TETANUS TOXIN FORMS CHANNELS IN PLANAR LIPID BILAYERS CONTAINING GANGLIOSIDES

HAMUTAL BOROCHOV-NEORI, EPHRAIM YAVIN, AND MAURICIO MONTAL
Departments of Biology and Physics, University of California at San Diego, La Jolla, California 92093

Although the agent responsible for the spasticity and convulsions characteristic of human tetanus was identifed at the turn of the century as the neurotoxin produced by the bacterium *Clostridium tetani* (1), the molecular basis for the action of the toxin at the central nervous system remains obscure (1, 2). Tetanus toxin (TT), a soluble protein of M_r ~150,000 in its native form, has no enzymatic activity identified thus far. It is known that TT binds specifically to gangliosides with a higher affinity to $GD1_b$ and $GT1_b$ (3). These sialo sphingolipids are especially abundant in nervous tissues. The question now arises: How is TT binding to gangliosides related to its neurotoxicity? We investigated the effect of the TT-ganglioside interaction in membranes by assaying the formation of transmembrane ionic channels in planar lipid bilayers. Asymmetric lipid bilayers (4) were formed by the apposition of a monolayer of asolectin (soybean phospholipids, AL) and a monolayer of AL supplemented with gangliosides (G). TT was added to one of the aqueous compartments and the current under voltage clamp was recorded. We found that TT forms channels only when added to the compartment in contact with the ganglioside face of the membrane. The TT channel is cation-selective and its residence time in the open state is longer than in the closed state. A preliminary account of this research appeared elsewhere (5).

The interaction of TT with asymmetric lipid bilayers was investigated in two conditions (Fig. 1). The conductance of unmodified lipid bilayers was low (≤4 pS). Addition of TT to the compartment limited by AL (lower panel) did not affect the membrane conductance even for periods longer than 1 h. In contrast, when TT was added to the compartment facing the gangliosides (upper panel), the membrane conductance increased in discrete steps and fluctuated between high and low conductance levels. This increase in conductance was due to the opening of ionic channels. Within minutes additional channels were inserted and the membrane conductance increased further. In addition, TT formed channels in symmetric bilayers containing gangliosides but not in symmetric bilayers composed exclusively of AL. Therefore, gangliosides are required for channel formation at neutral pH.[1]

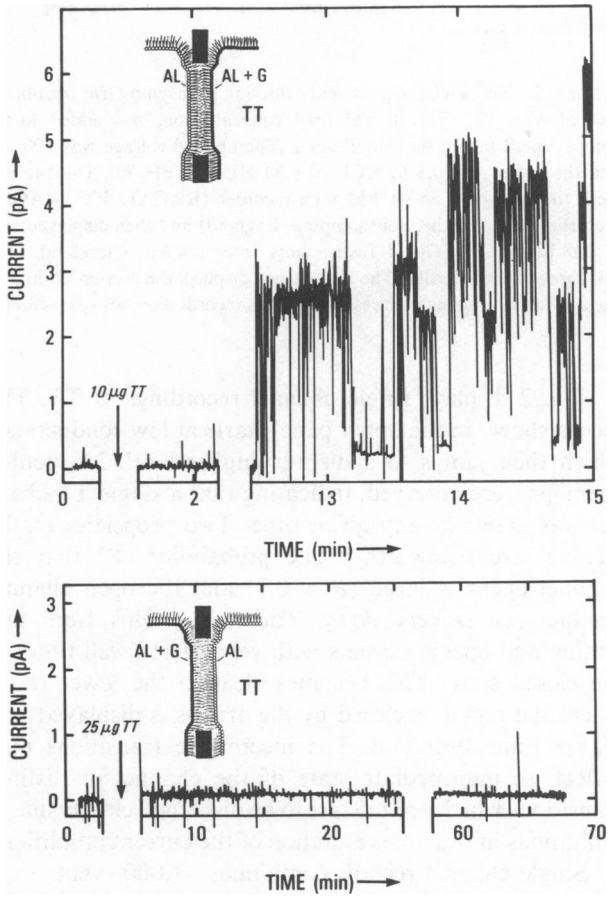

FIGURE 1 Membrane currents at a constant applied voltage through two asymmetric membranes treated with Tetanus toxin (TT). Asymmetric membranes were formed as described elsewhere (4) by the apposition of a monolayer of partially purified (6) asolectin (AL) (Sigma Chemical Co., St. Louis, MO) and a monolayer of AL supplemented with 5% purified total bovine brain gangliosides (Supelco, Inc., Bellefonte, PA) brain gangliosides (7) across a 200-μm hole in a 12-μm-thick Teflon partition separating two 1-ml capacity Teflon compartments. The hole was treated with a 0.5% solution of squalene (Sigma) in pentane. The aqueous medium was 0.1 M NaCl, 10 mM HEPES (Sigma), pH 7.0. Voltage was applied and the current measured using Ag/AgCl electrodes with a current-to-voltage converter (National Semiconductor, LF357AH) having a 1 GΩ feedback resistor; the amplifier time constant was 125 μs. The back compartment is defined as being at zero potential and positive current is the flow of positive ions into this compartment. The applied voltage was 100 mV, and purified TT (7) was added to the front compartment. The upper and lower traces are experiments where TT was added to the compartment facing the gangliosides and the compartment limited by AL, respectively. This is schematically illustrated in the *insets*. The experiments were performed at room temperature.

