strains. Conductance-voltage plots, Boltzmann analysis, and ion selectivity data were determined for channels derived from each strain; the results are summarized in Fig. 3 and Table I.

These studies demonstrate that microbial outer membrane proteins can be transferred spontaneously as functioning channels into model membranes. These channels can be identified in foreign membranes through the changes in specific electrical characteristics that they impart. In their transferability, conductance properties, ion selectivity, and voltage-dependent behavior, these porins produced by pathogenic *Neisserial* cocci differ markedly from those porins that have been described from *E. coli* studies (3).

The introduction of ion channels into host membranes by cytopathic organisms may be a central process to many diseases such as the cytolytic destruction of cells by the *Entamoeba histolytica* described by Lynch et al. (4). The insertion of ion channels into host cell membranes may also supply an electrical signaling mechanism that results in the resident uptake of a microorganism within a host cell phagosome. While the ability to transfer ion channel proteins into foreign membranes may be a general property of many pathogens, our studies suggest that only those highly virulent gram-negative cocci that can effect an intracellular invasion process (such as the *Neisseria meningitidis* and disseminated strains of *Neisseria gonorrhoeae*) exhibit a significant ability to effect an intermembraneous transfer of their outer membrane porin channels.

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Note added in proof: *N. gonorrhoeae*, strain 120176-2, used in our experiments, was originally isolated from the cervix but has the 32 KD Protein I that is characteristic of disseminated gonococcal strains.

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DIPHTHERIA TOXIN FRAGMENT CHANNELS IN LIPID BILAYER MEMBRANES

Selective Sieves or Discarded Wrappers?

STANLEY MISLER
Departments of Medicine and Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110 and Albert Einstein College of Medicine, Bronx, New York 10461

The relationships of protein structure to the gating and selectivity of transmembrane ion conducting channels can be investigated using sequenced and mutatable bacterial-toxin proteins that form channels in lipid bilayer membranes. This analysis might also offer some hints as to the role of channel-forming regions of these proteins in the mechanisms of action of the toxins.

Diphtheria toxin (DT) consists of two subunits joined by a peptide and a sulfhydryl bond: an enzymatic *A* subunit, which blocks protein synthesis by inhibiting nascent chain elongation, and a carrier *B* subunit, which introduces *A* into the cell. The COOH-terminal region of *B* binds to the host's plasma membrane surface; the NH_2-terminal region, called B45, contains highly hydrophobic sequences. The cytotoxicity of DT, after membrane binding, requires the presence of pH gradients or acidic vesicles within the cell (1). The B45 region itself appears to form ion channels of sufficient diameter to accommodate an unbranched polypeptide chain such as *A* (2). To make its devastating cytoplasmic appearance, subunit *A* might either traverse an open *B* channel, just as the signal sequence of a nascent polypeptide chain crosses the rough endoplasmic reticulum (3), or, at a most propitious moment, the *A* subunit might merely "pop out" of a cavity in *B*, into which it was packaged during toxin synthesis or membrane insertion.

Using standard bilayer techniques and asolectin bilayers (2, 4), I have examined aspects of the voltage and pH gating by single channels made by both the B45 region and CRM45, a mutant DT which consists of B45 plus the entire *A* subunit. CRM45 had either an intact or "nicked" sulfhydryl bridge between the *A* and *B* subunits. These DT fragments were kindly provided to the lab by Dr. A. M. Pappenheimer, Jr. Their structural stability was periodically checked by molecular weight determination using SDS polyacrylamide gel electrophoresis. These experiments have partially characterized the voltage and pH gating of B45 and CRM45 single channels and suggest that the addition of a large hydrophilic domain to the

hydrophobic channel-forming domain does not discernibly alter channel gating or ionic conductance. Some of the B45 data have been reported in more detail elsewhere (4).

RESULTS

Shortly after nanogram aliquots of freshly-diluted B45 or CRM 45 were added to one side of the bilayer chamber, single-channel current "bursts" were seen that consisted of rapid transitions (or "flickers") between a single "open" conductance level and the baseline. The total number of channels in each bilayer was at least two, perhaps due to the dimerization of toxin molecules in free solution. In a given condition of pH and ionic strength, the single channel conductance, γ_s, was constant over a wide range of transmembrane voltages ($V_m = -160$ to $+160$ mV). γ_s, which varied from 7 pS in symmetric 0.1 M KCl, pH 4.7, to 24 pS in symmetric 1 M KCl, pH 4.7, to 50 pS in symmetric 1 M KCl, pH 5.5, was always identical for B45 or CRM45. With both molecules, the average duration of the single channel "burst" as well as the total time a channel was actually "open" within a "burst," characteristically declined e-fold for each 15–20 mV increase in V_m, in the range 80–140 mV. This is the main cause for the e-fold decline in steady-state macroscopic conductance, G_{ss}, seen with each 22–25 mV increase in V_m over the same range. The macroscopic voltage dependence is less steep because of a small increase in the single-channel opening frequency, ν, over this V_m range. At lower values of V_m, ν probably shows a steeper voltage dependence. Assuming a kinetic model in which the channel proceeds through two closed states C_2 and C_1 before opening (state O),

