events may be discerned (Fig. 3*C*): at +60 mV, pores are mainly ''open,'' whereas at +100 mV, channels are mainly ''closed.'' The conductance of a single channel is around 75 pS, which is close to the value of 82 pS found for nonPEG-induced channels (Korchev et al., 1995*a*). The magnitude of the effect of PEG in inducing α-toxin channels is indicated in Fig. 3*D:* 10 ng/ml of α-toxin in the presence of PEG induced the same number of channels (i.e., the same current) as 10 μg/ml α-toxin without PEG, i.e., a 1,000-fold stimulation. When nonelectrolytes of different molecular weight were compared, it was found that maximal stimulation is achieved with PEG of 2,000 daltons (Fig. 3*E*), exactly as for liposomes (Fig. 1*B*); glycerol, glucose or sucrose are ineffective; nonelectrolytes at pH 4.5 induce no additional channels. Another similarity between the response of liposomes (made of POPC/cholesterol) and planar lipid bilayers (made of DPhPC) is indicated in Fig. 3*B:* if liposome pellets that have been treated with α-toxin and PEG are incorporated into planar bilayers, the ensuing current (filled diamonds) shows the sensitivity to voltage typical of PEG-treated DPhPC bilayers (filled circles), rather than that of nonPEG-treated DPhPC bilayers (open circles). In contrast, because PEGs induce an osmotic effect on cells, they protect rabbit erythrocytes against haemolysis caused by α-toxin (*see below*).

The stimulating effect of PEG on promoting channel formation is not restricted to α-toxin. Diphtheria toxin-treated planar lipid bilayers (Alder, Bashford & Pasternak, 1990) are sensitive, though planar lipid bilayers (or liposomes) treated with pneumolysin (Korchev, Bashford & Pasternak, 1992), melittin or triton ×100 (Alder et al., 1991) are not. Stimulation of channel formation by PEG is not confined to purely lipidic systems: planar bilayers composed of Lettré cell plasma membrane (Korchev et al., 1995*a*) show a large effect (Fig. 3*F*). In contrast neither Lettré cells ([^{3}H]-phosphocholine leakage: Bashford et al., 1986) nor rabbit or human erythrocytes (hemolysis) are affected by PEG added together with, before, or after α-toxin (*not shown*).

Estimation of Toxin Pore Size in Erythrocytes

α-toxin induced hemolysis proceeds by a colloid osmotic shock mechanism (Bhakdi et al., 1984). High molecular weight solutes protect red cells provided that they do not pass through the toxin-induced pores. Dextran 1500

