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# Interactions of diphtheria toxin with lipid vesicles: determinants of ion channel formation

John W. Shiver and James J. Donovan *

*Department of Comparative and Experimental Pathology, University of Florida, Gainesville, FL (U.S.A.)*

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**Lipid vesicles have been utilized to study the interactions of diphtheria toxin (DT) with membranes. The assay for DT ion channel formation was fluorescence-detected membrane potential depolarization of vesicles in which valinomycin-induced potassium diffusion gradients had been generated. The following requirements for ion channel formation have been identified: (1) acid pH (< 5); (2) *trans*-negative membrane potentials (35-fold increase in channel-forming activity from −6 mV to −59 mV); and (3) negatively charged phospholipid headgroups (about 100-fold more activity using vesicles formed from asolectin compared to soybean phosphatidylcholine). Concentration dependence plots of toxin activity showed a linear response with logarithmic slopes of nearly one for each lipid composition. These results show a close parallel to those obtained previously with planar lipid bilayers and thus provide guidelines for conditions which facilitate functional insertion of the toxin into vesicles.**

## Introduction

Diphtheria toxin (DT), a protein ($M_r$ 63000) secreted by certain strains of *Corynebacterium diphtheriae*, is a potent toxin for most eukaryotic cells [1–3]. DT has been shown previously to gain entry into cells via receptor-mediated endocytosis [1,2], and it has been hypothesized that the acidic environment of the endosome facilitates toxin access to the cytosol where it exhibits a lethal enzymatic activity which inhibits protein synthesis by ADP-ribosylation of elongation factor-2 (EF-2) [1–3]. The enzymatic activity of DT has been shown to reside in the 'A' fragment ($M_r$ 21000) and the membrane-interactive domain in the 'B' fragment ($M_r$ 40000) [3–6].

Ion channel formation in vitro by DT has been shown previously with planar lipid bilayers using both whole toxin [4,5], and a 'B' fragment derived from an immunologically cross-reacting mutant of DT, designated CRM45, which lacks the $M_r$ 17000 receptor-binding domain at the peptide carboxyl terminus [6]. DT forms channels in planar lipid membranes at low pH, and with an applied negative membrane potential (*trans*-side negative) [4–6]. These conditions are apparently required by numerous toxin-like molecules including botulinum toxin and colicins E1, Ia, Ib, A, and B [7–13]. DT channel formation also depends upon the

* Present address: Hepatitis/AIDS R&D, Abbott Laboratories, D-9ZR, AP-10 Abbott Park, IL 60064, U.S.A.

Abbreviations: DT, diphtheria toxin; DTn, the 'nicked' form of DT; DMG, dimethylglutaric acid; ANS, 8-anilino-1-naphthalenesulfonate; Mops, 4-morpholinepropanesulfonic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; EF-2, elongation factor-2; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; EDTA, ethylenediaminetetraacetic acid.

Correspondence (present address): J.W. Shiver, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, U.S.A.

lipid composition of the membranes, specifically requiring phospholipids with negatively charged headgroups, and especially phosphatidylinositols [5].

Previous results characterizing protein/membrane interactions of ion channels obtained with lipid vesicles have proved difficult to reconcile with results from planar membrane experiments. Colicin E1 channel formation demonstrates a very large potential dependence with planar bilayers, but only a small potential dependence with vesicles [8,14]. Similarly, Kagan and co-workers [6] found that a *trans*-membrane potential was not required for CRM45 'B'-induced $K^+$ efflux from large unilamellar vesicles formed from asolectin. The difference between the two systems has been ascribed variously as resulting from differences in experimental technique or from the differences in the behavior of curved versus planar membranes [14]. Planar lipid bilayer measurements of channel formation are performed under conditions of voltage clamp. Thus, any changes in the observed variable, the membrane conductance, can unambiguously be attributed to the formation of channels. In vesicle experiments the membrane potential changes during the course of an experiment because the first channel should be sufficient to depolarize the vesicle [8,14], and subsequent channels either do not form (because of the depolarization) or have a lesser effect. Also, it is difficult, if possible, to directly measure current flow across a vesicle membrane. Instead, the efflux of an entrapped solute from within the vesicular compartment or depolarization of the vesicle are measured [6,8,9,14,15,17]. These differences limit the variety of conditions under which vesicle and bilayer assays may be compared and raise the question as to whether the toxin inserts into vesicles in the same manner as in planar membranes and, thus, whether the results obtained with the two assays are comparable.

