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Tetanus toxin channel in phosphatidylserine planar bilayers: conductance states and pH dependence

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Abstract. Tetanus toxin (TeTx) forms ionic channel in phosphatidylserine bilayers. TeTx channels exhibit different modes of channel bursting activity, from a closed state to well defined open states of different amplitudes. At positive applied voltages, TeTx channels flicker continuously between a closed state and the various distinct open states. Furthermore, fast transitions into subconductance states are discernible within the bursts of channel activity. Elementary conductance steps submultiple of the open states were not identified in single channel records owing to rapid transitions between different states. However, statistical analysis shows that conductances cluster with amplitudes multiple of an elementary value: e.g. 25–30 pS at neutral pH. Single channel current amplitudes decrease with the pH of the bulk electrolyte solution. Conductance decrements can be accounted for by the relative decrease of permeant cation concentration at the membrane-water interface, by a relative enrichment of protons that block the channel or by the stabilization of a conformational state of the channel protein.

Key words: Tetanus toxin – Single channels – Phosphatidylserine – Planar bilayers

Introduction

Tetanus Toxin, a potent neurotoxin produced by the bacterium *Clostridium Tetanii*, causes paralysis in mammalian species, acting both at the central and the peripheral nervous systems (Bizzini 1979). It was proposed that, after entering the cytoplasm, the toxin undergoes retrograde axonal transport and uncouples signal transmission at the level of the postsynaptic membrane. Internalization into cytoplasmic endocytic vesicles seems to be an important intermediate step in this process (Simpson 1986).

TeTx increases membrane permeability both in planar lipid bilayers (Borochov-Neori et al. 1984; Gambale and Montal 1988; Hoch et al. 1985) and in phospholipid vesicles (Boquet and Duflot 1982; Menestrina et al. 1989). Tetanus toxin insertion into membranes depends on the pH of the ionic solution, the lipid composition of the membrane and the transmembrane potential (Boquet and Duflot 1982; Hoch et al. 1985; Gambale and Montal 1988; Menestrina et al. 1989).

Here, the occurrence of several conductance states of the TeTx channel in phosphatidylserine (PS) bilayers was characterized. We decided to use PS membranes because the single channel current amplitude is larger than in other lipids, thereby allowing the identification of possible substates. Furthermore, at positive applied transmembrane potentials, the TeTx single channel flickers continuously between a closed state and various open states, giving rise to current bursts which last for several seconds. A switch to negative voltages usually closes the channel in few milliseconds. This analysis raises defined suggestions concerning the mechanism of ionic conduction through the TeTx channel.

Materials and methods

Purified tetanus toxin was kindly provided by Dr. B. Bizzini (Pasteur Institute, Paris, France). The lipid used was bovine brain phosphatidylserine from Avanti Polar Lipids (Birmingham, AL).

Planar bilayers were formed by apposition of two phospholipid monolayers (Montal 1974). The ionic solution, 0.5 M KCl, was buffered with 10 mM Hepes and KOH at pH 7, and with citric acid and K_2HPO_4 at pHs ranging from 4 to 6. TeTx was directly added to the aqueous solution of one of the two compartments. The voltage of the TeTx-free compartment was defined as the reference voltage (see also Gambale and Montal, 1988).

The membrane current was monitored with a home made voltage-clamp amplifier and recorded on a video cassette recorder (Grundig VS 120) equipped with a PCM

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Sony F1, modified according to Bezanilla (1985). Data analysis was performed off line. Analog signals were filtered through a Kemo VBF8 filter (Kemo, Beckenham, U.K.) and digitally sampled (at a sampling rate of 140 µs per point) with a personal computer Olivetti M24 (Olivetti, Italy) equipped with a 12 bits A/D/A board Labmaster TM40 (Scientific Solutions Inc., Solon, Ohio). Current histograms were obtained from the frequency of occurrence of the 4,096 digital levels and the conductance values were calculated from current histograms best fitted with the sum of gaussian distributions (Labarca et al. 1984; Gambale and Montal 1988). Each histogram was obtained either from a single burst or by adding data collected from homogeneous contiguous bursts presenting equivalent current levels. The single channel conductance, γ, was calculated from the difference between the current peaks of the closed state and of the open state(s), divided by the applied voltage.

