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Ion-Conducting Channels Produced by Botulinum Toxin in Planar Lipid Membranes

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ABSTRACT: The interaction of botulinum neurotoxin (Botx) with planar lipid membranes was studied by measuring the ability of the toxin to form ion-conducting channels. Channel formation was pH dependent. At physiological pH, Botx formed no channels, whereas at pH 6.6, the toxin formed channels with a unit conductance of 12 pS in 0.1 M NaCl. The rate of channel formation increased with decreasing pH, reaching a maximum at pH 6.1, and then decreased at lower values of pH. The channels, once formed, were permanent entities in the membrane throughout the course of an experiment and fluctuated between an open and a closed state. The rate of channel formation depended upon the square of the toxin concentration, suggesting an aggregation step is involved in channel formation. The data were consistent with the hypothesis that Botx enters cells through endocytosis, followed by its release into the cytoplasm at low pH.

Botulinum toxin (Botx), an exotoxin produced by the bacterium *Clostridium botulinum*, is the etiological agent in botulism poisoning [for comprehensive reviews, see Simpson (1981) or Sugiyama (1980)]. Although there are seven distinct serotypes (designated A–G), they have the same general molecular properties. The neurotoxins are single-chain proteins with a molecular weight around 150 000. In most cases, the toxins produced in culture are purified in the "nicked" form, having been cleaved by protease(s) to constitute chains of M_r 50 000 (light chain) and M_r 100 000 (heavy chain). The chains are held together by a disulfide bond and noncovalent interactions, and there is evidence to suggest that nicking is necessary for the full expression of toxicity. While the nature of the receptor for Botx has not been defined, it may consist in part of a ganglioside.

It has been known for some time that the primary action of botulinum toxin is to inhibit the release of acetylcholine from the presynaptic membrane of the neuromuscular junction (Burgen et al., 1949). Unfortunately, little else is known about the molecular events that lead to intoxication. In the last few years, indirect evidence has suggested that the attack of Botx proceeds by several steps (Simpson, 1981). The first step is binding of the toxin to a receptor on the plasma membrane outer surface (Kitamura et al., 1980; Dolly et al., 1984). The second step seems to involve entry of the toxin into the cell, where it inhibits the release of acetylcholine through an unknown third step. The entry mechanism is as yet unclear, but an intriguing possibility was recently raised by Simpson (1984) when he showed that lysosomotropic amines, such as chloroquine, can protect the nerve from attack by Botx. This protection is similar to the protective action of amines from attack by other bacterial toxins, such as diphtheria toxin (Dorland et al., 1981; Leppla et al., 1980) and *Pseudomonas* A exotoxin (Fitzgerald et al., 1980). The similarity led Simpson to suggest that the mechanisms of entry of the toxins might be the same, i.e., that botulinum toxin may enter cells by a mechanism analogous to diphtheria toxin.

Diphtheria toxin enters mammalian cells by the process of receptor-mediated endocytosis (Middlebrook et al., 1978; Dorland et al., 1979). This involves the endocytosis of the receptor-bound toxin, followed by an escape from the endocytic compartment triggered by the low intravesicular pH. This escape, which arises from transport of diphtheria toxin across the membrane, occurs simultaneously with insertion of the toxin into the membrane and formation of an ion-conducting pore (Donovan et al., 1981, 1982; Zalman & Wisnieski, 1984). The actual transport of the toxic fragment of the molecule has been suggested to be through that pore (Boquet et al., 1976; Kagan et al., 1981). Were Botx to enter neurons by a similar mechanism, it would also be expected to form ion-conducting pores under conditions which prevail inside endocytic vesicles.

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Results by other investigators (Hoch et al., 1985; Boquet & Duflot, 1982; Borochov-Neori et al., 1983) indicate that clostridial toxin fragments or whole tetanus toxin can form ion channels in membranes. This study was begun to test the hypothesis that Botx could form ion-conducting channels in planar lipid bilayers and to characterize the conditions that are needed for channel formation. The experimental approach was to form planar membranes from pure phospholipids and to add Botx under various conditions of pH. Formation of ion-conducting channels by Botx at low pH would be suggestive that it is capable of inserting into the membranes of endocytic vesicles. It is possible that, by analogy with diphtheria toxin, insertion is followed by transport of Botx across the membrane.

