CHARACTERIZATION OF THE CHANNEL PROPERTIES OF TETANUS TOXIN IN PLANAR LIPID BILAYERS

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ABSTRACT A detailed characterization of the properties of the channel formed by tetanus toxin in planar lipid bilayers is presented. Channel formation proceeds at neutral pH. However, an acidic pH is required to detect the presence of channels in the membrane rapidly and effectively. Acid pH markedly lowers the single-channel conductance, for phosphatidylserine at 0.5 M KCl γ = 89 pS at pH 7.0 while at pH 4.8, γ = 30 pS. The toxin channel is cation selective without significant selectivity between potassium and sodium ($\gamma[K^+]/\gamma[Na^+] \geq 1.35$). In all the lipids studied γ is larger at positive than at negative voltages. The toxin channel is voltage dependent both at neutral and acidic pH: for phosphatidylserine membranes, the probability of the channel being open is much greater at positive than at negative voltage. In different phospholipids the channel exhibits different voltage dependence. In phosphatidylserine membranes the channel is inactivated at negative voltages, whereas in diphytanoylphosphatidylcholine membranes channels are more active at negative voltages than at positive. The presence of acidic phospholipids in the bilayers increases both the single-channel conductance as well as the probability of the channel being open at positive voltage. A subconductance state is readily identifiable in the single-channel recordings. Accordingly, single-channel conductance histograms are best fitted with a sum of 3 Gaussian distributions corresponding to the closed state, the open subconductance state and the full open state. Channel activity occurs in bursts of openings separated by long closings. Probability density analysis of the open dwell times of the toxin channel indicate the existence of a single open state with a lifetime $\geq$1 ms in all lipids studied. Analysis of intra-bursts closing lifetimes reveals the existence of two components; the slow component is of the order of 1 ms, the fast one is $\leq$0.5 ms.

The channel activity induced by tetanus toxin in lipid bilayers suggests a mechanism for its neurotoxicity: a voltage dependent, cation selective channel inserted in the postsynaptic membrane would lead to continuous depolarization and, therefore, persistent activation of the postsynaptic cell.

INTRODUCTION

Tetanus toxin (TeTx) is a neurotoxin produced by the bacterium *Clostridium tetani* and is the agent responsible for the spasticity and convulsions characteristic of human tetanus. TeTx is a water soluble protein with a molecular weight, M_r, of 150,000. The protein consists of two chains linked by a disulfide bond; a light chain with an apparent M_r of 50,000 and a heavy chain with an M_r of ~100,000. Mild proteolysis with papain produces two fragments; the amino terminus known as the B-fragment with the apparent M_r of 95,000, as well as the C-fragment which is the carboxy terminus of the protein (M_r 50,000) (Helting and Zwisler, 1977; Neubauer and Helting, 1981). Purified heavy and light chains are by themselves nontoxic. Likewise, purified fragment C is completely nontoxic whereas fragment B retains residual toxicity. Recently, overlapping clones encoding the entire gene of the toxin were isolated and the full amino acid sequence deduced from the nucleotide sequence (Eisel et al., 1986; Fairweather and Lyness, 1986).

Although it is widely accepted that the central nervous system is the target for TeTx (for reviews see: Bizzini, 1976; Mellanby and Green, 1981; Simpson, 1986), its mode of action at the molecular level remains elusive. A recent approach to investigate the mode of action of TeTx involves the study of its interaction with model lipid membranes. It was reported that TeTx forms transmembrane ion channels in lipid bilayers (Borochov-Neori et al., 1984). It was also demonstrated that acidic pH increases the insertion of this protein into lipid membranes (Boquet and Duflot, 1982; Hoch et al., 1985). This result has suggested analogies with the mechanism of internalization of other bacterial toxins such as diphtheria and botulinum toxins (Donovan et al., 1981; Donovan et al., 1982, 1985;

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Donovan and Middlebrook, 1986; Kagan et al., 1981; Hoch et al., 1985; Simpson, 1986).

Here, we present a detailed characterization of the TeTx channel, its mechanism of insertion into the lipid membranes, the modifications induced by pH as well as by lipid membrane composition, the regulation of the channel by the transmembrane voltage and an analysis of the conduction and gating properties of the TeTx channel. The results lead us to suggest a specific hypothesis for the neurotoxic action of TeTx, the essence of which is that the activity of the TeTx channel at postsynaptic membranes could account for the continuous depolarization and, therefore, persistent activation of the postsynaptic membrane. A preliminary account of this research was presented elsewhere (Gambale and Montal, 1987).

MATERIALS AND METHODS

Tetanus toxin, tetanus toxin fragment B, and tetanus toxin fragment C were all obtained from Calbiochem (La Jolla, CA). Purified TeTx samples were kind gifts of Dr. W. G. Habig (National Institutes of Health, Bethesda, MD) and Dr. B. Bizzini (Pasteur Institute, Paris, France). Toxins from the NIH and the Pasteur Institute appear on sodium dodecylsulphate polyacrilamide gel electrophoresis, under reducing conditions, as three bands corresponding to heavy chain, light chain, and unreduced toxin. Commercial toxins, instead, exhibited several bands of intermediate molecular weight corresponding presumably to partial proteolytic products. The following lipids were used: 1–2 diphytanoyl-3-sn-phosphatidylcholine (diphyPC), phosphatidylinositol (PI) from Bovine Brain and from soybean, phosphatidylserine (PS) from Bovine Brain, and L-α-lecithin (phosphatidylcholine, PC) from egg and from plant. These were all obtained from Avanti Polar Lipids, Birmingham, AL. Cholesterol, asolectin (Al, soybean lecithin) and dithiothreitol (DTT) were from Sigma Chemical Co., St. Louis, MO. Asolectin was partially purified according to Kagawa and Racker (1971). Highly purified (>99%) disialoganglioside (G_{D1b}) and trisialoganglioside (G_{T1b}) were a kind gift from FIDIA Research Laboratories, Abano Terme, Italy.

Planar Lipid Bilayers

Planar bilayers were formed by apposition of two monolayers initially formed at the air-water interface and the electrical properties were studied essentially as described previously (Montal, 1974). The monolayers at the air water interface were spread from a solution of the phospholipid under study in hexane at the final concentration of 2 mg/ml. DiphyPC, PS, plant PC, and egg PC were directly dissolved in hexane. For the phospholipid mixtures, the individual phospholipids (or cholesterol) were dissolved separately in chloroform: methanol 2:1, mixed and the solvent removed by evaporation. The residue was dissolved in hexane and adjusted to the indicated concentration. For the mixture of diphyPC and gangliosides, the procedure of McDaniel and McLaughlin (1985) was followed. G_{D1b} and G_{T1B} at weight fractions $\geq$20%–30% and PI at weight fractions $\geq$40% generated unstable membranes and, therefore, were not studied. The composition of the buffered salt solution was as follows: 0.5 M KCl, 10 mM Hepes, buffered with 2.2 mM KOH, pH 7. For the studies at pH 4.8, the solution was: 0.4 M KCl, 24.3 mM citric acid, 50 mM K_2HPO_4 to a total potassium concentration of 0.5 M. Planar lipid bilayers were formed across apertures with diameters ranging from 70 μm to 150 μm perforated in a 25 μm teflon film separating 2 1-ml capacity teflon chambers (Montal, 1974, 1986). The hole was coated with 2 μl of 0.5% (vol/vol) squalene (Sigma Chemical Co.) in hexane (Aldrich Chemical Co., Inc., Milwaukee, WI). The chambers were filled with 0.5 ml of the indicated buffer solution before spreading of the monolayers. The composition of the electrolyte solution in the two chambers was the same. Membrane formation was continuously followed by measuring the membrane capacitance. All the bilayers studied were stable for several hours and had a specific capacitance in the range of 0.7–0.8 $\mu F/cm^2$. All the planar bilayer experiments were performed at 22 ± 2°C.

Electrical Recordings and Data Processing

The majority of the records were obtained using a List Medical Electronics EPC7 amplifier (Medical Systems Corp., Greenvale, NY) in the voltage clamp mode. Some records were obtained with the current amplifier described previously (Labarca et al. 1984). Ag/AgCl pellet electrodes (E255 or E206 from In Vivo Metric Systems, Healdsburg, CA were used. Constant voltage was applied from a DC source (Omnical 2001; WPI Instruments, New Haven, CT). For the voltage ramps, a Wavetech signal generator (Wavetech, San Diego, CA) was used. The voltage of the TeTx-free compartment was defined as the reference voltage. For example, an applied voltage of +100 mV indicates that the compartment containing the toxin was held at +100 mV with respect to the opposite compartment.