$$C_2 \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} C_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} 0$$

and assuming that k_2 is smaller than all other k's, transition rate constants may be determined from t_{open}, $t_{closed,\ intermediate}$ ($t_{c,i}$) and the number of interruptions within a "burst." Over a range of V_m from 80–140 mV, k_{-1} and k_{-2} are the predominantly voltage-dependent rate constants, increasing ~e-fold for each 35 mV increase in V_m. Values for $t_{c,i}$ averaged 100–200 ms for both DT fragments. "Flickers" shorter than 10–20 ms were missed because of the limited bandwidth of the recording system. Raising the pH of the solution in the compartment not containing the

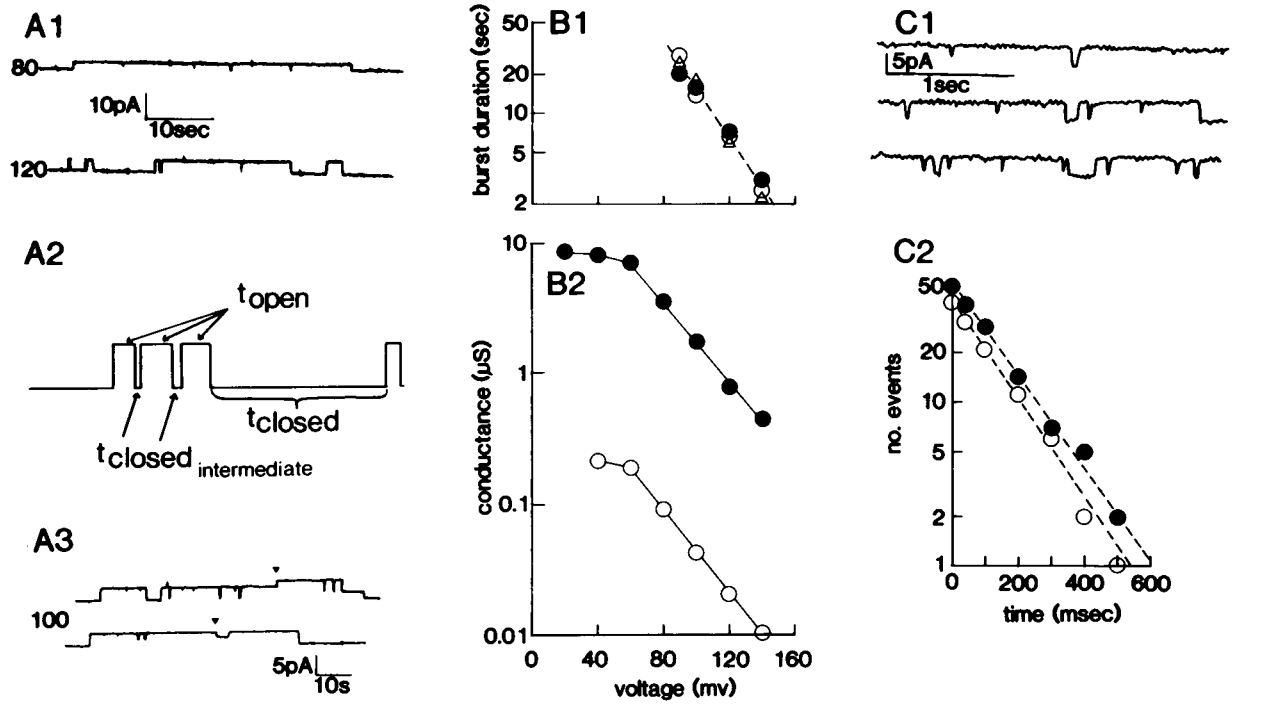

FIGURE 1 (*A1*) Single channel current "bursts" induced by freshly diluted CRM45. V_m = 80 mV and 120 mV. Solutions symmetric, 1 M KCl, pH 4.7. (*A2*) A schematic diagram of a "burst." (*A3*) "Lazy" channels and small channels (arrowheads) seen with CRM45 diluted several days before use. (*B1*) Decline in steady-state macroscopic conductance (G_{ss}) and (*B2*) single channel burst duration, seen with increasing membrane potential, V_m. Solutions contain 1 M KCl. ○, CRM45 with symmetric pH 4.7; ●, CRM45 with pH 4.7 *cis* and 7.2 *trans*; △, B45 with symmetric pH 4.7. Each pulse to V_m = 40–140 mV was preceded by a prolonged pulse to $V_m = -120$ mV. G_{ss} was achieved within 45 s of voltage transition for asymmetric pH and within two min for symmetric pH. G_{ss} declines more steeply with increasingly negative voltage pulses. At each V_m, a cumulative histogram of "burst" durations fit a single exponential whose time constant was taken as the channel "burst" duration. In this experiment, 0.3 ng CRM45 was added to the *cis* side in symmetric pH 4.7 to observe single channels, then 0.5 μg CRM45 was added to observe the macroscopic conductance, after which the *trans* pH was raised to 7.2. The remainder of the points are from different experiments. (*C1*) Data traces containing some intermediate closed flickers seen in multiple channel record of CRM45 at V_m = 100 mV. (*C2*) Comparison of cumulative histograms for the intermediate closed times ($t_{c,i}$) within channel bursts induced by B45 (○) and CRM45 (●) at V_m = 120 mV.

toxin (*trans*) from 4.7 to 7.2 (*cis*, side pH constant at 4.7) increased G_{ss} from 20- to 50-fold. In cases where a large G_{ss} was already present in symmetric pH solutions, the rate of approach to G_{ss} increased fivefold. In two experiments, returning the *trans* pH to 4.7 reduced the rate of approach to G_{ss} but not its ultimate value. Single-channel "burst" duration and flicker characteristics within a "burst" were unchanged, suggesting that by the scheme presented above the only rate affected would be the unmeasured k_2 (see Fig. 1).

DISCUSSION