Vesicles offer the possibility of numerous experiments impractical with planar bilayers such as peptide mapping with proteases or antibodies, and solute accessibility or marker efflux from within the vesicular compartment. One of the authors has already utilized vesicles to demonstrate that EF-2 entrapped within vesicles is accessible for ADP-ribosylation by DT [17]. In addition, the importance of diphtheria toxin in the construction of immunotoxins, potential 'magic bullets' for cancer therapy [18], requires that the functional activities of the toxin be well-characterized. In this report, the approach has been to study the formation of ionic channels in lipid vesicles by observing the collapse of an imposed membrane potential. The results indicate that the factors which influence channel formation rates in planar membranes (potential, pH, the presence of phosphoinositides) similarly modulate channel formation in lipid vesicles.

## Materials and Methods

*Protein.* Diphtheria toxin was obtained from Connaught Laboratories (Willowdale, OR) and purified by DE-52 chromatography as described previously [19]. The resulting protein solution contains two forms of DT: the major fraction of toxin is a single polypeptide chain with two disulfide bridges, and a smaller amount of the toxin is 'nicked', in which the peptide is cleaved into the two fragments, 'A' and 'B', which are still joined by a disulfide bond [19]. It has been shown that 'nicking' increases the potency of the toxin [19], increases conductance by an order of magnitude relative to 'un-nicked' toxin as measured with planar bilayers [4], and may be essential for in vivo activity [20]. Intact toxin may be converted to the 'nicked' from by gentle treatment with trypsin [21]. This form of the toxin, designated as DTn, was used in all the experiments described in this text.

*Vesicle formation and assays.* Large, mostly unilamellar vesicles (LUV) were prepared from asolectin, purified by the method of Kagawa and Racker [22], or soybean phosphatidylcholine (PC), either alone or with 20% soybean phosphatidylinositol (PI) added (Avanti Polar Lipids, Birmingham, AL). 5 mg of lipid, solubilized in chloroform and stored at $-20\,^{\circ}C$, was dried to a thin film under a stream of nitrogen and placed under vacuum for several hours. The lipid was then suspended in 0.25 ml of 0.1 M KCl, 10 mM DMG, 1 mM $CaCl_2$ at pH 5.0, sonicated to clarity, and subjected to two freeze-thaw cycles in a dry ice/ethanol bath. For the membrane potential depolarization experiments, vesicles were di-

luted 125-fold to 0.16 mg/ml in 0.1 M choline chloride, 10 mM DMG, 1 mM $CaCl_2$ at the desired pH. For neutral pH solutions, 10 mM Mops was used rather than DMG. *Trans*-negative potassium diffusion potentials (−118 mV for this solution) were established by addition of valinomycin (15 nM) by a 1:200 dilution from a methanolic stock solution [15]. For membrane potential dependence experiments, the potassium-containing vesicles were diluted into solutions of equal ionic strength but different concentrations of KCl and choline chloride. The resulting chemical potentials were calculated using the Nernst equation

$$\Delta E = -RT/F \ln \frac{[K^+]_{in}}{[K^+]_{out}}$$

where $\Delta E$ is the potential difference across the membrane in the absence of any net ionic current; $[K^+]_{in}$ and $[K^+]_{out}$ are the potassium ion concentrations inside and outside the vesicles, respectively; $T$ was 298 K and $R$ and $F$ have their standard meanings. *Trans*-positive potentials were established by formation of vesicles in 0.1 M choline chloride, 0.001 M KCl solution and dilution of the vesicles into 0.1 M KCl (+118 mV). The membrane-potential sensitive dye, 8-anilino-1-naphthalenesulfonate (ANS, Eastman), which has been shown to be an indicator of the magnitude of the diffusion potential [15], was used for fluorescence detection of DTn-mediated membrane depolarization by addition of the dye to 10 μM from a 10 mM methanolic stock solution. Fluorescence was measured by an Aminco-Bowman spectrophotofluorometer using excitation and emission wavelengths of 380 nm (4 nm bandpass) and 480 nm (10 nm bandpass), respectively. Vesicles (0.25 ml) were maintained at 25°C in a stirred cuvette. Channel formation was quantified as the initial change in fluorescence intensity per unit time after addition of toxin to vesicles. The initial rates were expressed as a percentage of the largest value measured for a given set of experiments.