Materials and Methods

Botulinum toxin, serotype C, the gift of Dr. Leonard Smith, had been purified by the method of Syuto and Kubo (1977). The preparation was found to be better than 95% pure and 60% nicked by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. An isoelectric focusing gel revealed three bands with p*I*'s of 5.9, 6.6, and 6.9 with densitometric ratios of approximately 40:40:20. Asolectin [partially purified soybean phospholipids of defined composition (Miller & Racker, 1976)] was prepared by the method of Kagawa and Racker (1971) from L-α-lecithin (Sigma) and stored as a $CHCl_3$ solution at −20 °C until use.

Planar lipid membranes were made by the apposition of two lipid monolayers as described (Montal, 1974; Donovan et al., 1981). Two adjacent compartments in a Teflon chamber were separated by a thin (12.5-μm) Teflon partition (Yellow Springs Instruments) in which a small (200-μm) aperture had been punched. The aperture was treated with a 1% solution of squalene (Sigma) in *n*-pentane. Then, buffer solution (0.1 M NaCl/5 mM PO_4) was introduced into the compartments such that they were filled to below the level of the aperture. A phospholipid monolayer was formed by spreading 10 μL of a 0.5% solution of phospholipid in pentane on the buffer in each compartment and waiting for the solvent to evaporate. Thereafter, buffer solution was injected under the monolayers to raise them over the aperture, thus forming a bilayer. Bilayer formation was continuously monitored by the increase in electrical capacitance between the two compartments. All bilayers were formed and studied at room temperature.

The compartment to which the toxin was added (the cis side) was defined as being at ground potential, and voltages were applied to the side of the membrane opposite to the toxin (the trans side). Positive current is the movement of positive ions from the toxin-free to the toxin-containing side of the membrane (trans to cis). Current was measured with Ag/AgCl electrodes and was converted to voltage with a Keithley 427 current amplifier. The signal was digitized and stored in an IBM microcomputer and also recorded on a stripchart recorder.

The effect of pH on the toxin-induced current was measured by first adding toxin to the compartment on one side of the membrane at pH 7.5 and stirring. A base-line current was established, and then 0.1 M citric acid was added to the toxin-containing side of the membrane to lower the pH. The amount of acid required to lower the pH to a given value had been determined previously by titrating the buffer with the citric acid solution in the absence of toxin. After 1 min, stirring was stopped while the current was monitored. The rate of increase of the conductance was then calculated by measuring the slope of the current trace as a function of time and dividing by the applied potential. The values of pH given below are

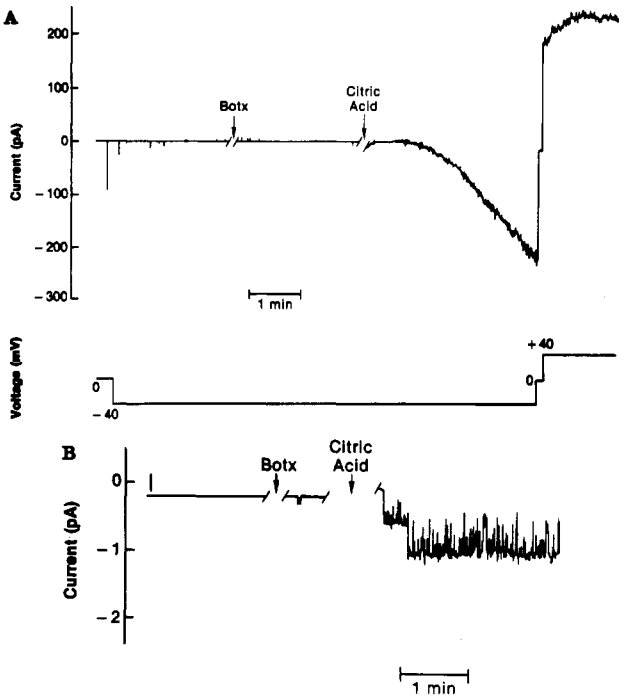

FIGURE 1: Formation of ionic conductances by Botx. Membranes were formed from asolectin, and Botx was added to one side as indicated. The pH of the chamber was then dropped to 6.6 by addition of 100 mM citric acid. (A) Botx concentration was 1.0 μg/mL. Applied potential is shown in the lower trace. (B) Botx concentration was 0.2 μg/mL and was added before the trace begins. The current scale is expanded relative to (A) to show the individual conductance steps. Applied potential was −40 mV throughout.

the pHs of the buffer in the toxin-containing side; the pH of the toxin-free side of the membrane was always 7.5. The single-channel conductance was calculated by measuring the size of the unit current jump and dividing by the applied potential.