Dr. Yavin's permanent address is Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel.

[1]After this work was completed, P. Boquet and E. Duflot (*Proc. Natl. Acad. Sci. USA.* 79:7614–7618, 1982) reported that TT fragment B increased the passive permeability of AL lipid vesicles to K^+ only at pH lower than 5.0.

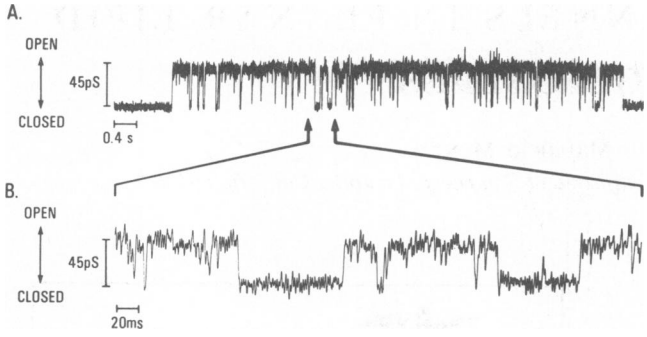

FIGURE 2 Single-channel currents through an asymmetric membrane treated with TT. TT, 10 nM final concentration, was added to the compartment facing the gangliosides. The applied voltage was 100 mV and the buffer was 0.5 M KCl, 10 mM HEPES, pH 7.0. The currents were first recorded on an FM tape recorder (RACAL 4DS, RACAL Recorders Ltd., Hythe, Southampton, England) and then displayed on a Gould Brush 220 (Gould Instruments International, Cleveland, OH) ink-spray chart recorder. The lower trace displays the section limited by the arrows at a higher time resolution. The records were low-pass filtered at 1 kHz.

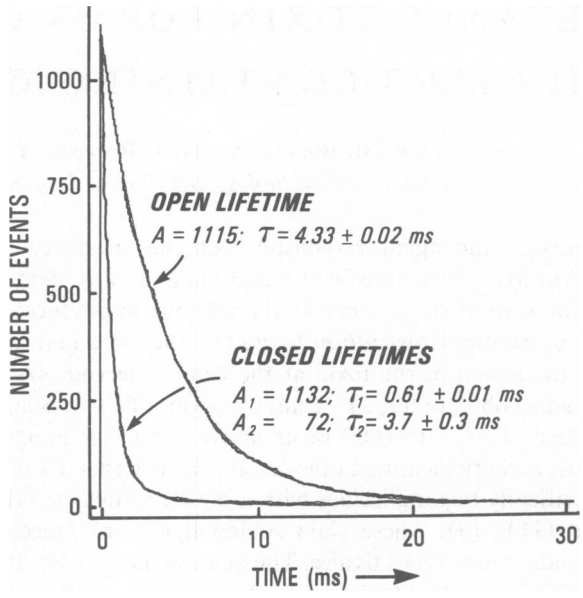

FIGURE 3 Frequency histograms of single channel open and closed times recorded at an applied voltage of 100 mV. Zero-time amplitudes (A) and time constants (τ) were determined by fitting one or two computer-generated exponentials to the data points displayed simultaneously on an oscilloscope screen. The fitted curves (smooth curve) were superimposed on the histograms of the actual data (noisy curve).

Fig. 2 displays single channel recordings of TT. The record shown in the upper panel starts at low conductance which then jumps to a distinct higher level. No double openings were observed, indicating that a single TT channel was active at any given time. Two properties of the channel are noteworthy: The probability (P) that the channel opens is large ($P \geq 0.7$) and the open channel conductance is very noisy. The noise stems from fast closing and opening events with very short dwell times in the closed state. This becomes clear in the lower trace, where the region enclosed by the arrows is displayed at a higher time resolution. The incomplete transitions may reflect an intermediate state of the channel or distinct transitions which are too fast to be discerned clearly due to limitations in the time resolution of the current amplifier.

Single-channel records containing ~1,000 events were analyzed.[2] Frequency distribution histograms of the single channel currents were bimodal: the average opening probability was ≥ 0.7. The single-channel conductance was 46 ± 3 pS in 0.5 M KCl and 40 ± 3 pS in 0.5 M NaCl. Frequency histograms of channel open times followed a single exponential function, while histograms of channel closed times were best fitted by the sum of two exponentials (Fig. 3). These results suggest that the TT channel has a single open state and two distinct closed states. The TT channel is cation-selective: reversal potential measurements yield a transference number of 0.83 for K^+. The TT channel is voltage-dependent: it activates as the compartment containing the toxin becomes more positive.