Membrane current was recorded directly on a storage oscilloscope or amplified and stored in a RACAL 4DS tape recorder (FM bandwidth DC-40 kHz, Hythe, Southhampton, England) or in a video cassette recorder (Sony-Beta Max) equipped with a digital audioprocessor (Sony PCM 501ES) modified according to Bezanilla (1985) and commercially available from Unitrade, Inc. (Philadelphia, PA). The recordings were filtered at 3 kHZ (−3 dB), unless otherwise indicated, with an 8-pole-Bessel low-pass filter (Frequency Devices, Haverhill, MA) and digitized at 100 μs per point using an INDEC-L-11/73-70 microcomputer system (Indec, Sunnyvale, CA). Channel open and closed conductance states were discriminated using a pattern recognition program described previously (Labarca et al., 1984). Unless otherwise indicated, all the conductance values were calculated from the current histogram best fitted by the sum of two Gaussian distribution. The statistical program also evaluates the standard error of the estimates of each parameter. The difference between the current values corresponding to the two current peaks was divided by the applied voltage to obtain the single-channel conductance. The number of events analyzed to obtain each conductance and lifetime value was always >500. If not otherwise indicated, data were derived from at least three experiments performed with a freshly dissolved lipids on different days. The conductance values reported in the text are given as mean, plus/minus standard error. The records obtained were displayed either on a Gould 2400S recorder (Gould, Cleveland, OH) or as a direct printout from the Microcomputer System.

RESULTS

Tetanus Toxin and Tetanus Toxin B Fragments Form Ionic Channels in Lipid Bilayers at Neutral pH

Highly purified TeTx and commercial preparations of TeTx (containing a mixture of forms of the protein) induce the formation of ionic channels in planar lipid bilayers, as will be described in detail. In contrast, isolated C fragment had no effect on the electrical properties of lipid bilayers when studied under equivalent conditions. It was reported (Hoch et al., 1985) that the light chain does not affect membrane permeability while the B fragment increases permeability (Boquet and Duflot, 1982; Hoch et al., 1985).

To explore if the presence of the light chain in the B fragment was necessary for channel formation, the TeTx B fragment was incubated for 15 min in 0.1 M DTT (Dithio-

threitol) in 0.5 M KCl at pH 7 in order to reduce the disulfide linkage between the light chain and the amino terminus of the heavy chain (Matsuda and Yoneda, 1975). Thereafter, the protein was desalted by passing it through Sephadex G 15 column and dialyzed overnight against 0.5 M KCl at pH 7.0 to remove residual DTT and salt. Addition of reduced TeTx B fragment to lipid bilayers produces transmembrane channels indistinguishable from those measured with either intact TeTx or its B fragments both at neutral and at acidic pH. Therefore, the channel forming domain of TeTx is located in the amino terminus fragment of the heavy chain. Most of the results presented in this paper were obtained with the B fragment of TeTx. However, some results were obtained also with intact toxin. In the description that follows, we will not make specific distinction between the preparations and will refer to the channel as the TeTx channel.

The currents flowing through individual TeTx channels recorded at an applied voltage, V, of +80 mV are illustrated in Fig. 1 *A* (CalBiochem TeTx) and Fig. 1 *B* (reduced TeTx B fragment). As indicated, an upward deflection denotes a channel opening. The records were purposely filtered at 3 kHz to illustrate two features characteristic of the TeTx channel (Borochov-Neori et al., 1984). First, channels appear in bursts and within a burst, the probability (P) that the channel is open is large (usually $P \geq 0.7$). Second, the open channel conductance exhibits considerable current noise due to rapid flickering of the channel between the open and closed states. These features of the TeTx channel will be discussed in more detail later. The single-channel conductance, γ, measured from the amplitude of the single-channel current divided by the applied voltage is 89.01 ± 0.30 pS in 0.5 M KCl at pH 7.0 (see Table I). These records were obtained in symmetric PS membranes, in which γ is largest. As previously reported (Hoch et al., 1985) there is dispersion in single-channel conductance. Transitions to smaller, non-integer multiples of the maximum conductance were detected infrequently in all the lipids studied, but were not characterized.

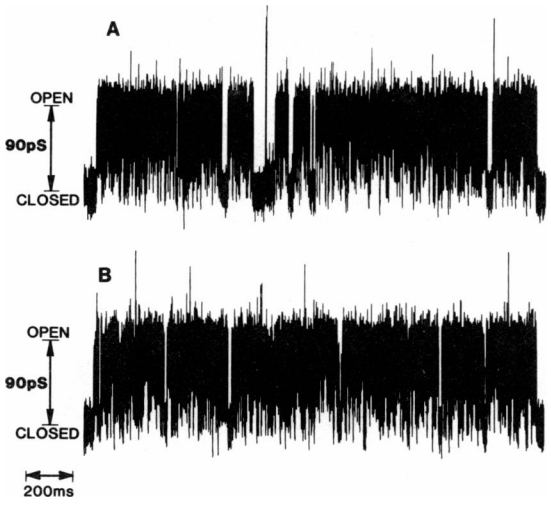

FIGURE 1. Single-TeTx channel current records in PS membranes at neutral pH 7. *A* and *B* correspond to TeTx (25 μg/ml) and its B fragment (25 μg/ml), respectively. Applied voltage was $V = +80$ mV. The signals were low-pass filtered at 3 kHz, digitized at a 100 μs sampling-interval and played-back in a paper oscillographic recorder at a 100 times lower speed. The composition of the aqueous solution was 0.5 M KCl, 10 mM Hepes, pH = 7.

Acid pH Promotes the Insertion of the Toxin Channel into the Membrane

As described for diphtheria toxin (Donovan, et al. 1981; Kagan, et al. 1981), botulinum and tetanus toxin (Donovan and Middlebrook, 1986; Hoch, et al. 1985), channel formation is sharply increased by lowering the pH (pH ≃ 4) in the compartment containing the toxin. As shown in Fig. 2, we confirmed the result. Addition of 25 μg/ml of toxin in PS membranes at pH 7 results in the occurrence of channels after a silent period of 54 min. (Fig. 2 *A*), while under identical conditions, this period is reduced to only 84 s at pH 4.8 (Fig. 2 *B*). In addition, the sensitivity of the membranes to TeTx concentration is greatly increased by conducting the experiment at acidic pH. As illustrated in Fig. 2 *C*, at pH 4.8 and at a concentration of 0.8 μg/ml of toxin, channels are incorporated after a latency of only 13 min. Thus, acid pH promotes the insertion of the channel by decreasing the time required for channel insertion in the membrane as well as reducing the concentration required to effectively detect the presence of channels in the bilayer.

Acid pH Lowers the Single-Channel Conductance of the Tetanus Toxin Channel

It is evident in Fig. 2 that the single-channel conductance of the TeTx channel at pH 4.8 is considerably lower than that at pH 7 under otherwise identical conditions. At pH 7,

TABLE I
TETANUS TOXIN SINGLE-CHANNEL CONDUCTANCE IN BILAYERS COMPOSED OF DIPHYPC OR PS AT pH-7 AND 4.8

Lipid	pH 7		pH 4.8	
	V	γ	V	γ
	mV	pS	mV	pS
diphyPC	−86	25.18 ± 0.14	−100	12.66 ± 0.13
diphyPC	+86	31.41 ± 1.39	+60	16.63 ± 0.19
PS	−80	65.58 ± 0.96	−70	21.43 ± 0.16
PS	+80	89.01 ± 0.30	+70	30.12 ± 0.33

Single-channel currents were filtered at 3 kHz. Single-channel conductances were determined from conductance histograms as those illustrated in Fig. 3 and 11. The tabulated values were obtained from fits of the histograms to the sum of 2 Gaussian distributions. The total number of transitions analyzed was 19,065; the minimum number for one point was 695; and maximum number for one point was 11,559. Other conditions were as for Fig. 2 legend.