Results

When Botx was added to the solution on one side of an asolectin membrane at pH 7.5, no observable change in the membrane conductance occurred. As the pH was lowered, the conductance increased after Botx addition, and did so in a series of discrete steps (Figure 1). Figure 1A shows the current through a membrane as a function of time both before and after the addition of Botx. At the beginning of the trace, with no potential applied across the membrane in symmetric NaCl solutions, the current through the membrane was zero. After a potential of −40 mV was applied, the current increased a small value (<0.5 pA) which was indistinguishable from zero on the scale shown. This small current, which arose from the ion current through the lipid matrix of the membrane, was stable with time and did not change after the addition of Botx serotype C to a concentration of 1.0 μg/mL. However, after the addition of citric acid sufficient to bring the pH of the chamber to 6.6, the current changed. At first, there was a transient deflection arising from the electrical disturbance caused by the addition itself. Then, following a lag time of less than 1 min, the current increased (became more negative with the applied negative potential) and continued to increase linearly for several minutes. When the pH was dropped to 6.6 in the absence of Botx, no conductance change occurred. Neither was there any change if the trans pH was lowered with Botx on the cis side. When the pH was lowered on both cis and trans sides (so that no pH gradient was present), the conductance increased, but at a slower rate.

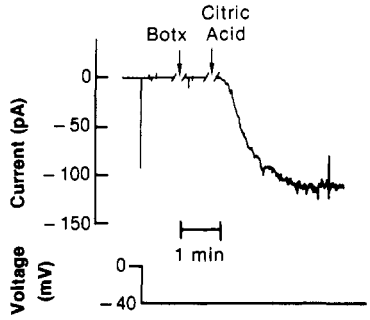

FIGURE 2: Saturation of Botx-induced conductance with time. The membrane was formed from asolectin in 0.1 M NaCl, pH 7.5. Botx was added to a concentration of 1.0 μg/mL, and the pH was dropped to 5.6 by the addition of 100 mM citric acid. Applied potential is shown in the lower trace.

This increase depended not only upon the presence of the toxin and the low pH but also upon the application of a negative membrane potential. When the potential was switched to zero and then to +40 mV, the current jumped immediately from −200 pA to zero and then to +200 pA. It then relaxed to a slightly higher value and remained there, no longer increasing with time. When the voltage was switched back to a negative value, the current returned to −200 pA; it then increased linearly as before (not shown).

Although the individual conductance steps are not visible at the resolution of Figure 1A, the increase in noise of the signal is indicative of the formation of ionic channels (Neher & Stevens, 1977). The individual conductance steps themselves are visible in Figure 1B, which shows the current at higher amplification through a different membrane at a lower toxin concentration, 0.2 μg/mL. At this low concentration, the rate of channel formation was slower, making it possible to distinguish the individual events. The toxin had been added and a 40-mV potential applied at the start of the trace. After the addition of citric acid, the current increased in discrete steps. Each step had a conductance of 12 pS. After the channels opened, they appeared to fluctuate between two states, one of high conductance and one of low conductance. As shown in Figure 1B, the channels spent most of the time in the high conductance state. Although visual inspection of Figure 1B suggests only two conductance states, others may also exist.