In conclusion, a consequence of the binding of TT to gangliosides is the insertion of the protein into and across the membrane. A portion of the polypeptide chain must span the lipid bilayer to act as a channel. The TT channel is cation-selective and is preferentially open. We suggest that the TT channels may account for some aspects of the neurotoxic action of TT in situ: It is known that TT perturbs synaptic function by causing synaptic disinhibition (1, 2). Thus, the activity of the TT channel described here at postsynaptic membranes would lead to persistent activation via continuous depolarization.

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ATYPICAL GRAMICIDIN A CHANNELS APPEAR TO HAVE INCREASED FIELD STRENGTH AT ONE BINDING SITE

DAVID BUSATH AND GABOR SZABO
Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

The population of discrete transmembrane channels formed by gramicidin A, whether purified or synthetic, exhibits a heterogeneous distribution of conductances characterized by a single narrow peak typically containing 65% of the channels, together with a broad dispersion of the remaining channels encompassing all conductance values below the main peak. The "standard" channels in the narrow main peak appear to result from NH_2-terminus to NH_2-terminus dimerization of left-handed, single-stranded $\beta^{6.4}$ helices (Urry et al., 1983, Bamberg et al., 1978, Weinstein et al., 1980). The "variant" channels in the low-conductance range were initially ignored (Hladky, 1972) and later attributed to the presence of double-stranded helical conformers (Ovchinnikov and Ivanov, 1977; but see Bamberg et al., 1978). Consideration of hydrogen-bonding patterns in the various dimer structures indicates that double-stranded channels should have much longer lifetimes than single-stranded, end-to-end dimer channels (Bamberg et al., 1978). We have shown that the variants have approximately the same lifetimes as standard channels, implying that variants must also be comprised of end-to-end combinations of single $\beta^{6.4}$ helices. That standard and variant channels are structurally homologous is further supported by our observation that standard channels may spontaneously change to a variant conductance state and revert back to the standard state (Busath and Szabo, 1981). It is difficult to imagine double-stranded to single-stranded conformational changes taking place so rapidly and without loss of channel conductance. Thus it is likely that variants result from secondary conformational changes which do not substantially alter the basic helical structure. At present it is not known, however, whether the arrangements of the peptide backbone or the side chains are altered. The hypothesis of altered side-chain conformation is particularly plausible because it readily allows for multiplicity of channel states with discrete interconversions, which are rare due to substantial steric hindrance (Urry et al., 1981).

This paper presents preliminary results concerning the mechanisms by which the energetics of ion permeation are altered for channels in low-conductance states. Specifically, we present evidence that most variants are caused by an increased binding affinity for cations at one end of the channel. The increased binding shows ion specificity that appears to be in qualitative agreement with the predictions of Eisenman's theory (Eisenman, 1962).

METHODS

To assess the transport energetics of individual variant channels, we measured their current voltage (*I-V*) relation. A lipid bilayer was formed on the aperture of a pipette as previously described (Prasad et al., 1982). Val^1-gramicidin A, purified from gramicidin D (ICN Pharmaceuticals, Cleveland, OH) by HPLC and stored in reagent-grade methanol at 3°C, was added to the chamber. Transmembrane current was measured while applying voltage ramps to the membrane from a dispersion (50 mg/ml) of glyceryl monooleate (GMO) (Nucheck) in squalene (Eastman Organic Chemicals Div., Rochester, NY) or hexadecane (Aldrich Chemical Co., Inc., Milwaukee, WI). The single channel *I-V* was obtained by subtracting the *I-V* measured before and after the opening of a single channel from that measured while the channel was open. For any given channel, increasing and decreasing voltage ramps gave identical *I-V*, as expected. These were therefore combined.

In symmetric bathing solutions (i.e., the same solution on both sides of the membrane) all channels have vanishingly small reversal potentials. Standard channels have symmetric *I-V* whereas variant channels, randomly interspersed between standard channels, always have asymmetric *I-V* (Busath and Szabo, 1981). We made use of the frequently employed three-barrier/two-site version of Eyring rate theory (Begenisich and Cahalan, 1980; Urban and Hladky, 1979) to assess variations in the energetics of ion transport through variant channels. In this model, ions are not allowed to cross paths within a channel. Steric restraints, energetic contributions of the aqueous solvent, and repulsion between ions are implicit in the barrier height and ion-ion interaction terms of the model.

RESULTS AND DISCUSSION

Using a nonlinear least-squares algorithm, we first determined parameters which provide good fits to the *I-V* of standard channels in aqueous 3 M, 1 M, and 0.1 M KCL solutions. These are given in Table I. The positions of the barriers and wells in the electric field determine the voltage dependence of the rate constants for ion translocation as suggested by Eyring et al. (1949). In the symmetric standard channel, $\alpha_4 = 1 - \alpha_2$ and $\alpha_5 = 1 - \alpha_1$. The simple two-site/three-barrier model gave a qualitatively reasonable but statistically unacceptable fit ($P < 0.005$). Nevertheless, for the purpose of qualitatively determining the nature of the energetic changes occurring in the variant