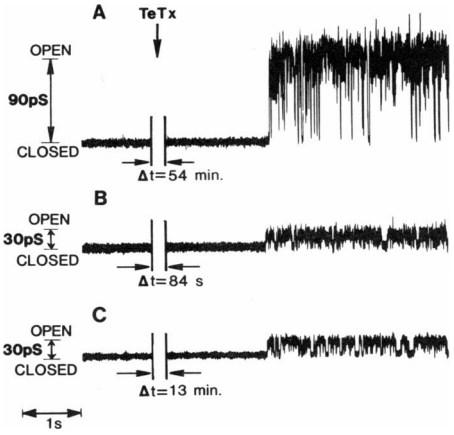

FIGURE 2 TeTx single-channel records in PS membranes at pH = 7 (*A*, applied voltage $V = +80$ mV) and at pH 4.8 (*B*, applied voltage $V = +50$ mV) in the presence of 25 μg/ml of TeTx. In *C* at pH 4.8, the TeTx concentration was 0.8 μg/ml ($V = +50$ mV). Signals were low-pass filtered at 200 Hz. The aqueous solution composition was: 0.5 M KCl, 10 mM Hepes, pH = 7 for *A* and 0.4 M KCl, 50 mM K_2HPO_4, 24.3 mM citric acid, pH = 4.8 for *B* and *C*.

$\gamma = 89.01 \pm 0.30$ pS while at pH 4.8, $\gamma = 30.12 \pm 0.33$ pS for symmetric PS bilayers in 0.5 M KCl (see Table I and Fig. 4). This effect is clearly illustrated in Fig. 3, where the same studies were performed in neutral lipid bilayers composed of diphyPC. The left hand panel (A) illustrates a single-channel record obtained at pH 7 while the right hand panel (C) shows a record obtained at pH 4.8, under otherwise identical conditions. The lower panels (B and D) illustrate the corresponding current histogram. At pH 7, two peaks are clearly distinguishable which correspond to the closed and open states of the channel. The data were fitted to the sum of 2 Gaussian distributions. From the fitted curves (smooth curves), γ, is calculated by the current difference between the two peaks divided by the applied voltage. For the current record shown in Fig. 4, $\gamma =$ 29 pS. In contrast, at pH 4.8, the two peaks corresponding to the closed and open states of the channel are not clearly discerned. The data again were fitted to the sum of 2 Gaussian distributions and from the fitted curves, γ was calculated to be 13 pS. Table I presents a summary of the results obtained in membranes prepared in the neutral phospholipid diphyPC and the negatively charged phospholipid, PS. It is clear that, in both systems and at both negative and positive applied voltages, the effect of lowering the pH from 7 to 4.8 is to reduce the single-channel conductance.

Selectivity of the Tetanus Toxin Channel

The channel discriminates between cations and anions with an apparent transferrence number for cations >0.7 (see Borochov-Neori, 1984; Hoch et al., 1985). Furthermore, γ in 0.5 M KCl or 0.5 M NaCl solutions buffered at pH 7 was different. In asolectin membranes, at $V = +64$ mV, $\gamma(K^+) = 45.97 \pm 0.24$ pS while $\gamma(Na^+) = 34.16 \pm 0.13$ pS. At $V = -64$ mV, $\gamma(K^+) = 39.42 \pm 0.28$ pS while $\gamma(Na^+) = 28.51 \pm 0.07$ pS. Therefore, the TeTx channel discriminates between K^+ and Na^+, with a higher selectivity for K^+ as inferred from the ratio of γ in K^+ over Na^+. Particularly, $\gamma(K^+)/\gamma(Na^+) \geq 1.35$ and 1.38 for $V = +64$

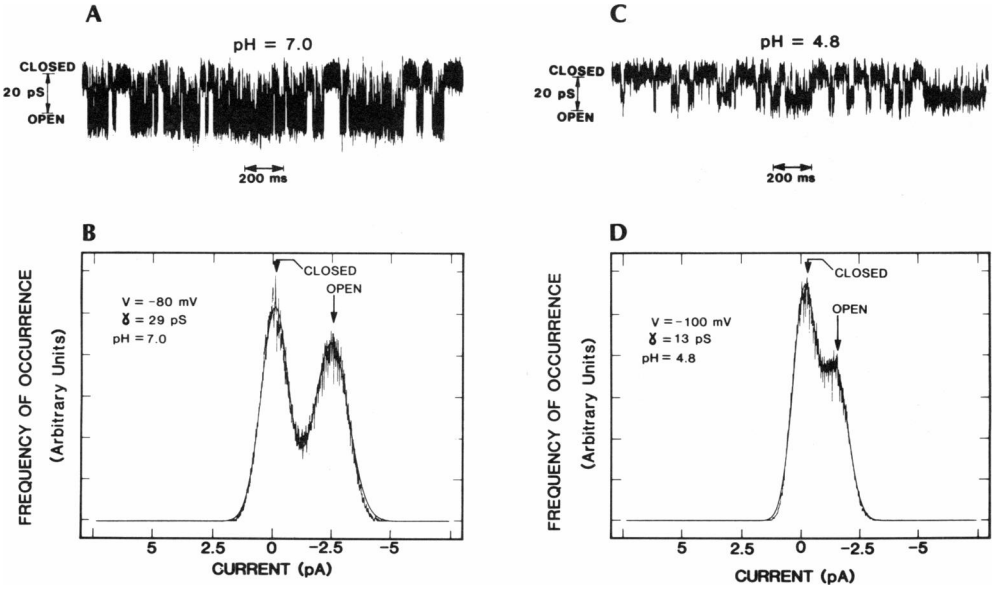

FIGURE 3. TeTx channel records in diphyPC membranes at pH = 7 (*left hand trace*) in the presence of 25 μg/ml of TeTx (applied voltage was $V = -80$ mV) and at pH = 4.8 (*right hand trace*) in the presence of 1.25 μg/ml of TeTx (applied voltage was $V = -100$ mV). The lower panels are the corresponding single-channel current histograms calculated from the analysis of 3,276 (filtered at 3 kHz) and 1,536 events (filtered at 0.5 kHz), respectively. The fitted gaussian distributions (*smooth curves*) indicate the difference between the mean current amplitudes at the two indicated pH's. Other conditions were as in Fig. 2 legend.

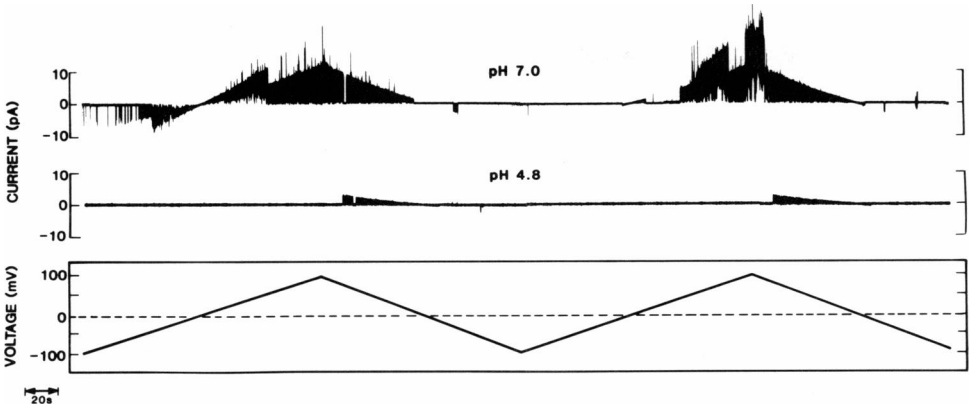

FIGURE 4 Single-channel current through PS membranes at pH = 7 (*upper trace*) and at pH = 4.8 (*lower trace*) in the presence of 16 μg/ml and 0.8 μg/ml of TeTx, respectively. The applied voltage was continuously cycled from −100 mV to +100 mV. The signals were low-pass filtered at 100 Hz. Other conditions were as in Fig. 2 legend.

mV and $V = -64$ mV, respectively. These values approach the conductance ratio expected if the permeabilities of the two ions were limited by diffusion in aqueous solution. Thus, the TeTx pore is poorly selective (see also Hoch et al. 1985). No apparent difference in the open and closed channel lifetimes in the two different buffered salt solutions was measured (data not shown).