The frequency of occurrence of conductances at each pH (Fig. 4) was obtained from histograms such as that illustrated in Fig. 3b: the time subtended by each gaussian was assigned to the corresponding conductance and the frequency of occurrence was obtained by dividing over the total time of observation at each pH. Occurrences comprised within the same bin (bin size = 3 pS) were cumulated.

For each pH a grand mean conductance, $\langle\gamma\rangle$, was obtained by averaging the conductances measured at that pH: $\langle\gamma\rangle$ was obtained by assigning to each γ a weight proportional to the frequency of occurrence at that pH.

Single channel currents were plotted either by replaying data digitized on the personal computer system or by using an INDEC 11–23 minicomputer (INDEC, Sunnyvale, CA). For the latter, analog signals were digitized at a sampling rate of 100 µs per point.

Results

TeTx spontaneously inserts into lipid bilayers and forms transmembrane channels; channel open-closed transitions occur in current bursts. Distinct full open states are clearly identified in all membranes. Three channel bursts recorded within few minutes in the same PS membrane at pH 7, are displayed in Fig. 1. Full-open conductance levels of 113 pS, 53 pS and 24 pS were resolved in the top, middle and lower trace, respectively. Occasionally, bursts of different amplitude proceed in sequence and thus allow a transition between two distinct full open states to be discerned. This is illustrated in Fig. 2 (panel a) where a burst with two consecutive distinct full open levels is displayed. In panel b) the section of trace a), beginning where indicated by the first arrow-head, is illustrated at faster time resolution. The conductance suddenly increased from ≈30 pS to 120 pS and remained at this value for a few milliseconds. Transitions from the low conductance level to the full open level and back to the original level are clearly visible. Panels c) and d) are reproductions (at higher time resolution) of two consecutive intervals of 100 ms each, beginning in correspondence with the second arrow-head; the channel flickers between the

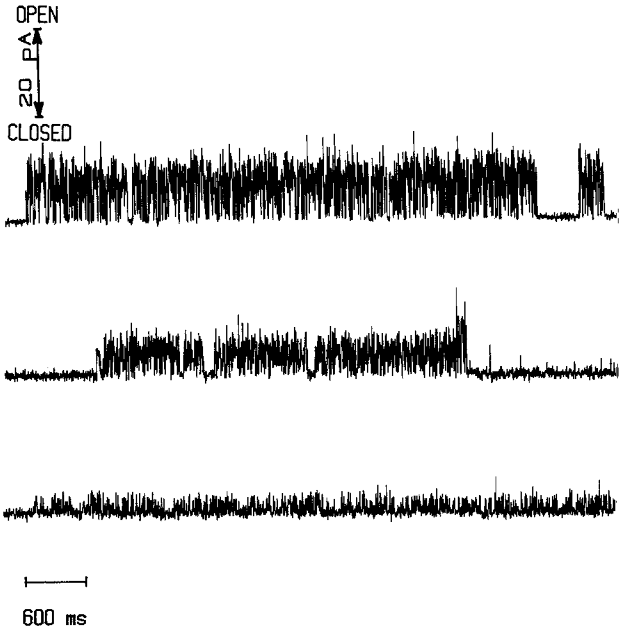


Fig. 1. TeTx single channel bursts recorded in a PS membrane at pH 7. The three bursts were recorded within few minutes in the same membrane. Full open states with conductances of 113 pS, 53 pS and 24 pS were identified. Applied voltage was $V = +100$ mV. The signal was low pass filtered at 3 KHz. The aqueous solution composition was KCl 0.5 *M*, HEPES 10 m*M*, pH 7 by addition of KOH. The tetanus toxin concentration was 25 µg/ml