## Results

*pH dependence of DTn membrane potential depolarization*

Fig. 1 shows the effect of pH on the ability of

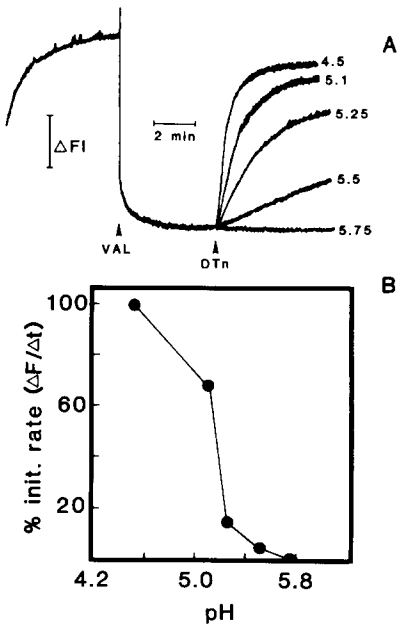


Fig. 1. pH dependence of DTn-mediated depolarization of asolectin vesicles. DTn (0.5 μg) was added to vesicles (40 μg) with an imposed membrane potential of −118 mV (A). Each trace following DTn addition represents a different experiment at the indicated pH. ΔFl = 10%. (B) DTn-mediated vesicle depolarization as a function of pH. The rates of channel formation were normalized to the rate observed at pH 4.5.

DTn to depolarize asolectin vesicles with imposed *trans*-negative membrane potentials of −118 mV. The decrease in ANS fluorescence that occurred after valinomycin addition to the vesicle suspension indicated the presence of a $K^+$-diffusion potential. The depolarization was seen as an increase in fluorescence that occurred after addition of toxin. DTn showed no activity at pH > 5.75. As the pH was lowered below this value, DTn activity increased, especially under pH 5.25. Optimal activity occurred at pH < 5.0 although a maximum value was not obtained.

Sandvig and Olsnes [20] have shown that exposure to acidic pH can inactivate DTn's cytotoxic activity. When toxin (0.5 μg) was added to diluted asolectin vesicles (0.16 mg/ml) in 0.1 M choline chloride solution at pH 5.0 at least 5 min before a $K^+$ diffusion potential was established, subsequent valinomycin addition resulted in formation of a membrane potential with a magnitude equal to that established in the absence of DTn (result not shown). This experiment implies that at

low pH either DTn does not insert into the membrane or, if inserted, does not form an open channel unless a membrane potential also exists and that while in one of these two states acidic pH can inactivate the toxin. This experiment appears to contrast with a previous planar bilayer membrane binding experiment [4] with DT, which did not result in a pH-mediated loss in toxin activity. However, in the planar bilayer experiments, a *trans*-positive potential of +50 mV was maintained during binding rather than 0 mV as was used here.

*Potential dependence of DTn channel-forming activity*

DTn was added to asolectin vesicles at pH 5.0 under conditions which varied the applied potassium diffusion potential as described in Methods. Fig. 2(A–D) shows DTn channel activity with vesicle potentials of −59, −41, −23.5, and −5.6 mV, respectively. The results presented in Fig. 2 clearly show that the initial rate of depolarization after toxin addition decreased as the applied potential was decreased. The initial rate of depolarization at −59 mV (A) was approx. 35-fold greater than at −6 mV (D), and no change in fluorescence was observed at 0 mV. A plot of these and additional data is depicted in Fig. 3 which shows that the rate of channel formation increased exponentially from 0 to −59 mV. Only a small increase in activity was observed between −59 and −118 mV. This may represent the limit of resolution of this technique under the given conditions rather than an actual plateau of initial rates. The change in fluorescence intensity of ANS after addition of valinomycin to vesicles is a linear function of applied potential (see Fig. 2 and [15]). In order to plot the potential dependence of DTn channel activity (Fig. 3), each measurement of initial slope induced by toxin addition to vesicles at a given potential was multiplied by the ratio of valinomycin-mediated fluorescence change at −118 mV to the valinomycin-mediated fluorescence change at the potential of interest. Some dependence of ANS fluorescence on pH was also observed over a broad pH range (>2 units) but was insignificant for the pH values used in Fig. 1. The pH inside and outside the vesicles was 5.0 for all the experiments shown in Figs. 2 and 3 so that no pH gradient would contribute to the chemical potential. These results demonstrate that the initial rate of channel formation was strongly depen-