The Tetanus Toxin Channel is Voltage Dependent

The membrane potential has a considerable effect on the rate of development of the conductance. This is evident in Fig. 4 where the probability of the channel being in the open state is illustrated. The figure shows the membrane current in response to a continuously cycled voltage from −100 to +100 mV in symmetric PS bilayers treated with TeTX at pH 7 (upper panel) or pH 4.8 (middle panel). The record obtained at pH 7 illustrates the presence of two channels into the membrane, during both the negative and the positive branch of the first cycle. At around +50 mV, one channel closes and remains closed for the rest of the cycle. During the positive branch of the second cycle, the presence of two open channels is again clearly recognized. The record at pH 4.8 illustrates the presence of one channel mainly at positive voltages. This type of measurement has the salient advantage of providing an instantaneous current voltage relationship. Two results are noteworthy. First, γ at acidic pH is greatly reduced. Second, both at neutral and acid pH, the probability of the channel being open is higher at positive voltages. During the sections of the cycle corresponding to negative applied voltages the channel is practically closed all the time. The channel asymmetry with respect to the applied voltage is also evident in the data shown in Fig. 5 *E* (for PS). Similar results were also obtained at pH 4.8 (not shown). A statistical analysis of the percent of time that the channel is open at voltages of different polarities (±86 mV) is summarized in Fig. 9. It is clear, therefore, that the voltage dependence of TeTx channel is not affected at acidic pH but is likely an inherent property of the protein molecule.

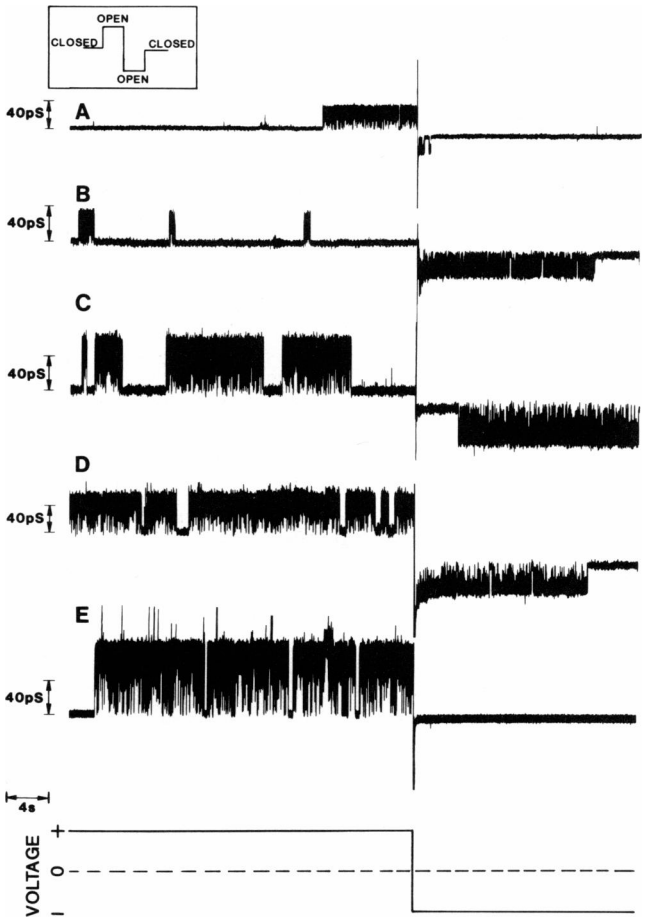

FIGURE 5 Single-TeTx-channel responses (*A–E*) to the inversion of the applied voltage (*lower trace*) in membranes of different phospholipid composition. The voltage pulse protocol is illustrated in the lower record. The insert shown in the upper left hand section illustrates the channel states at different polarities. The applied voltage was (*A*) $V = \pm 64$ mV for egg PC, (*B*) $V = \pm 86$ mV for diphyPC, (*C*) $V = \pm 86$ mV for diphyPC:PI (40%), (*D*) $V = \pm 64$ mV for diphyPC:PS (40%), and (*E*) $V = \pm 80$ mV for PS. The signals were low-pass filtered at 200 Hz. The aqueous solution was 0.5 M KCl, 10 mM Hepes, pH 7.0.

TABLE II
TETANUS TOXIN SINGLE CHANNEL CONDUCTANCE IN BILAYERS OF DIFFERENT LIPID COMPOSITION AT pH 7.0

Lipid	V	γ	V	γ	γ^+/γ^-
	mV	pS	mV	pS	
diphyPC	−86	25.18 ± 0.14	+86	31.41 ± 1.39	1.25
eggPC	−86	25.38 ± 0.11	+86	27.75 ± 0.20	1.09
plant PC	−64	22.10 ± 0.19	+64	29.00 ± 0.21	1.32
diphyPC:cholesterol (20%)	−60	27.76 ± 0.18	+60	38.62 ± 1.00	1.39
diphyPC:PI (40%)	−86	35.56 ± 0.13	+86	53.83 ± 0.05	1.51
diphyPC:PS (40%)	−86	37.33 ± 0.09	+86	60.20 ± 0.20	1.61
diphyPC:G_{Dib} (20%)	−60	25.56 ± 0.42	+60	39.06 ± 0.19	1.53
diphyPC:G_{Tib} (30%)	−86	26.09 ± 0.55	+86	30.02 ± 0.45	1.15
Al	−86	32.26 ± 0.22	+86	51.88 ± 0.13	1.61
PS	−80	65.58 ± 0.98	+80	89.01 ± 0.30	1.36

Single-channel currents were filtered at 3 kHz and γ determined as described in Table I legend. The total number of events was 103,569, the minimum number for one point was 550, and the maximum number for one point was 18,212. For plant PC and egg PC data were derived from one experiment and two experiments, respectively. γ^+/γ^- is the ratio of γ measured at positive and negative voltages. Other conditions were as for Fig. 5 legend.

The Properties of the Tetanus Toxin Channel are Exquisitely Sensitive to the Lipid Composition of the Membrane

To establish the specificity of the interaction between TeTx and lipids, the properties of the TeTx channel in a variety of phospholipid membranes were systematically studied. The studies were performed both at pH 7 and at pH 4.8. At this point we restrict the description to the data obtained at pH 7. Fig. 5 illustrates the results obtained with five different phospholipids. The voltage pulse protocol of the experiments is illustrated in the lower most trace. The insert shown in the upper left hand section illustrates the channel states at different polarities. Initially, a positively applied voltage is imposed on the membrane, thereafter, the applied voltage is switched to the opposite polarity and held constant for several seconds. The results shown correspond to the following phospholipid compositions: egg PC, diphyPC, diphyPC:PI (40%), diphyPC:PS (40%), and PS, as A, B, C, D, and E, respectively. In egg PC, the residence time of the channel in the open state at positive applied voltages is significant while it is very brief at negative applied voltages. In diphyPC, (Fig. 5 *B*) channel opening at positive applied voltages occurs in brief bursts of activity separated by long closed periods. At negative applied voltages, the channel is open most of the time. Supplementing diphyPC with an acidic lipid, either PI (40%), as in Fig 5 *C* or with PS (40%), as in Fig. 5 *D*, greatly increases the probability of the channel being in the open state at both positive and negative voltages. In PS membranes (Fig. 5 *E*), the channel is open virtually all the time at positive applied voltages and closed at negative applied voltages. The lipid composition not only affects the probability of the channel being in the open state, but also modifies the single-channel conductance (see Table II).

TABLE III
RELATIONSHIP BETWEEN WEIGHT FRACTION OF CHARGED PHOSPHOLIPID AND MEMBRANE CHARGE DENSITY

Phospholipid	Charge density
	charges/Å^2
diphyPC:PI (5%)	$0.69 \cdot 10^{-3}$
diphyPC:PI (10%)	$1.39 \cdot 10^{-3}$
diphyPC:PI (20%)	$2.79 \cdot 10^{-3}$
diphyPC:PI (40%)	$5.61 \cdot 10^{-3}$
diphyPC:PS (40%)	$5.93 \cdot 10^{-3}$
PS	$14.29 \cdot 10^{-3}$
Al	$\simeq 3.40 \cdot 10^{-3}$

Charge density for lipid mixtures used in Figs. 6 and 8. Acidic phospholipid molar fraction, Mf, was derived from weight fraction, Wf, according to the following relation: $Mf = Wf \cdot 100/(\alpha[100\text{-}Wf] + Wf)$ where α = (acidic phospholipid molar weight)/(diphyPC molar weight) and Wf is given in percent units. Charge density, Cd, was obtained assuming a mean molecular area of 70 Å^2 for all the lipids. For example, $Cd = 5.6 \cdot 10^{-3}$ charges/Å^2 for diphyPC:PI (40%) and $Cd = 5.93 \cdot 10^{-3}$ charges/Å^2 for diphyPC:PS (40%). Mean molecular weights were estimated from the fatty acid composition of different lipids, provided by the supplier (Avanti Polar Lipids, Birmingham, AL). Particularly: (diphyPC) MW = 846.27, (PI) MW = 870.68, (PS) MW = 795.43. Cd for Al membranes was calculated on the basis that Mf of charged phospholipid is 24%.