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Fig. 3. Effect of PEG on α-toxin induced ion current across lipid bilayers. Planar lipid bilayers were made from DPhPC (panels *A, B, C, D* and *E*), POPC/cholesterol liposomes (panel *B*) or Lettré cell plasma membrane (panel *F*) and ion current measured as described in Materials and Methods. *Panel A:* Stimulation of ion current from many α-toxin channels by PEG. Bilayers composed of DPhPC were exposed to α-toxin (10 μg/ml) at pH 7.4 for 5 mins followed by addition of PEG 4,000 (final 20% w/v) to both chambers as indicated by the first arrow. When current had reached a plateau, after approx. 7 min, the solutions in each chamber were changed to ones without PEG 4,000. The pH in each chamber was changed to pH 4.5 at the point indicated by the second arrow. *Panel B:* Voltage-dependence of α-toxin induced currents (many channels). The relationship between current at different voltages as equilibrium is approached (*I*) and the initial (maximal) current at these voltages (I_{max}) is plotted against different voltages. Bilayers composed of DPhPC were exposed to α-toxin (30 μg/ml) at pH 7.4 (◯), to α-toxin (1 μg/ml) at pH 7.4 followed by PEG 4,000 (20% w/v) at pH 7.4 followed by its removal when current had reached a plateau as in panel *A* (●), or to α-toxin (1 μg/ml) at pH 7.4 followed by PEG 4,000, followed by its removal when current had reached a plateau, followed by exposure to pH 4.5 for 5 min (⊕). Liposomes composed of POPC/cholesterol (30 μg/ml; w/w) and exposed to α-toxin (2 μg/mol) and PEG 2,000 (10% w/v) as in Fig. 1 were pelleted after calcein leakage had reached a maximum and used to form a bilayer (◆). *Panel C:* Induction of single α-toxin channels by PEG. Bilayers composed of DPhPC were exposed to α-toxin (3 ng/ml) at pH 7.4 at the point indicated by the first arrow (no detectable current) and removed after 5 mins. PEG 4,000 (final 20% w/v) was added to both chambers at the point indicated by the second arrow and removed at the point indicated by the third arrow. *Panel D:* Dependence of PEG-induced currents on concentration of α-toxin. Bilayers composed of DPhPC were exposed to α-toxin at pH 7.4 at the concentration shown for 5 mins followed by its removal. PEG 4,000 (20% w/v final) was then added (filled symbols) followed by its removal when the numbers of channels (at low toxin concentration or in the absence of PEG) or the current (at higher toxin concentrations in the presence of PEG, where individual channels cannot be resolved) had reached a plateau. Open symbols refer to channels in the absence of PEG. All experiments were performed at +60 mV. The ordinate shows the number of channels computed from traces such as that of panel *C* (for low toxin concentration or in the absence of PEG) or from the current (for higher toxin concentration in the presence of PEG) on the assumption that at +60 mV each channel has a current of 10 pA (*see* panel *C*). Means of a number of experiments, with error bars (SEM) where applicable, are plotted. *Panel E:* Dependence of nonelectrolyte induced currents on molecular weight. Bilayers composed of DPhPC were exposed to α-toxin (10 μg/ml) at pH 7.4 for 5 mins followed by its removal. Nonelectrolytes (all at 20% w/v final) at pH 7.4 (filled symbols) or pH 4.5 (open symbols) were added, followed by their removal when a plateau had been reached. The nonelectrolytes used were glycerol (circles), glucose (squares), sucrose (upward triangles), PEGs of various molecular weight (downward triangles) or dextran (diamonds). All experiments were carried out at +60 mV and the number of channels (derived from the current in the case of high molecular weight nonelectrolytes at pH 7.4 as for panel *D*) recorded. Means of a number of experiments, with error bars (SEM) where applicable, are plotted. *Panel F:* Stimulation of ion current from single to many α-toxin channels by PEG. Bilayers formed from purified Lettré cell plasma membranes were exposed to α-toxin (10 μg/ml) in 80 mM KCl at pH 4.5 until a single channel current was observed. Excess toxin was removed; the channel remained stable at pH 4.5 for more than two hrs. The medium was then replaced by 80 mM KCl, pH 7.4 with PEG 2,000 (final 20% w/v). The time scale indicator applies to both sections of the record.