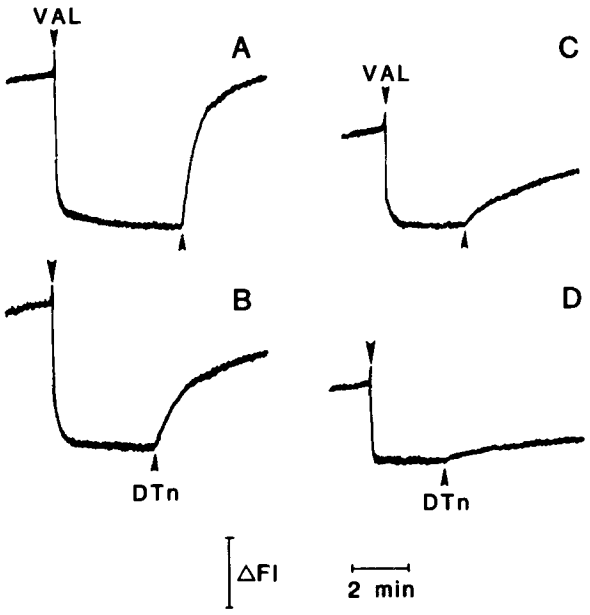


Fig. 2. Potential dependence of DTn-mediated depolarization of asolectin vesicles. DTn (0.5 μg) was added to vesicles (40 μg) at pH 5.0 after valinomycin addition to impose calculated potentials of −59 mV (A), −41 mV (B), −23.5 mV (C), and −5.6 mV (D). The corresponding ratios of $K^+$ inside and outside the vesicles were 100/10, 100/20, 100/40, and 100/80, respectively. $\Delta Fl = 10\%$.

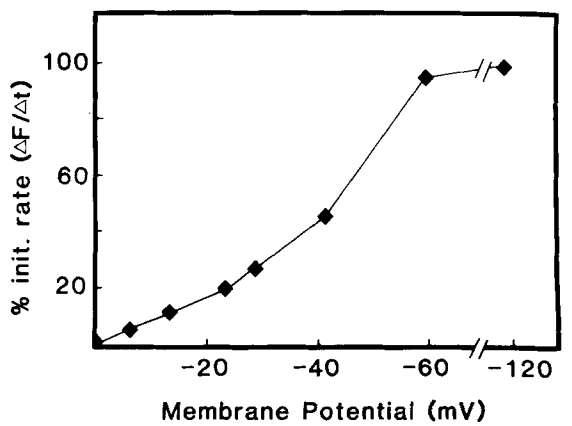


Fig. 3. Initial rate of DTn-mediated depolarization of asolectin vesicles as a function of applied potential. The rates of channel formation were normalized to the rate observed at −118 mV.

dent upon the imposition of a negative membrane potential.

The polarity of the membrane potential was found to be important for channel formation rates as well as the magnitude. Fig. 4 shows the activity of DTn with asolectin vesicles with a *trans*-positive applied diffusion potential of +118 mV (0.1 M chloride/0.001 M KCl inside, 0.1 M KCl outside the vesicles). At pH 5.0 and +118 mV the initial rate of depolarization was 13-fold smaller (Fig. 4A) than at pH 5.0 and −118 mV. This calculation probably underestimates the difference in channel formation rates at these two potentials because, as described above, our limit for resolving rates of channel formation at negative potentials occurred at −58 mV. However, this result demonstrates that channel formation does occur at *trans*-positive potentials but at much slower rates than with *trans*-negative potentials. At neutral pH no activity was observed with a *trans*-positive potential (Fig. 4B), indicating that at this applied voltage DTn channel activity was still pH dependent.

*Effect of lipid composition on DTn channel-forming activity*

Vesicles varying only in lipid composition were tested to determine whether DTn channel formation exhibited lipid specificity. Fig. 5 shows that DTn exhibited the greatest activity with vesicles composed of asolectin (A), intermediate activity with 20% PI/80% PC (B), and least activity with pure PC (C). Thus, DTn activity was enhanced when reacted with vesicles containing inositides,