Acidic Phospholipids Increase the Single-Channel Conductance

The effect of acidic phospholipids on the single-channel conductance of the TeTx channel was studied in more detail. Membranes were formed from mixtures of the neutral lipid diphyPC with either PI or PS at different weight fractions of the charged phospholipid, 100% PS, or Al. Asolectin is a natural mixture of phospholipids primary composed of PC, PI, PE, PS, and cardiolipin. In Table III, the surface charge density for membranes formed from the phospholipid mixtures used in Figs. 6 and 7 is reported.

The results of the study are summarized in Fig. 6 and in Table II. Increasing the weight fraction of charged phospholipid in the membrane increases γ. The effect is apparent at both positive and negative applied transmembrane voltage. In diphyPC, $\gamma = 31.41 \pm 1.39$ pS. With 40%

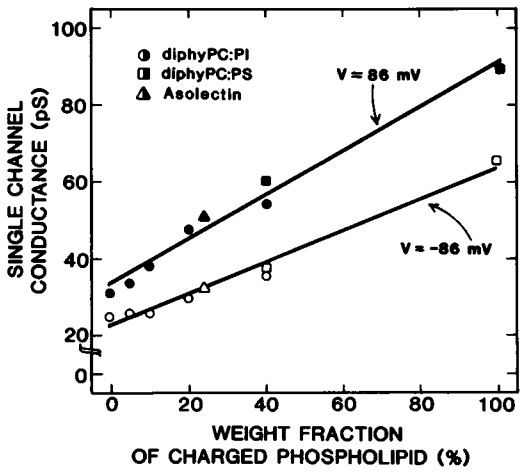

FIGURE 6 TeTx channel conductance as a function of the weight fraction of charged phospholipid in the membrane. Applied voltage was $V = -86$ mV (O, △, □) and $V = +86$ mV (●, ▲, ■). The two straight lines are the least square fits to the data points. The corresponding slopes are 22.36 pS at $V = -86$ mV and 33.67 pS at $V = +86$ mV. The weight fraction of charged lipid in A1 was estimated to be 24% (O'Brien et al., 1977; Montecucco et al., 1986). Other conditions were as in Fig. 5 legend.

PS it increases to 60.20 ± 0.20 pS and at 100% PS it becomes 89.01 ± 0.30 pS, at $V = +86$ mV. The triangles indicate the data obtained with Al and they are illustrated as single points in Fig. 6. It is interesting to note that the γ in Al would correspond to a weight fraction of acidic phospholipid of ~24%. This is in close agreement with the reported content of charged phospholipids in Al as determined by chemical analysis (Montecucco et al., 1986; O'Brien et al., 1977). Table II shows that γ in three different classes of PC membranes is practically the same. For diphyPC, egg PC and plant PC, γ = 31.41 ± 1.39 pS, 27.75 ± 0.20 pS and 29.00 ± 0.21 pS, respectively. The results obtained in Fig. 6 clearly point to a positive correlation between the increase in the negative surface charge of the lipid bilayer with an increase in γ. Estimates of the apparent surface charge introduced by supplementing negatively-charged phospholipids into the lipid bilayer suggest that this is the case. This effect may arise from the enrichment of ions at the entrance of the channel produced by the negative surface charge.

The results obtained with mixtures of diphyPC and G_{D1b} or G_{T1b} are also reported in Table II. A ganglioside weight fraction in the order of 30% is the maximum compatible with the generation of stable lipid membranes. Even at this weight fractions of the gangliosides, γ for G_{T1b} was comparable with that of diphyPC alone, while the G_{D1b}, γ increased to 39.06 pS.

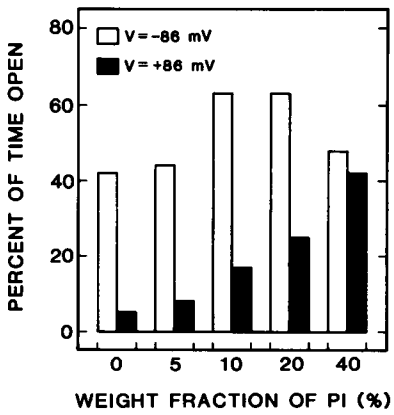

FIGURE 7 Percent of time that the TeTx channel is in the open state in membranes formed from diphyPC and different weight fraction of PI at $V = -86$ mV (*empty bars*) and at $V = +86$ mV (*solid bars*). Other conditions as in Fig. 5 legend.

Bilayer Phospholipid Composition Affects the Probability of Channel Opening

Inspection of Figs. 4, 5 and 8 shows that the frequency of channel opening depends on the composition of the lipid bilayers and on the applied voltage. In order to quantitate the probability of channel opening, records 30-s long were analyzed and the percent of time that the channel was open during that interval was determined at $V = \pm 86$ mV. Every record contained, at least, one burst of channel activity. The data were determined from at least three segments for each one of the conditions. The percent of time open, f_{op}, was calculated according to Eq. 1:

$$f_{op} = t_{bursts} \times \tau_b / T, \quad (1)$$

where t_{burst} is the length of the measured burst, τ_b is the percent of time that the channel is in the open state during the burst and T is the total recording time at a particular applied voltage, in this case 30 s. The results of the studies are summarized in Fig. 9. At $V = 86$ mV, f_{op} is drastically increased from 5 to 42% and 41%, respectively, by supplementing diphyPC with PI (40%) or PS (40%). This effect

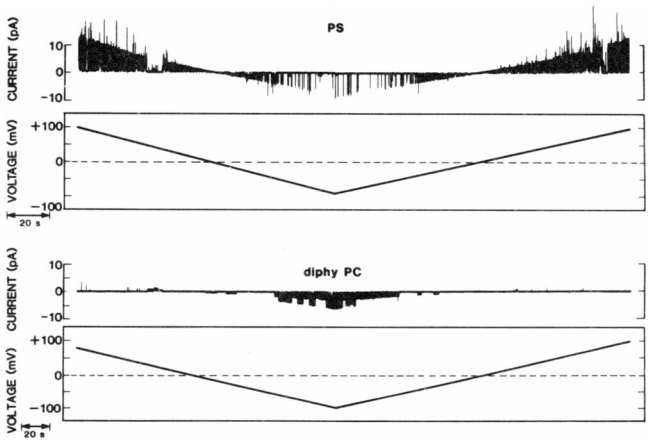

FIGURE 8 Membrane current through single channels in PS (*upper trace*) and diphyPC (*lower trace*) membranes in the presence of 16 μg/ml and 25 μg/ml of TeTx, respectively. The applied voltage was continuously cycled between −100 mV and 100 mV. The signal was low-pass filtered at 100 Hz. Other conditions were as for Fig. 5 legend.

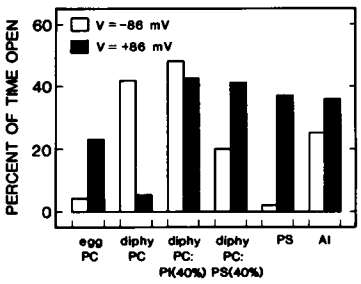

FIGURE 9 Percent of time that the TeTx channel is in the open state in membranes of different phospholipid composition. Applied voltage was $V = -86$ mV (empty bars) and $V = +86$ mV (*solid bars*). Records containing at least one current burst were studied. Segments of the records 30 s long were analyzed. The mean values obtained from at least three sections are displayed. Other conditions were as for Fig. 5 legend.

of the charged lipid is also prominent in membranes composed exclusively of PS or an Al membranes. The results obtained at $V = -86$ mV for diphyPC alone or supplemented with PI and PS (40%), or in asolectin membranes, where the percentage of charged lipid is ~24%, do not show a significant variation. However, in membranes composed exclusively of PS and held at $V = -86$ mV, the channel was rarely open.

To further investigate the effect of the charged phospholipid on the percent of time open, the same analysis was performed on diphyPC membranes supplemented with PI at different weight fractions. The results of this study are shown in Fig. 7 at $V = -86$ mV and $V = +86$ mV. Increasing the weight fraction of PI from 0 up to 40% increases f_{op} from 5% up to 42% at $V = +86$ mV. In contrast, at $V = -86$ mV, $f_{op} \geq 40\%$ at all weight fractions studied. These results indicate that, in addition to the surface charge effect introduced by the inclusion of acidic phospholipid in the bilayer, there may be a specific effect arising from the chemical nature of the phospholipid head group. This is particularly prominent when a comparison is made between the data obtained in membranes composed exclusively of PS and in membranes containing 40% PI. Unfortunately, membranes composed exclusively of PI are rather unstable and a rigorous comparison between PI and PS was not possible. The data, therefore, show that acidic phospholipids have a profound effect on the probability of channel opening.