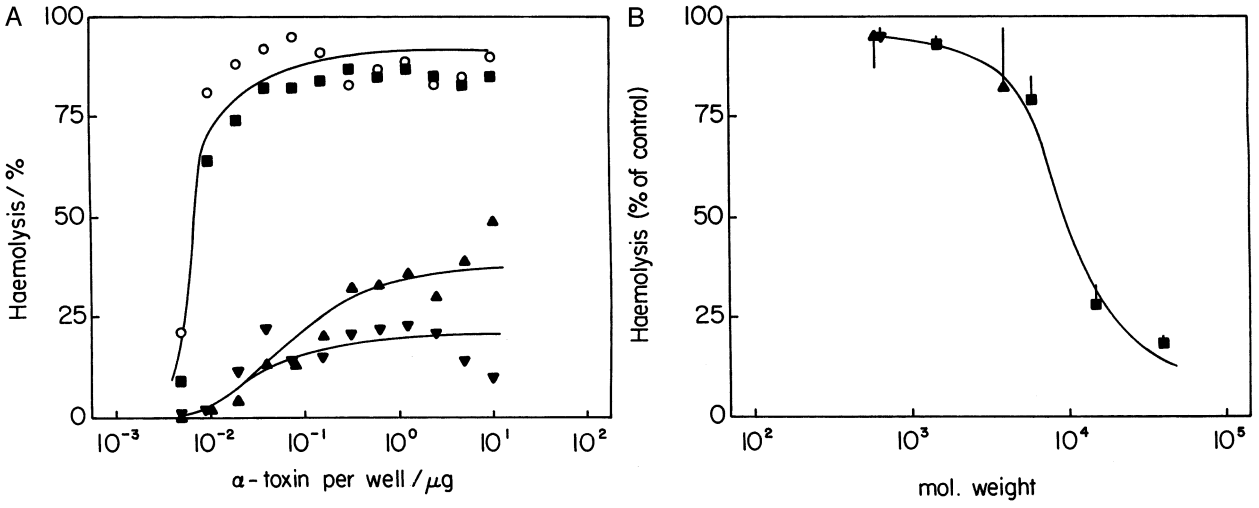

Fig. 4. Protection by nonelectrolytes of α-toxin induced hemolysis. Hemolysis of rabbit erythrocytes was assessed as described in Materials and Methods. *Panel A:* Dependence on α-toxin concentration. Hemolysis in the absence of (○) or presence of 10 mM dextran 1500 (■), 10 mM dextran 15,000 (▲) or 10 mM dextran 40,000 (▼) was measured. *Panel B:* Dependence on molecular weight of nonelectrolytes. Hemoloysis by α-toxin (10^{-3} to 10^{-1} μg/well) in the presence of 10 mM PEGs (▲), 10 mM stachyose (▼) or 10 mM dextrans (■) of molecular weights as indicated was measured as described in panel *A* and is expressed as the % of hemolysis by α-toxin in the absence of nonelectrolyte. Error bars (SEM) refer to hemolysis at different α-toxin concentrations and in 8–10 different experiments.

(and lower molecular weight species) has little effect on α-toxin induced hemolysis (Fig. 4*A*) whereas Dextran 15000 and especially Dextran 40000 exert considerable protection. Data from several experiments for solutes of differing size, are collected in Fig. 4*B.* Interpolation indicates that 50% protection is achieved with polymer of molecular weight around 8500. Note that the α-toxin/hemolysis dose-dependence is not significantly shifted by the presence of dextrans (Fig. 4*A*). A polymer of 8500 daltons has a radius of approx 1.7 nm (in the 50% hydrated form) which may be compared with an estimated pore size of 0.7 nm radius from measurements of conductance across lipid planar bilayers (Korchev et al., 1995*b*). The reason for this apparent discrepancy is not clear, but we note that at low toxin concentrations leakage of ions and intermediary metabolites, but not uptake of trypan blue or erythrosin, is observed; higher concentrations of α-toxin are required to elicit such leakage (Korchev et al., 1995*a*). At even higher concentration, lactate dehydrogenase begins to leak (Bashford et al., 1986).

DISCUSSION

To the best of our knowledge, this is the first direct demonstration that the hexamer of α-toxin is indeed the predominant form present within the hydrophobic milieu of membranes undergoing leakage (Fig. 2). Previous attempts with photoactivable probes (Thelestam et al., 1983; Harshman et al., 1989) labeled only monomer, though it was assumed that hexamer is the active species (Harshman et al., 1989). To observe extensive labeling of hexameter we used nonelectrolytes like PEG or dextran that increase (i) the amount of toxin associated with liposomes (Fig. 2) and (ii) the degree of toxin-induced leakage across liposomes (Fig. 1) or planar bilayers (Fig. 3). The fact that leakage across α-toxin-treated planar bilayers can be increased by as much as 1000-fold by the addition of PEG (Fig. 3) suggests that >99% of α-toxin is normally associated with membranes in a nonpore-forming configuration—perhaps on, but not in, the membrane—with infrequent conformation changes that cause part of the molecule to span the membrane. Somewhat similar conclusions have been reached with regard to melittin (Terwilliger, Weisman & Eisenberg, 1982) and colicin (Van der Groot et al., 1991). The action of PEG is then to catalyze the insertion process, presumably by dehydrating the area of contact between α-toxin and lipid bilayer, in the way that nonelectrolytes dehydrate molecular complexes such as hemoglobin (Colombo, Rau & Parsegian, 1992). The fact that erythrocytes treated with α-toxin are insensitive to PEG may indicate that in this instance a receptor protein fulfils a similar function. Another situation exists, in which a protein (annexin V) appears to form specific (Ca^{2+}) channels across lipid membranes (Demange et al., 1994) without inserting into the bilayer structure; addition of PEG in this system causes no additional channels to form (Y.E. Korchev and S. Liemann, *unpublished experiments*).

The second point that this study has revealed concerns ion conductance through α-toxin-induced channels. We have shown that channels induced in Lettré cells or across planar bilayers composed of Lettré cell plasma membrane are much more sensitive to ''closure'' by voltage, and show a higher degree of rectification, than channels across planar bilayers composed of

DPhPC (Figs. 5 and 7 of Korchev et al., 1995*a*). One might postulate that membrane proteins are responsible for this. However, the fact that PEG-induced channels across DPhPC bilayers have the same sensitivity to voltage and degree of rectification as Lettré cell membranes indicates that it is the α-toxin hexamer itself that appears to exist in several states, one of which is more voltage-sensitive and shows higher rectification than others. The difference between such states is likely to be elucidated through studies with site-directed mutants in the way that these have contributed to voltage sensitivity of—for example—the potassium channel (Schoppa et al., 1992).

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