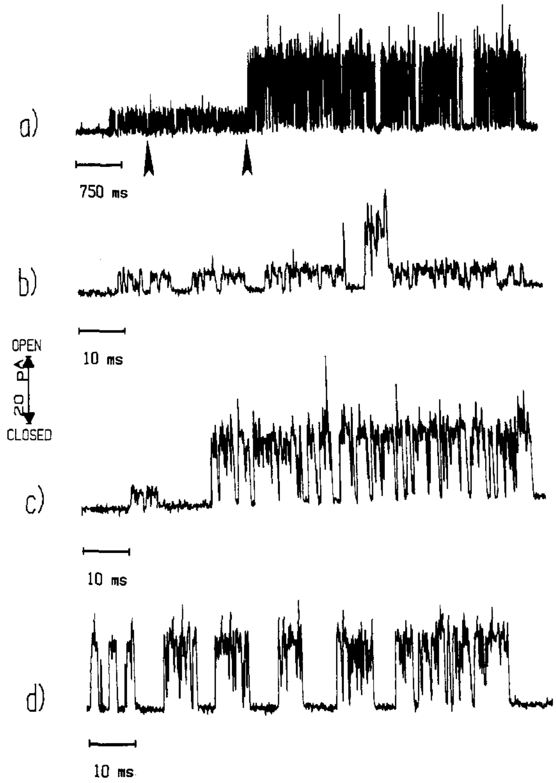
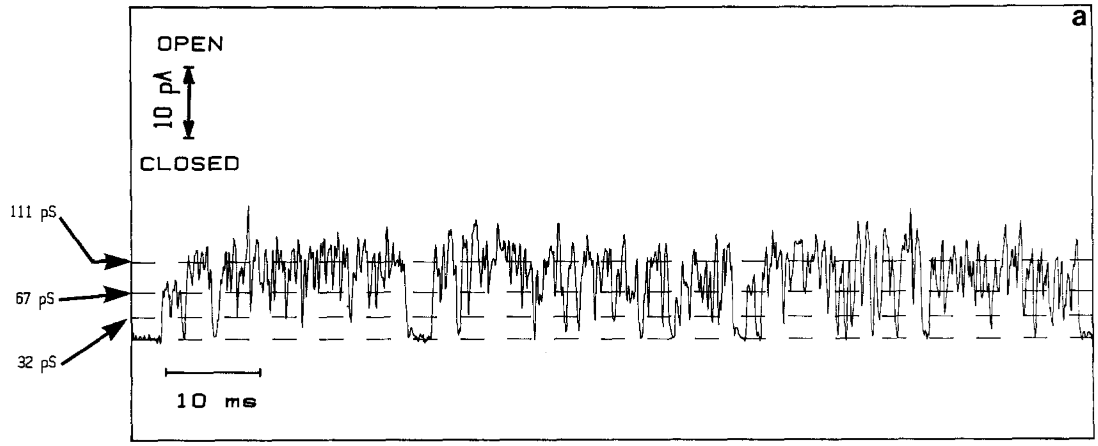


Fig. 2a–d. Signal recorded in a PS membrane at pH 7. The TeTx channel undergoes a transition from a low to a high full-conductance state (**a**). In **b**, **c**, **d** the sections of the signal beginning where indicated by the first and second arrow, respectively, are displayed at higher time resolution. Applied voltage was $V = +140$ mV. Other conditions were as for Fig. 1



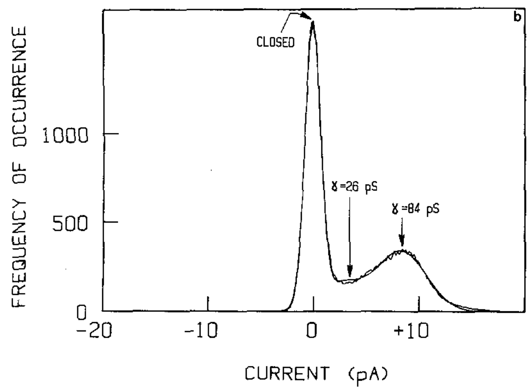


Fig. 3. **a** TeTx single channels in PS membranes at pH 7. Broken lines indicate three distinct open levels corresponding to conductance values of 32 pS, 67 pS and 111 pS. The best fit of the current histogram with the sum of three gaussian distributions as in Fig. 3b gave a full open state of 113 pS and a subconductance level of 72 pS. Other conditions were as for Fig. 1. **b** Current histogram of a TeTx single channel burst recorded in a PS membrane at pH 6 in the presence of ≈1 μg/ml of TeTx. The smooth curve represents the best fit of experimental data with the sum of three gaussian distributions, corresponding to two open states of 84 pS and 26 pS; the peak at zero current corresponds to the closed state. The signal was low-pass filtered at 1 kHz. The aqueous solution composition was: K^+ 0.5 *M*, Cl^- 0.31 *M*. pH 6 was obtained by addition of appropriate amounts of citric acid and K_2HPO_4

closed state and a new higher open level. Several direct jumps between the two extreme conductances are illustrated in d.