Variations in the fatty acid composition of the lipids may influence f_{op}, as shown by the difference between egg PC and diphyPC. In contrast, no significant effects were measured when diphyPC membranes were supplemented with cholesterol (up to 20%, data not shown).

Fig. 8 shows the current voltage relationships for the TeTx channel in negatively-charged bilayers composed exclusively of PS (upper panel) and in neutral membranes composed of diphyPC (lower panel). The Figure illustrates the membrane current through the bilayers in response to a continuously cycled voltage from +100 to −100 mV. For PS, f_{op} is distinctively large in the positive branch of the voltage cycle and is considerably lower in the negative segment of the cycle. In contrast, for diphyPC, channel activity is especially prominent during the negative phase of the voltage cycle. In this particular experiment (diphyPC), two channels appear superimposed. The two records are displayed at the same current sensitivity to emphasize the effect of the negatively charged lipid in increasing the conductance of the channel. In agreement with the data of Fig. 5 *B* and *E*, the data show that the TeTx channel is voltage dependent in both diphyPC membranes and PS membranes but with an entirely opposite sensitivity to the polarity of the applied voltage.

Conductance States of the Tetanus Toxin Channel

At higher magnification of the current, records displayed in Figs. 4 and 8 show that the current-voltage relationships for the TeTx channel deviate from linearity. This effect was further investigated and the results are illustrated in Fig. 10. In A, the current flowing through individual TeTx channels at the indicated voltages for a diphyPC:PI (20%) membrane is shown.

Single-channel currents were determined at each voltage from amplitude histograms as in Fig. 3. Panel B displays the single-channel current voltage curves for the TeTx channel in symmetric diphyPC:PI (20%) mem-

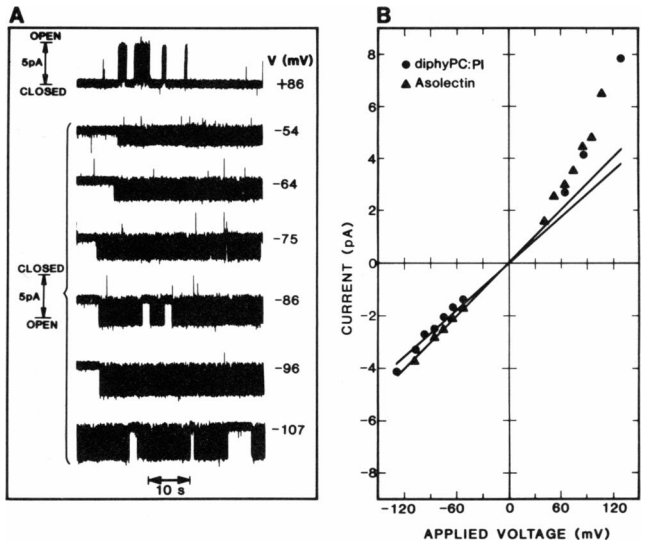

FIGURE 10 (*A*) Representative single-channel current records at the indicated voltages in diphyPC:PI (20%) membranes in the presence of 12 μg/ml TeTx. (*B*) Single channel current-voltage characteristics for diphyPC:PI (20%) (●) and A1 (▲) membranes. Each point is the mean value derived from the current histograms of at least 1,616 events. Total number of events analyzed for A1 membranes was 66,372. For diphyPC:PI (20%) membranes the total number of events was >56,000. The signals were filtered at 3 KHz. The average standard deviation was 0.01 pS. The data points at negative voltages were fitted by straight lines passing through the origin. The corresponding slopes are 29.48 pS for diphyPC:PI and 33.45 pS for A1. For diphyPC:PI (20%) data were derived from the same membrane, while A1 data were obtained from five different membranes. Other conditions were as for Fig. 5 legend.

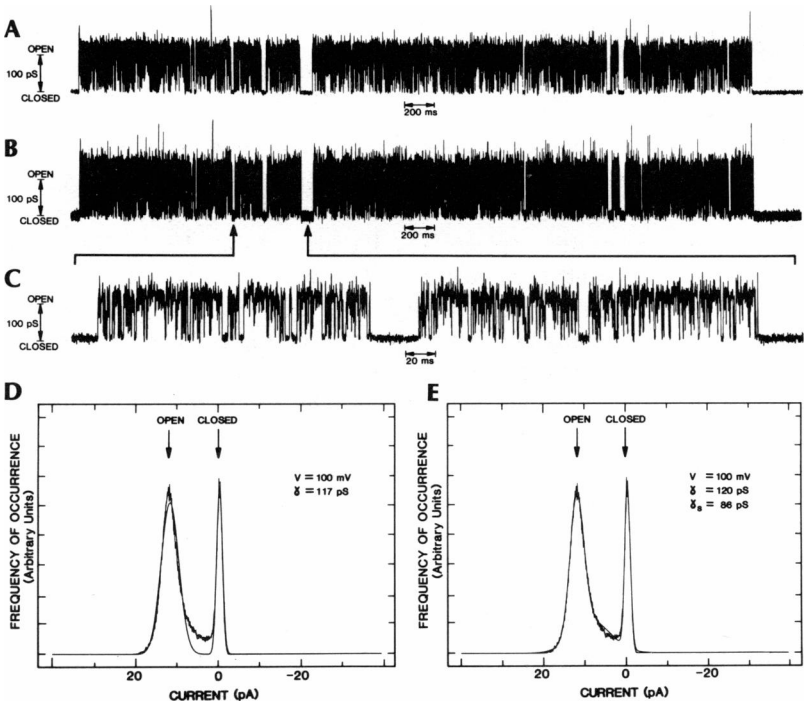

FIGURE 11 Single-TeTx-channel records in PS membranes in the presence of 25 μg/ml TeTx. In *A* and *B* the same current record was filtered at 1 KHz and 3KHz, respectively, digitized at 100 μs sampling-interval, and played back into paper oscillographic recorder at a 100 times lower rate. *C* shows the section of the record delimited by the arrows and filtered at 3 KHz at higher time resolution (note the change in time calibration). Current histograms obtained analyzing 5,980 transitions (filter at 3 KHz) were best fitted by a sum of 2 (*D*) or 3 (*E*) Gaussian distributions. In *E* a substate presenting a conductance which is 0.72 the amplitude of the fully open state has been identified. The relative frequency of occurrence was 2:1.3:1 for the open state, subconductance state and closed state, respectively. The applied voltage was $V = +100$ mV. Other conditions as for Fig. 5 legend.

branes and in Al membranes (~24% of acidic phospholipids). For negatively applied voltages the I-V curve is linear and ohmic. γ, calculated from the slope of the line in the negative voltage branch of the figure, was 29.48 pS for diphyPC:PI (20%) and 33.45 pS for Al. In contrast, the I-V relationship for positive applied voltages is supralinear.

As shown in Fig. 1, the open channel conductance of TeTx is very noisy. Fig. 11 shows single-channel currents recorded from planar lipid bilayers in the presence of TeTx at pH 7. The single-channel recordings filtered at 1 kHz are illustrated in panel A. The records show that the channel spends most of the time in the open state with a few interruptions in the closed state. However, the open state shows very fast transitions to the closed state giving the appearance of flickering between the open state and short-lived closed state. In order to resolve the fast transitions, the records are illustrated at a filter frequency of 3 kHz in panel B. It is evident that the open state of the channel shows considerable current noise due to the fast flickering transitions between the open state and the closed states. A section delimited by the arrows is displayed at high time resolution in panel C. The characteristic opening and closing events are clearly resolved, showing the occurrence of closings with very short lifetimes. A quantitative analysis of the relative residence time of the channel in the closed and the open state is determined from conductance histograms. This is illustrated in panels D and E. The conductance histogram is fitted by the sum of 2 Gaussian distributions, one for the closed state and the second one for the open state of the channel (panel D). The theoretical curve is displayed as the smooth curve superimposed on the actual data (noisy signal). The conductance histogram, however, is fitted by the sum of 3 Gaussian distributions as illustrated in panel E. The channel, therefore, appears to fluctuate with apparent conductances of 86 and 120 pS. Since the conductance of the most populated state, namely, the open state of the channel agrees well for the data fitted with the sum of 2 Gaussians or with the sum of 3 Gaussians, we are led to suggest that the intermediate