These data may have implications for the mechanism of entry of DT into cells. Because imposing a pH gradient across the bilayer modifies the steady-state conductance more than it does the rate of channel opening, the data suggest that pH predominantly affects channel insertion into the bilayer. The undetectable effect of the hydrophilic *A* subunit on the single-channel conductance or "flicker" characteristics of the hydrophobic *B* channel suggests that if the *A* subunit actually crosses the open *B* channel, it might have to do so in an electrically "silent" way, i.e., not clogging the channel for more than several ms. Given that local anesthetics, which are less bulky, cause "flickering" of acetylcholine channel currents in the millisecond timescale (5), the inability to see a new class of short channel flickers with CRM45 makes this explanation less appealing. A more reasonable hypothesis is that the hydrophilic *A* subunit is folded into the *B* pore during molecular synthesis or membrane insertion, and that the orientation of the inserted toxin is such that *A* merely needs to flip out into the cytoplasm. Is the *B* channel merely a wrapping sleeve left behind after the toxic *A* subunit has popped out?

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Note added in Proof: An alternative and perhaps more definitive approach to the question posed by this note may be found in the paper by Kagan et al. in this volume.

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VIBRATIONAL ANALYSIS OF THE STRUCTURE OF CRYSTALLINE GRAMICIDIN A

VAMAN M. NAIK AND S. KRIMM
Biophysics Research Division, The University of Michigan, Ann Arbor, Michigan 48109

For the channel-forming dimeric species of gramicidin A (GA), single and double-helical structures have been proposed. Single-stranded helical structures are formed by regular intramolecular NH···O hydrogen bonds whereas double helices are formed by the association of two conformationally identical peptide strands wound about a common helix axis and are stabilized by interchain intermolecular NH···O hydrogen bonds. The present x-ray work on GA and its monovalent cation complexes has not been able to distinguish between these two types of structures in the crystalline state (1, 2). The observed dimer chain lengths and channel diameters in both ion-bound and ion-free GA crystals can be explained by single helical structures dimerized in a head-to-head or tail-to-tail fashion and by parallel or antiparallel double helical structures (3). D,L-alternating oligovalines have been studied recently with regard to their ability to form such helices. One such oligomer, namely Boc-(L-Val-D-Val)$_n$-OMe, with $n = 4$, is shown to exist in an antiparallel double stranded structure ($\uparrow\downarrow \beta^{5,6}$) by a single-crystal x-ray analysis (4). The x-ray powder diffraction patterns of Boc-(L-Val-D-Val)$_6$-OMe indicate a similar structure for this compound in the crystalline state (5).

We have studied GA and its Cs^+ and K^+ ion complexes and the L,D oligovalines using Infrared (IR) and Raman spectroscopy. Crystals of GA (commercial mixture of gramicidin A, B and C from ICN Life Sciences group, ICN Nutritional Biochemicals, Cleveland OH) and its Cs^+

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