A clear graphic identification of the intermediate conductance states between the closed state and the full open state is difficult because of the rapid transitions that are characteristic of TeTx channel (Gambale and Montal 1988). However, few relatively long-lived substate steps can be discerned when the current signal is digitized at 100 μs per point and then displayed on the digital video display. In the record shown in Fig. 3a at least three distinct conductive levels are resolved in PS bilayers at pH 7. Broken lines correspond to conductance amplitudes of 32 pS, 67 pS and 111 pS.

Histograms representing the frequency of occurrence of current levels of TeTx bursts were usually best fitted by the sum of three gaussian distributions, thus confirming that the TeTx channel undergoes transitions between more than one conductance state (Gambale and Montal 1988). A typical current histogram obtained from a channel burst recorded in PS membrane at pH 6 is shown in Fig. 3b. The data were best fitted with the sum of three gaussian distributions (smooth curve) identifying a closed state (peak at zero current) and two open states with conductances of 26 pS and 84 pS.

The frequency of occurrence of the distinct TeTx single channel conductances, γ, in PS planar bilayers is a function of pH. A summary of results obtained at pHs ranging between 4 and 7 is illustrated in Fig. 4. Bursts collected from 16 different experiments and for a total recording time >1200 seconds were analyzed. At pH 7 the TeTx single channel conductance ranged between 20 and 120 pS (higher conductances were occasionally observed). Conductances seem to occur in clusters centered around multiples of 25–30 pS. Conductance histograms obtained at pHs 6, 5 and 4 displayed a similar pattern but with higher frequencies of lower amplitudes. A concomitant shift to the left of the histogram can be recognized, so that, at pH 4 γ ranged between 12 and 42 pS. Accordingly, the fraction of experiments presenting conductances related to groups of higher amplitude decreased on decreasing the pH. As an example, for the four clusters present in Fig. 4 and for clusters of increasing conductance, these fractions were: 1.0, 0.7, 0.3, 0.7 and 0.7, 1.0, 0.3, 0.2, at pH 7 and 5, respectively.

A quantitative evaluation of the TeTx single channel conductance at different pHs, is reported in Table 1, where the averaged conductance, $\langle\gamma\rangle$, is reported together with the potassium and proton concentration at the membrane-water interface.

Discussion

Single channel current bursts undergoing transitions between fully open states and subconductance levels have been recorded in a variety of systems (see as an example Geletyuk and Kazachenko 1985; Hamill and Sakmann 1981; Krouse et al. 1986; Mauro et al. 1988; Nelson et al. 1984). Since bursts are thought to represent open-closed transitions of the same physical entity, clusters of several units sharing common closing-opening mechanisms were

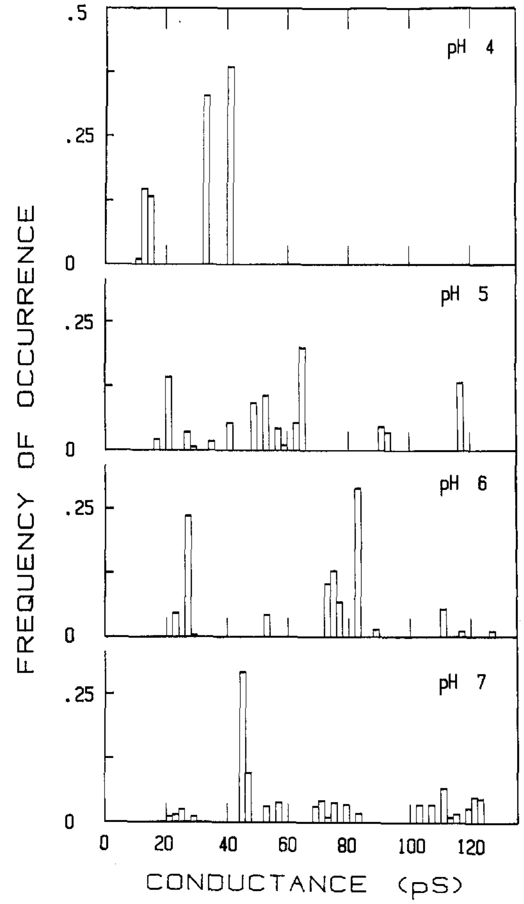


Fig. 4. TeTx single channel conductance histograms obtained at the indicated pH. Data were obtained assigning to each conductance level (obtained from a current histograms such as that in Fig. 3b) a frequency of occurrence proportional to the area of the corresponding theoretical gaussian distribution. At each pH, data were normalized with respect to the fraction of time spent by the channel in the open states. Bursts for a total observation time of 1,200 s were analyzed. Ionic solutions were KCl 0.5 *M*, HEPES 10 m*M* adjusted to pH 7 with KOH. At pHs 4, 5 and 6, K^+ was maintained constant at 0.5 *M* and appropriate amounts of citric acid and K_2HPO_4 were added to adjust the pH. TeTx concentrations were 25 μg/ml, 1 μg/ml, 0.5 μg/ml and 0.25 μg/ml at pHs 7, 6, 5 and 4 respectively