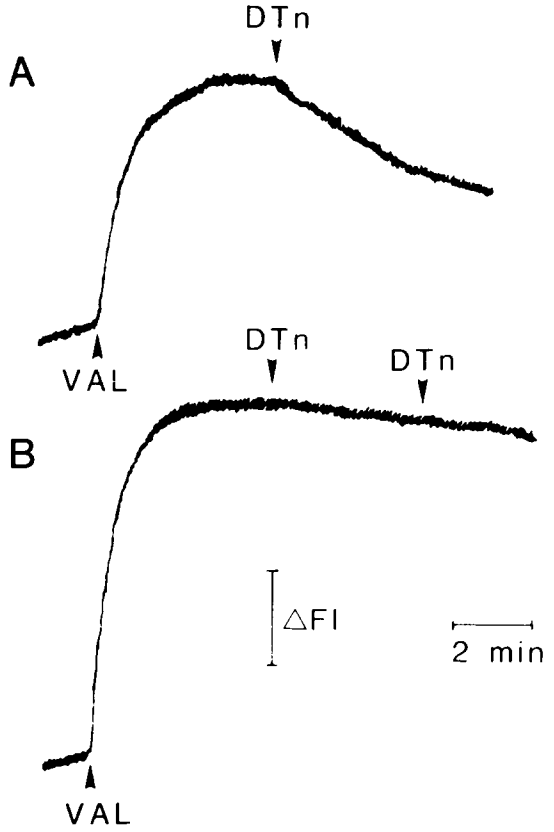


Fig. 4. The effect of DTn on vesicles with *trans*-positive diffusion potentials. Asolectin vesicles (40 μg) containing 0.1 M choline chloride, 0.001 KCl were diluted into 0.1 M KCl solution at pH 5.0 (A) and pH 7.0 (B). Valinomycin and DTn (0.5 μg) were added as indicated. ΔFl = 10%.

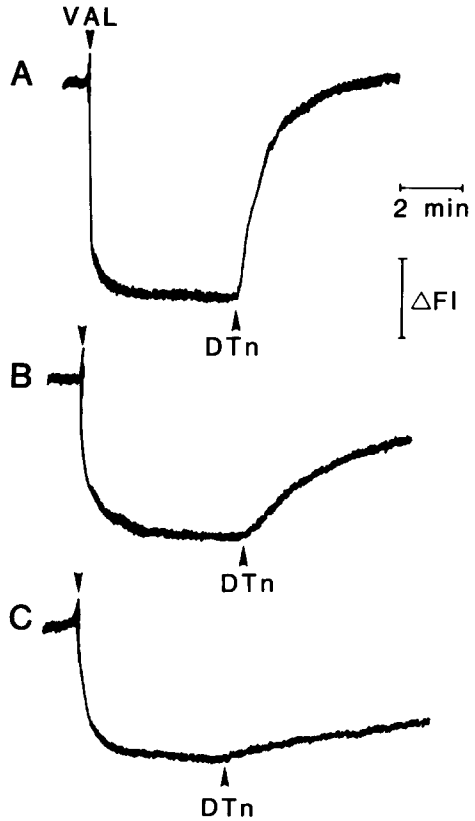


Fig. 5. The effect of lipid composition on DTn-mediated vesicle depolarization. DTn (0.5 μg) was added to vesicles (40 μg) composed of asolectin (A), 20% PI in PC (B), and PC (C) at pH 5.0 with applied membrane potentials of −118 mV. ΔFl = 10%.

as reported with planar bilayers [5]. Concentration dependence experiments show that DTn was able to completely depolarize membranes of all three lipid compositions when used in sufficient quantity (Fig. 6). There was approximately one order of magnitude difference in depolarization activity between each type of vesicle at a given DTn concentration. For complete depolarization of PC vesicles, the lipid to protein ratios were so small (about 4 : 1, w/w) that non-specific lipid–protein interactions could be responsible for membrane damage severe enough to dissipate a $K^+$ diffusion potential. However, the similarity of the plots obtained for each vesicle type shown in Fig. 6 suggests that this was not the case.

The concentration dependence data of Fig. 6 was used to calculate the molecularity of the DTn channel. The rate of channel formation nearly doubled when the amount of DTn added to a vesicle sample was doubled yielding linear concentration dependences with logarithmic slopes of 0.8 for asolectin pure soybean PC and 1.0 for 20% PI/80% PC vesicles (uncertainties = $\pm 0.1$, correlation coefficients = 0.99). This result implies that the DTn channel was composed of a single channel-forming species: either from monomeric DTn or from a water-soluble DTn multimer.

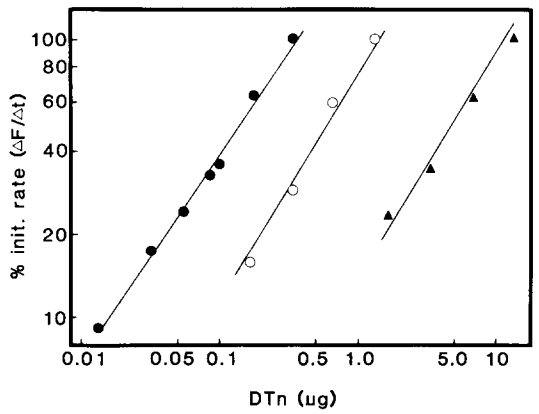


Fig. 6. Concentration dependences of DTn-mediated depolarization of vesicles of different lipid compositions. Varying amounts of DTn were added to vesicles formed from asolectin (●), 20% PI in PC (○), and PC (▲) at pH 5.0 with applied membrane potentials of −118 mV. The rates of channel formation were normalized to the rate observed for the largest addition of toxin for each lipid composition.