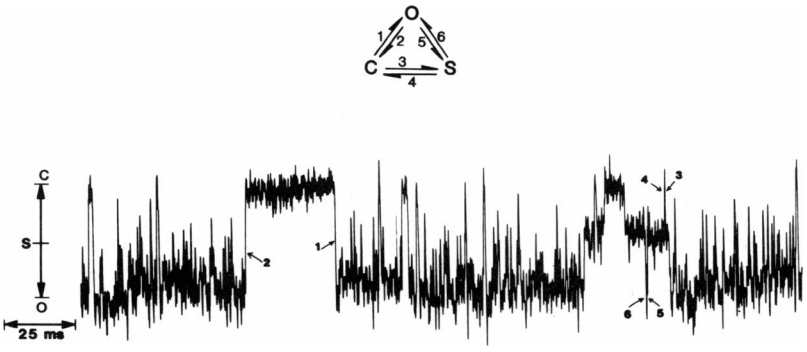

FIGURE 12 TeTx channel current record in PS membranes displayed at high time resolution. The signal was low-pass filtered at 3KHz, digitized at a 100 μs sampling-interval, displayed on a HP 1345A digital display (2,048 × 2,048 points) and hard-copied on a digital *x-y* plotter. Transitions from the closed to the fully open state and from the substate to the closed and open state can be identified. The applied voltage was $V = -60$ mV. These intermediate states are usually short lived and exhibit a conductance which may be ⅓ or ⅔ of the amplitude of the fully open state. Other conditions were as for Fig. 5 legend.

conductance state corresponds to a conductance substate of the toxin channel. The relative frequency of occurrence of each state is determined from the area under each Gaussian distribution: for data in Fig. 11, the corresponding figures are 23% for the closed state, 30% for the open subconductance state and 47% for the fully open state.

Fig. 12 displays a section of record at higher time resolution where transitions between the three indicated states are clearly discerned. Channel opening is indicated by a downward deflection and the approximate value of the open subconductance state is marked. The insert to the figure illustrates the transition between a closed (C), open substate (S) and the fully open (O) state. The arrows with specific numbers point to the transition indicated in the insert. Proceedings from left to right, a transition from the fully open to the closed state (2) and from the closed state back to the fully open state (1) are followed by transitions from the fully open state to the open subconductance state (5) and from the subconductance state to the closed state (4). Subsequently, the transition from the closed state to the open subconductance state (3) is followed by a transition from the subconductance state back to the fully open state (6). Thus, the subconductance state inferred from the theoretical fits to the conductance histogram is indentifiable in the single-channel recordings and the transitions into the state and out of this state can be clearly recognized. Transitions 3 and 4 are less frequent than transitions 1 and 2 and especially than 5 and 6 which continuously affect the open state level.

Channel Gating Kinetics

Fig. 13 illustrates the probability distribution of the open and closed dwell times of the record shown in Fig. 11. The data are illustrated as the noisy curve superimposed on which is shown the dwell time histogram fitted by a multi-exponential probability density function of the form:

$$f(t) = \sum_{i=1}^{N} a_i e^{-t/\tau_i}, \qquad (2)$$

with N being the number of exponential, while a_i and τ_i are respectively the amplitude and the time constant of the exponentials. The fits were calculated by χ^2 minimization algorithm (please see Labarca et al., 1984). The open channel lifetime was 1.42 ms while two closed lifetimes, τ_{c1} and τ_{c2}, were identified: τ_{c1} = 0.26 ms and τ_{C2} = 1.34 ms. This analysis was performed on single-channel recordings obtained from TeTx channels in lipid bilayers of different phospholipid composition. The results of this study are summarized in Table IV where data obtained at $V = -86$ and $V = +86$ mV are presented. It is evident that in all lipid membranes the open channel lifetime is longer than 1 ms while the slow component of the closed lifetime is in the order of 1 ms. The values of the fast component are not reported in Table IV because they are unreliable due to the limited time resolution of the system. Presumably, the fast component is related to the very fast transitions in and out of the substate. In fact, the choice of a unique threshold to identify closings of the channel (when it is in the open state) and the relatively low probability of transitions from the fully closed state to the subconductance state (and vice versa) imply that the intermediate level is usually recognized by the pattern recognition program (Labarca et al., 1984) as a closed state. It is worth noting that the open lifetimes depend on the polarity (Fig. 14) but not on the magnitude ($0 \leq V \leq 100$ mV) of the applied voltage.

Acidic Phospholipids Affect the Open but not the Closed Channel Lifetimes

Fig. 14 shows the effect of supplmenting diphyPC with progressively increasing concentration of PI on both the open channel lifetime and the closed channel lifetime. It is

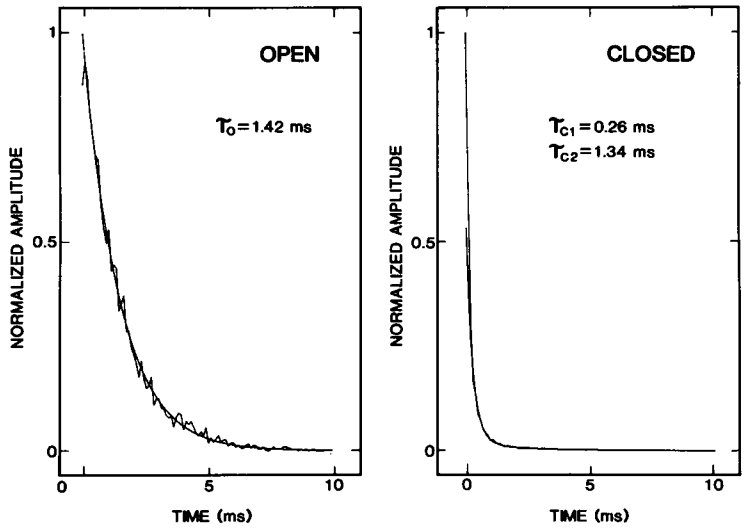

FIGURE 13 Probability density distribution of dwell times in the open (*left side*) and closed (*right side*) state of TeTx channels in PS membranes. The data were filtered at 3 KHz. Data were well fitted by a single exponential distribution for open time and a two exponential distribution for the closed time. Due to limited time resolution of the system, the contribution of short durations (≤200 μs) was systematically disregarded. The fitted curve (*smooth curve*) is superimposed on the histograms of the actual data (*noisy curve*). τ_0 and τ_{C1}, τ_{C2} are the open intermediate and closed time constants, respectively. Other conditions were as for Fig. 5 legend.

TABLE IV
OPEN AND CLOSED CHANNEL LIFETIMES OF TETANUS TOXIN CHANNELS IN BILAYERS OF DIFFERENT LIPID COMPOSITION

Lipid	V	τ_0	τ_{c2}	V	τ_0	τ_{c2}
	mV	ms	ms	mV	ms	ms
diphyPC	−86	2.41 ± 0.05	0.95 ± 0.07	+86	1.26 ± 0.04	1.00 ± 0.01
diphyPC:PI (40%)	−86	3.19 ± 0.05	1.05 ± 0.06	+86	2.36 ± 0.05	0.88 ± 0.05
diphyPC:PS (40%)	−86	2.11 ± 0.03	0.92 ± 0.05	+86	2.27 ± 0.04	0.94 ± 0.10
Al	−86	2.92 ± 0.06	0.63 ± 0.04	+86	2.36 ± 0.05	0.62 ± 0.03
PS	−80	1.01 ± 0.05	1.72 ± 0.41	+80	1.40 ± 0.02	0.74 ± 0.05

Single-channel currents were filtered at 3 kHz. Channel lifetimes were determined by probability density analysis as shown in Fig. 13. The open-state time constant (τ_0) and the longer component for the closed-state lifetime distribution (τ_{c2}) are tabulated. The total number of events was 28,496, minimum number for one point was 550; and the maximum number for one point was 4,676. Other conditions were as for Fig. 5 legend.

clear, that increasing the weight fraction of negatively charged lipid does not affect the channel closed lifetime. In contrast, both at $V = -86$ mV and $V = +86$ mV, there is a small yet significant prolongation of the open channel lifetime with increasing the weight fraction of PI in the membrane. At $V = -86$ mV, the open channel lifetime in diphyPC:PI (40%) membranes is 3.2 ms while in diphyPC exclusively, or supplemented by 5% PI, the open channel lifetime is 2.4 and 2.2 ms, respectively. This effect clearly contributes to the previously described effect of acidic phospholipid in increasing the probability of the channel being in the open state (Figs. 9 and 7). However, PS shortens τ_0. Thus, negative surface charge is not the only factor that modulates channel opening.