To quantify the pH dependence of the insertion of Botx into the membrane and resulting channel formation, the rate of increase of the conductance (dG/dT) was measured at different values of the pH. This approach yields a measure of the rate of channel formation, since each channel contributes equally to the conductance, which is symbolized by the letter g. The parameter used for making a comparison between different values of the pH was the maximal rate of increase of conductance. The maximal rate was chosen for two reasons. First, at moderate values of acidity (pH ≥6.5), the current increased constantly in the presence of toxin when a negative potential was applied to the membrane. This continued increase apparently occurred because the channels formed were permanent entities in the membrane on the time scale of these experiments (a few hours); that is, channels never "popped out" of the membrane or completely disappeared. Since channels continually formed, they did not always reach an equilibrium with a defined number of channels, and only a slope could be measured. This slope was reproducible from membrane to membrane. Second, at the lowest values of pH studied, the behavior changed; the current increased very rapidly for a short (<2 min) time (Figure 2) and then saturated (Figure 2). This was in contrast to the behavior evident in Figure 1, where the conductance continued to increase linearly. The plateau value of the conductance varied from membrane to membrane, whereas the maximal rate of conductance increase was found to be reproducible. The latter, therefore, was taken as the best available measure of the channel formation process.

Table I: Effect of pH on the Maximal Rate of Increase of Conductance Induced by Botx at −40 mV[a]

pH	dg/dt (pS/min)
7.5	0 (2)
7.1	15 ± 6 (2)
6.6	120 ± 36 (4)
6.1	1540 ± 790 (3)
5.6	220 (1)

[a] Results are mean ± SEM. The numbers in parentheses are the number of replicates.

The results of the experiments at different values of pH are shown in Table I. At pH 7.5, no increase in conductance was measurable within the time of these experiments. As the pH was dropped to 7.0 or less, the rate of channel formation increased up to a maximum around pH 6. At this point, the biphasic nature of the conductance increase was evident, and the current plateaued as described previously. As the pH was dropped even lower, the maximal rate of conductance increase dropped. The maximal rate was reached very quickly under these conditions, and a plateau was attained within 1 min of the addition of acid. Also, the magnitude of the conductance increase was reduced at pHs below 6.0, since the maximum occurred before the conductance had much time to change.

The plateau in the current occurred when the pH was less than or equal to the isoelectric point of the toxin. Generally, a protein becomes less soluble in water at its isoelectric point and may precipitate. Thus, the saturation might have resulted from a simple precipitation which decreased the concentration of free toxin. To test whether precipitation could account for the effect, Botx was exposed to pH 5.5 for 15 min and centrifuged (3000g, 10 min), supernatants were removed and neutralized, and the Botx concentration was checked by using a mouse toxicity assay (Schantz & Kautter, 1978). The supernatant of the acid-treated toxin was equally toxic as Botx which had not been exposed to a low pH (results not shown), indicating that the toxin had not precipitated. Saturation of the current at pH 6 and below must therefore arise from some other process.

At constant pH, the rate of conductance increase was dose dependent (Figure 3). As the concentration of toxin was raised, the conductance increased more rapidly. When plotted on a log–log scale, the data could be fit to a straight line with a slope of 2.2 (Figure 3). The slope of ~2 in the log plot indicates that the rate of conductance increase depended upon the square of the toxin concentration. If individual toxin molecules formed channels independently, the channel formation, and hence the conductance, would be expected to increase linearly with concentration. The square relationship suggests that an aggregation step is involved in channel formation.

Discussion

Serotype C of botulinum toxin does form ion-conducting channels in pure lipid membranes in analogy with the actions of diphtheria toxin. The channels formed by Botx in planar lipid membranes are very similar to those formed by diphtheria toxin. Both channels form only at low pH and with a negative membrane potential applied to the membrane. The macroscopic conductances depend upon voltages in similar ways, and