## Discussion

This work investigated in vitro conditions which were required for diphtheria toxin to insert into membranes and form ion channels in lipid vesicles. For each of the conditions examined, the rate of channel formation in vesicles depended upon the same conditions as the rate of channel formation observed in planar lipid bilayers [4–6]. Channels formed fastest at low pH, with *trans*-negative membrane potentials, with inositides in the membrane, and with increasing amounts of toxin present. The magnitudes of the effects were also similar with several exceptions. The potential dependence plot of channel formation in Fig. 3 shows that the initial rate of channel formation increased exponentially with potential up to −60 mV, but became insensitive to further changes in applied potential at larger voltages. As stated in Results, this is probably because depolarization occurred more rapidly at potentials greater than −60 mV than our measurements were capable of resolving. The most important factor influencing potential-dependent DTn channel formation in vesicles as described in this text (see also Refs. 6, 14) appears to be the inclusion of calcium during vesicle formation and in external bathing solutions. Calcium may promote formation of larger vesicles by fusion of SUV [25], which would then be better able to maintain a membrane potential, and enhanced voltage dependence when included in the vesicle bathing solution. It has been shown previously that protein insertion into SUV may be facilitated compared to LUV, ostensibly due to differences in lipid packing density [26]. Thus, a vesicle preparation containing a significant population of SUV may nonspecifically leak sufficient solute in the absence of a membrane potential after addition of toxin so that the response appears to be voltage independent. Whether these criteria explain the observations of DT- or colicin E1-mediated voltage independent efflux of solute from vesicles as described by other researchers [6,14] is uncertain. Vesicles were prepared by a substantially different technique by Kagan and co-workers [6] while Davidson et al. [14] included EDTA in their final vesicle formation solution. Also, it may be inappropriate to extend the results obtained for DT with vesicles as described in this

text to colicin E1 despite apparent similarities between their respective channel activities with planar lipid bilayers.

The lipid specificity of DTn channel formation depicted in Figs. 5 and 6 shows that the presence of phosphatidylinositol in the membrane increased the rate of channel formation by 10-fold compared to vesicles composed of neutral lipids. However, DTn-mediated depolarization activity was much greater with asolectin vesicles than those formed from PI/PC mixtures, a result also found with planar membranes [5]. Asolectin is a mixture of lipids including PE, PC, PI, and PS, with 20% net negatively charged headgroups, most of which is PI [5,24]. The natural membrane composition of asolectin may provide additional factors such as membrane fluidity, or lipids other than inositols which enhance DTn channel activity.

The only result described in this text in disagreement with planar bilayer data is the concentration dependence of DTn channel formation. The data in Fig. 6 suggest that channel formation is a unimolecular event but planar bilayer experiments found that the rate of channel formation depended on the square of toxin concentration, supporting a dimeric channel structure [4,6]. There are several possible causes for this difference. First, the limits of resolution for high values of initial slope, as discussed previously in the text, which were obtained when larger amounts of toxin was used: the highest rates may be underestimated, which would have the effect of removing the square dependence of the relationship between toxin concentration and channel formation. A second possibility, also described above, is that planar bilayers measure the rate of channel formation with subsequent channels following the first. In the vesicle experiments, because the first channel should be sufficient for depolarization what is actually measured is the rate at which the first channel forms in a vesicle. The square relationship for channel formation observed in planar membranes implies interaction or cooperativity between toxin molecules as they form channels. This may occur as an aggregate formation prior to insertion into the membrane, or as a cooperative effect between channels, i.e., that one channel in the membrane may aid in the formation of subsequent channels. If this were the case, it would explain the difference in concentration dependence experiments between vesicles and planar membranes: since the first channel is sufficient to depolarize the membrane, subsequent channels that would be promoted by the first would not have much effect.

The most relevant factor for differences between vesicles and planar bilayer experiments seems to be that the membrane potential is constant in one case, but changes with time in the other. The membrane events appear to be similar but the size of the internal compartments, and hence their abilities to maintain a constant potential, account for the differences between the systems.

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