DISCUSSION

Addition of tetanus toxin to lipid bilayers of different lipid composition induces the formation of ionic channels. Channel incorporation is more effective at acidic pH, but occurs also at neutral pH. Channels observed with different preparations, i.e., highly purified (National Institutes of Health and Pasteur Institute) toxin, commercial TeTx, or its B fragment are indistinguishable. Furthermore, if the toxin is partially proteolyzed and the disulfide bridge between the heavy and the light chain is reduced, channel activity can be routinely measured at physiological pH, but at lower efficiency than that observed at acidic pH (see Fig. 2). Thus, channel activity is independent of the integrity of the dichain protein (Hoch et al., 1985). Neither the light chain (Hoch et al., 1985) nor the C fragment (this paper) increase the ionic conductance of planar bilayers. Therefore, the channel forming domain of TeTx must be located in the amino terminus fragment of the heavy chain (the 45 kd fragment also known as B45).

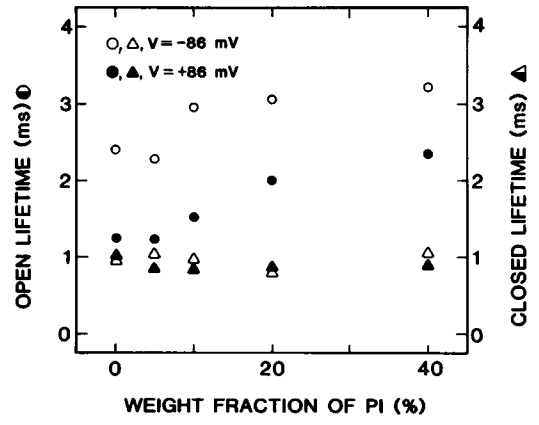

FIGURE 14 Mean open ● and closed channel lifetimes ▲ in diphyPC:PI membranes as function of the weight fraction of PI. Signals were filtered at 3 KHz and the mean lifetimes calculated as shown in Fig. 13. For the closed lifetime, only the slow component is shown. Each value is the result obtained from the analysis of at least 999 events. The total number of events analyzed was 38,394. Other conditions were as for Fig. 5 legend.

The rate of insertion of TeTx channels into lipid bilayers is promoted by both acid pH in the bulk aqueous solution and the presence of acidic phospholipids in the membrane (Montecucco et al., 1986). This suggests that the insertion step is dependent on the surface pH at the membrane–water interface which is determined by both the solution pH and the nature of the phospholipid polar headgroups. In addition, acidic pH lowers γ both in neutral and negatively charged membranes (Table I). Evidently, acidic pH determines a different organization of the protein in aqueous solution (see also Boquet and Duflot, 1982; Boquet et al., 1984; Roa and Boquet, 1985) (presumably exposing extensive hydrophobic domains), which may persist when incorporated into lipid bilayers and account for the reduction in γ.

The other properties of the TeTx channel hitherto studied are not affected by the acidic pH of the aqueous solution: the higher conductance measured at positive than at negative voltages, and the percent of time the channel is open in a variety of lipids. Therefore, this must reflect inherent properties of the protein. If acid pH determines changes in the folding of the protein, then such conformation retains the channel properties characteristics of TeTx at physiological pH. The asymmetry of γ measured in all membranes irrespective of lipid composition (summarized in Table I) suggests that the pore forming unit is asymmetric and tends to insert into the membrane with the same orientation.

The probability of the channel being open depends both on the applied voltage and on the lipid composition of the membrane. Bursts recorded in diphyPC (very short at positive voltage, very long at negative voltage) are distinct from those recorded in egg PC bilayers, which were similar to those recorded in PS membranes (Figs. 5, 9). This implies that the lipid hydrocarbon tails may play a role in determining the burst length. Synthetic diphyPC has peculiar branched acyl chains (3,9,11,15-tetramethylhexadecanoic acid) whose backbone is comprised of 16 carbon atoms. Instead, the natural lipids used have the following percentages of fatty acids which length is $\leq$ of 16 carbon atoms: Bovine Brain PS 1%, liver PI 2%, Soy PI 31%, egg PC 37%. The extent of penetration of the pore forming unit in the bilayer hydrocarbon core may determine the transition probabilities between channel states.

The increase in γ with larger fractions of negatively-charged phospholipids can be accounted for by the enrichment of counterion concentration at the membrane surface. Considering Gouy-Boltzmann (Chapman) distribution (McLaughlin et al., 1971) the surface potential of membranes composed of diphyPC:PS (40%) and exclusively PS in 0.5 m KCl is estimated to be -49 mV and -89 mV, respectively. Considering the Gouy-Chapman-Stern formalism (McLaughlin, 1982), the effective ionic concentration at the membrane–water interface is calculated to be 2 M and 6 M, respectively. These values are ~10-fold higher than in neutral membranes. Since the TeTx channel is cation selective, the enrichment in cationic activity at the surface of negatively-charged bilayers would determine a larger γ. The results obtained on ganglioside-supplemented bilayers render support to this notion. Stable membranes cannot be formed at high concentrations of gangliosides. Considering that, at a molar fraction of 10%, the mean area occupied by a ganglioside molecule is 140–160 Å^2 (Maggio et al., 1981; Usai et al., 1983) it follows that a surface potential of only -6 mV would exist under our experimental conditions. Moreover, the ganglioside electric charges lay in a plane which is at least 10 Å away from the water–bilayer interface (Hill and Lester, 1972; McDaniel et al., 1986) and, since the Debye length in 0.5 M KCl is 4.3 Å, it follows that the surface potential due to gangliosides is negligible at the level of phospholipid headgroups. Therefore, the cationic concentration at the membrane surface is virtually equivalent to that in the bulk solution, as in membranes that do not contain negatively charged lipids. These facts, make the TeTx channel practically insensitive to the presence of G_{D1b} and G_{T1b}. This does not imply that, in the native system, gangliosides do not affect the behavior of the channel, because it is well known that glycolipids exhibit a high tendency to cluster and, therefore, to form microscopic domains where the local sialic acid concentration and, therefore, the electric charge, is particularly high.

The subconductance state observed at neutral pH within the most probable conductance level may be interpreted in terms of fast transitions between different steric conformations of the channel forming unit: Low pH would stabilize a low-conductance conformation of the channel which, at neutral pH, appears occasionally (low conductance level) and is highly unstable with respect to the most probable state (higher conductance). The possibility that a H^+-blocking mechanism accounts for this result cannot be excluded (see e.g., Prod'hom et al. 1987)

These results suggest a possible mechanism of action for the neurotoxicity of TeTx. It is known that TeTx perturbs synaptic activity by causing synaptic disinhibition at spinal cord motorneurons (for review see Mellanby and Green, 1981). It is generally accepted that gangliosides are necessary to bind and internalize TeTx into cells (Fishman 1982; Gawade et al., 1985; Helting et al., 1977; Ledley et al., 1977; Van Heyningen and Miller, 1961; Yavin et al., 1982). Penner, et al. (1986) showed that intracellularly injected TeTx inhibits exocytosis and, therefore, neurosecretion in chromaffin cells. Furthermore, it is established that TeTx migrates by retrograde axonal transport from the periphery into the central nervous system (Büttner-Ennever et al., 1981; Mellanby and Green, 1981; Schwab et al., 1979). These facts, together with our results on the channel activity of TeTx can be intergrated into the following hypothesis: Gangliosides on the extracellular surface are the receptor sites for TeTx. The ganglioside toxin complex is internalized and sequestered into an endocytic vesicle (Montesano et al., 1982; for review see Simpson, 1986). Low pH in the vesicle, activates the insertion of the channel in the vesicle membrane (Boquet and Duflot 1982; Hoch et al., 1985). Retrograde axonal transport would carry the toxin to the central synaptic area. Thereafter, a low pH vesicle would fuse with the plasma membrane of the postsynaptic cell; the voltage dependence of the channel would be such that it would promote channel opening (Borochov et al. 1984; see Fig. 1) at netural pH. Channel opening would lead to the further depolarization of the postsynaptic membrane. Thus, the activity of the TeTx channel described here at postsynaptic membranes would lead to persistent activation via continuous depolarization (Borochov et al., 1984). We are in the process of testing different aspects of this hypothesis.

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