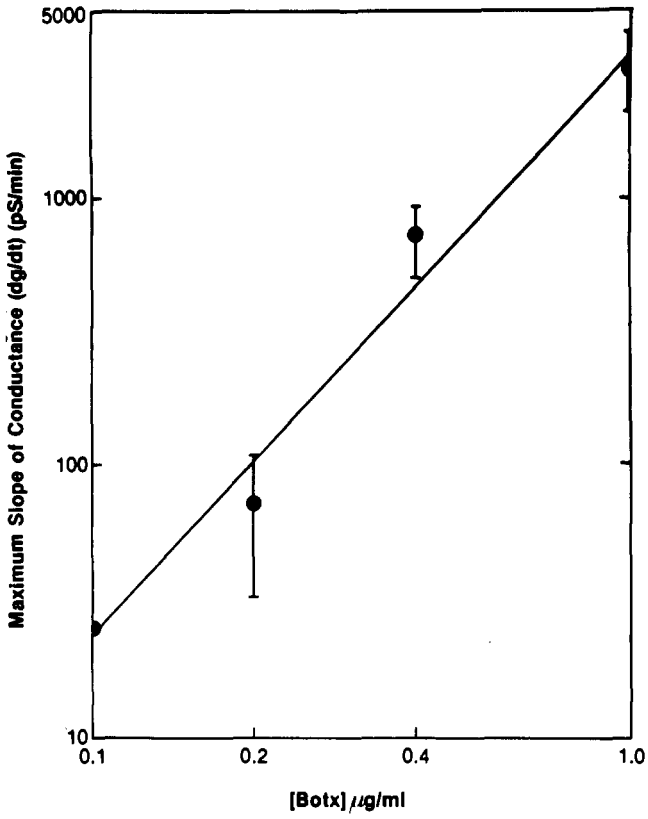

FIGURE 3: Dependence of conductance on Botx concentration. Membranes were formed under the conditions in Figure 1 except that the Botx concentration was varied. The maximal slope of the conductance vs. time (dg/dt) was measured and plotted as a function of Botx concentration. The line is a simple linear regression of the points (all points weighted equally) and has a slope of $m = 2.2$. The number of replicates for each point is four for 1000 ng/mL, three for 400 ng/mL, two for 200 ng/mL, and one for 100 ng/mL. The error bars mark the SEM of the points.

the unit conductances of the individual channels are similar (7 pS for diphtheria toxin, 12 pS for Botx).

One important difference between the insertion of Botx or diphtheria toxin into membranes is the pH dependence of the processes. Diphtheria toxin shows very little channel activity at pH values greater than 5.0, whereas Botx exhibits a great deal of activity at pH 6.6. The rate of channel formation for diphtheria toxin increases monotonically with decreasing pH down to pH 4.0. For Botx, on the other hand, an optimum is reached at around pH 6, and channel formation decreases at lower pH. At values of pH optimal for diphtheria toxin, Botx shows very little activity. This difference may be a result of the difference in the isoelectric points of the two molecules. The isoelectric point of diphtheria toxin is around 5.1 (Collier, 1975), which is much lower than that of Botx, 6.5. This suggests, then, that the critical pH for channel formation for bacterial toxins is the isoelectric point, or slightly below. The possible importance of the isoelectric point may partially result from the charge on the membranes used. When a protein is bathed in a medium below its isoelectric point, it has a net positive charge. Since the charge on the membranes used in this study was negative, it would attract a positively charged protein.

These results are similar to those of Hoch et al. (1985), who used fragments of Botx. They observed channels of 20 pS in 0.2 M NaCl at pH 4.0 induced by the heavy but not the light chain of Botx. Interestingly, they saw no activity for whole Botx, in contrast to our results. One possible explanation for this apparent discrepancy is the serotype used; Hoch et al. employed type B, whereas we used type C. Another explanation for the difference may be the conditions under which the experiments were performed. Hoch et al. worked at much lower values of pH than we did, 4.0 and 4.6. Since we observe an optimum for channel formation at pH 6, we would expect that channel formation would be greatly depressed at pH 4.6 for the whole toxin.

These results are also similar to those obtained by investigators studying tetanus toxin (Hoch et al., 1985; Borochov-Neori et al., 1983). The effect of pH is not clear in these studies, however. Hoch et al. find that a low pH is essential for channel formation, whereas Borochov-Neori et al. report no effect of pH. Rather, the latter find that gangliosides must be present in the membrane for tetanus toxin to form channels. Because of this controversy, it is difficult to relate the pH dependence of Botx channel formation to tetanus toxin channel formation.

The maximal rate of conductance increase in Figure 2 was taken as a measure of the effect of pH on the insertion of the toxin into the membrane. This method was adopted because two regions of behavior seem to exist, one in which the current increases continuously with time and another at lower pH in which the current plateaus after a few minutes. The maximal rate of conductance increase was one parameter that could be extracted from the data in both regions, and the only one that was reproducible.