postulated to explain the behaviour of channels presenting multiple conductances (Krouse et al. 1986; Geletyuk and Kazachenko 1985; Mauro et al. 1988; Meves and Nagy 1989).

Table 1. Tetanus toxin channel conductance at different pH. The grand mean of the TeTx single channel conductance, $\langle\gamma\rangle$, at each pH was obtained by averaging the conductance amplitudes calculated from the best fit of current histograms (similar to the one reported in Fig. 3b) and assigning to the conductance a weight proportional to the area of the corresponding theoretical gaussian distribution. $[K_0]$ is the potassium concentration at the membrane surface evaluated using the Gouy-Chapman-Stern equation (McLaughlin 1982; Eisenberg et al. 1979), assuming a maximum surface charge due to PS of one elementary charge per 70 $Å^2$ and intrinsic dissociation constant for K^+ and H^+ equal to 6.66 *M* and $2.5 \cdot 10^{-4} M$ (Eisenberg et al. 1979; Tsui et al. 1986). $[H_0]$ is the proton concentration at the membrane surface calculated with the same equation. Contributions due to surface charges of the toxin were not considered but they cannot be excluded

Bulk pH	$\langle\gamma\rangle$ (pS)	$[K_0]$ (*M*)	$[H_0]$ (μ*M*)
7	70.2	5.09	1.02
6	65.6	4.99	9.99
5	60.2	4.23	84.71
4	31.0	2.13	425.62

TeTx forms ionic channels with a characteristic pattern of occurrence of single channel activity: current bursts presenting full open states of dissimilar amplitudes and, within the same burst, different subconductance levels. Consider the record shown in Fig. 2. The probability that the higher conductance results from superposition of several small independent channels is quite low since the high conductance level is at least 4 times larger than lower conductance. Several transitions occur between the full open state and the closed state. Considering a mean open time of 2 ms (Gambale and Montal 1988) the probability of 4 "minichannels" closing simultaneously, within the 100 μs digital sampling time, would be $(1-\exp(-100/2{,}000))^4$ i.e. $5 \cdot 10^{-6}$. Therefore, the occurrence of highly conductive states may arise from cooperative transitions of several molecules, interacting in the membrane phase. Accordingly, subconductances and burst transitions between full levels of different amplitudes could be different aspects of the same phenomena: a more labile or stable coordination of several interacting units.

Rapid flickering between the different states complicates the identification of an elementary current step submultiple of the higher current levels in TeTx single channels. However, histograms describing the channel conductance distribution at different pHs (Fig. 4) indicate that amplitudes are distributed along a wide range of conductance values with a few conductance peaks. For PS membranes at pH 7 four clusters are centered around conductances multiple of an elementary value of 25–30 pS. The conductances of the less conductive states are likely to be underestimated on account of the lower signal to noise ratio of these states. This suggests that the maximum conductance level may be accounted for by 4 coordinate units. Rarely and only at neutral pH, single channel conductances higher than 200 pS were observed (Gambale and Montal, unpublished results).