This behavior could be explained by assuming that the toxin may undergo two reactions, one of which is binding of the toxin to the membrane, followed by channel formation, and the other which involves an unknown conformational change that prevents membrane binding. At lower pH, the conformational change would occur more rapidly, depleting the chamber of active toxin. Because the two reactions are competing, some of the toxin is never available for membrane insertion and channel formation, and at long times, all of the toxin has been converted to the inactive state. If this model is correct, then the maximal rate of conductance increase is an underestimate of the actual effect of pH on channel formation, since the inactivating reaction has decreased the concentration of active toxin. It is, however, the only measure available.

The data shown in Figure 3 suggest an aggregation step in channel formation. These data are insufficient to indicate whether the channel itself arises from toxin monomers, dimers, or aggregates, since the parameter measured is the rate of formation and not the equilibrium number of channels. All that can be said with certainty is that the rate-limiting step in channel formation involves aggregates of the toxin, possibly dimers.

These results are consistent with the model that Botx enters nerve terminals through receptor-mediated endocytosis. Other evidence for such a model includes the results of Simpson (1984) that lysosomotropic agents can protect from Botx intoxication and the results of Dolly et al. (1984) that Botx binds specifically to nerve terminals and is internalized with time. Dolly et al. also found that metabolic inhibitors decrease the internalization of Botx, probably by inhibiting endocytosis. In light of the data reported in this paper, such inhibitors may also have a second effect, that of diminishing the pH gradient across the endocytic vesicles by inhibiting the active transport of protons into the vesicles. This too would be expected to inhibit the transport of the toxin and its entry into the cytoplasm.

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Anomalous Driving Force for Renal Brush Border H^+/OH^- Transport Characterized by Using 6-Carboxyfluorescein†

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ABSTRACT: The pH, ΔpH, and membrane potential dependences of H^+/OH^- permeability in renal brush border membrane vesicles (BBMV) were studied by using the entrapped pH indicator 6-carboxyfluorescein (6CF). Quantitative H^+/OH^- fluxes (J_H) were obtained from a calibration of the fluorescence response of 6CF to intravesicular pH using vesicles prepared with varying intravesicular and solution pHs. Intravesicular buffer capacity, determined by titration of lysed vesicles, increased monotonically from 140 to 260 mequiv/L in the pH range 5–8. J_H was measured by subjecting voltage-clamped BBMV (K^+/valinomycin) to preformed pH gradients over the pH range 5–8 and measuring the rate of change of intravesicular pH. For small preformed pH gradients (0.4 pH unit) J_H [6 nequiv s^{-1} (mg of protein)$^{-1}$] was nearly independent of pH (5–8), predicting a highly pH dependent H^+ permeability coefficient. J_H increased in a curvilinear manner from 6 to 104 nequiv s^{-1} (mg of protein)$^{-1}$ as ΔpH increased from 0.4 to 2.5. J_H increased linearly [1.6–7.3 nequiv s^{-1} (mg of protein)$^{-1}$] with induced K^+ diffusion potentials (21–83 mV) in the absence of a pH gradient. These findings cannot be explained by simple diffusion of H^+ or OH^- or by mobile carrier models. Two mechanisms are proposed, including a lipid diffusion mechanism, facilitated by binding of H^+/OH^- to fixed sites in the membrane, and a linear H_2O strand model, where dissociation of H_2O in the membrane fixes H^+ and OH^- concentrations in strands, which can result in net H^+/OH^- transport.

The passive transport of H^+/OH^- across biological membranes and phospholipid bilayers has been the subject of considerable interest. Passive H^+/OH^- transport is important biologically because it causes the dissipation of pH gradients required for normal cell function; it is of mechanistic interest because H^+ behaves anomalously when compared to the passive transport of other monovalent cations across membranes. The H^+ permeability of biological membranes and phospholipid bilayers is many orders of magnitude larger than would be predicted from the permeabilities of other monovalent cations. In addition, H^+ conductances measured in pure lipid bilayers do not vary linearly with applied voltage, suggesting that the mechanism for passive H^+/OH^- transport across membranes is not a simple diffusional process (Cafiso & Hubbell, 1983; Deamer & Barchfield, 1984; Gutknecht, 1984; Nichols & Deamer, 1980).

Fluorescence methods have been used to study H^+/OH^- fluxes in a wide variety of membranes. Many of these studies have been based on the partitioning of the fluorescent dye

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