At pH 4, the lower conductances are centered around 13 pS, a value never observed at neutral pH. The propensity of the conductance to shift to lower values with decrements of pH can be quantitatively represented by the decrease of the grand mean conductance, as reported in Table 1. The decrement of $\langle\gamma\rangle$ and the shift to the left of the conductance histograms (Fig. 4) produced by acidification, particularly the smaller elementary conductance evident at pH 4, may be ascribed to two factors: decrease of potassium concentration at the membrane surface, $[K_0]$, or a blocking mechanism due to the increase of surface proton concentration, $[H_0]$ (see Table 1). The relative contribution of these two factors is difficult to quantitate in PS membranes because of the concomitant decrease of $[K_0]$ when $[H_0]$ increases (see Table 1). Further, a comparable analysis in neutral membranes is difficult because, at positive transmembrane voltages (which favour the incorporation of TeTx), the channel exhibits very brief bursts of considerably lower conductance. A mechanism accounting for both aspects was also invoked by Prod'hom and coauthors (Prod'hom et al. 1987; Colquhoun 1987) to explain the unusual block of L-type calcium channel by protons. The binding of protons to a protonable site external to the pore would decrease the channel conductance reducing the local surface charge created by this group. For the TeTx channel in PS membranes the surface potential could arise from contributions of both phospholipid and protein charged groups. Several features of the TeTx channel are similar to those of the L-type calcium channel (Prod'hom et al. 1987): pH dependence of the single channel conductance, fast flickering of the current and existence of short-lived intermediate states. Alternatively, the correlation between decrements of conductance with acidification could be attributed to a conformational transition of the protein (Boquet and Duflot 1982; Menestrina et al. 1989). Studies are in progress to test these models.

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References

Bezanilla F (1985) A high capacity data recording device based on a digital audioprocessor and a video cassette recorder. Biophys J 47:437–441

Bizzini B (1979) Tetanus toxin. Microbiol Rev 43:224–240

Boquet P, Duflot E (1982) Tetanus toxin fragment forms channels in lipid vesicles at low pH. Proc Natl Acad Sci USA 79: 7614–7618

Borochov-Neori H, Yavin E, Montal M (1984) Tetanus toxin forms channels in planar lipid bilayers containing gangliosides. Biophys J 45:83–85

Colquhoun D (1987) A new type of ion-channel block. Nature 329:204–205

Eisenberg M, Gresalfi T, Riccio T, McLaughlin S (1979) Adsorption of monovalent cations to bilayers membranes containing negative phospholipids. Biochemistry 18:5213–5223

Gambale F, Montal M (1988) Characterization of the channel properties of tetanus toxin in planar lipid bilayers. Biophys J 53:771–783

Geletyuk VI, Kazachenko VN (1985) Single Cl^- channels in molluscan neurones: multiplycity of conductance states. J Membrane Biol 86:9–15

Hamill OP, Sakmann B (1981) Multiple conductance states of single acetylcholine receptor channels in embryonic muscle cells. Nature 294:462–464

Hoch DH, Romero-Mira M, Ehrlich BE, Finkelstein A, DasGupta BR, Simpson LL (1985) Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: relevance to translocation of proteins across membranes. Proc Natl Acad Sci USA 82:1692–1696

Krouse ME, Schneider GT, Gage PW (1986) A large anion-selective channel has seven conductance levels. Nature 319:58–60

Labarca P, Lindstrom J, Montal M (1984) Acethylcholine receptor in planar lipid bilayers. Characterization of the channel properties of the purified nicotinic acethylcholine receptor from Torpedo Californica reconstituted in planar lipid bilayers. J Gen Physiol 83:473–496

Mauro A, Blake M, Labarca P (1988) Voltage gating of conductance in lipid bilayers induced by porin from outer membrane of *Neisseria gonorrhoeae*. Proc Natl Acad Sci USA 85:1071–1075

McLaughlin SGA (1982) Divalent cations, electrostatic potential, bilayer membranes. In: Martonosi A (ed) Membranes and transport. Plenum Press, New York, pp 51–55

Menestrina G, Forti S, Gambale F (1989) Interaction of tetanus toxin with lipid vesicles. Effect of pH, surface charge, and transmembrane potential on the kinetics of channel formation. Biophys J 55:393–405

Meves H, Nagy K (1989) Multiple conductance states of the sodium channel and of other ion channels. Biochim Biophys Acta 988:99–105

Montal M (1974) Formation of bimolecular membrane from lipid monolayers. Methods Enzymol 32b:545–554

Nelson DJ, Tang JM, Palmer LG (1984) Single-channel recordings of apical membrane chloride conductance in A6 Epithelial cells. J Membrane Biol 80:81–89

Prod'hom B, Pietrobon D, Hess P (1987) Direct measurement of proton transfer rates to a group controlling the dihydropiridine-sensitive Ca^{2+} channel. Nature 329:243–246

Simpson LL (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. Annu Rev Pharmacol Toxicol 26:427–453

Tsui FC, Ojcius DM, Hubbel WL (1986) The intrinsic pK_a values for phosphatidylserine and phosphatidylethanolamine in phosphatidylcholine host bilayers. Biophys J 49:459–468