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Mechanism of Tetanolysin-Induced Membrane Damage: Studies with Black Lipid Membranes

ROBERT BLUMENTHAL[1]* AND WILLIAM H. HABIG[2]

Section on Membrane Structure and Function, National Cancer Institute,[1] and Division of Bacterial Products, Office of Biologics, U.S. Food and Drug Administration,[2] Bethesda, Maryland 20205

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Tetanolysin produced similar rates of leakage of K^+ and hemoglobin from erythrocytes. When studied by using cholesterol-containing black lipid membranes, this hemolysin induced conductance steps with a broad frequency distribution. These findings are inconsistent with the formation of structural channels and suggest that tetanolysin acts by causing lipid perturbations.

Tetanolysin is a cytolytic toxin produced by *Clostridium tetani*. It is closely related to a group of oxygen-labile hemolysins that include streptolysin O, cereolysin, and at least 12 others (4). These toxins cause permeability changes in liposomes and biological membranes, resulting in cell lysis. The membrane "receptor" for these toxins is cholesterol (4, 18), and cells which lack this lipid are insensitive to the action of these toxins. The interaction of tetanolysin and the other hemolysins with erythrocyte membranes or liposomes results in the formation of characteristic arc- and ring-shaped structures seen by electron microscopy (7, 9, 17). The rings probably represent either aggregates of toxin or toxin-cholesterol complexes, but they have also been considered as sites of pore formation (4).

Two models for the permeability caused by this toxin have been proposed. Rottem et al. (18) hypothesized that the target membrane becomes unstable by complexation of the toxin with cholesterol, thereby perturbing the interaction of cholesterol with phospholipids. Alving et al. (1) found that in cholesterol-containing liposomes, the lytic activity of tetanolysin was not influenced by temperature or by the fatty acyl chain length of liposomal phospholipids. They concluded that cholesterol-dependent lysin-mediated damage was caused by formation of a pore consisting of a complex of toxin and cholesterol.

Tetanolysin was highly purified from crude culture filtrates of *C. tetani*, as described previously (1). Tetanolysin is a single polypeptide chain of about 45,000 daltons. Our preparations contain two isozymes of equal specific activity with isoelectric points at pH 6.1 and 6.4. These properties are similar to those described by Mitsui et al. (13). The tetanolysin had a specific activity of greater than 10^6 hemolytic units (HU)/mg of protein. The definition of an HU is arbitrary (see reference 1).

To examine the mechanism of lysis, we first studied leakage of molecules from erythrocytes. In many cases erythrocyte lysis is caused by a colloid osmotic mechanism (19). For instance, complement employs this mechanism of erythrocyte lysis. According to that mechanism, the lytic agent initially causes a permeability change for small ions or molecules. The ions or small molecules then lose their ability to serve as an osmotic balance against the macromolecules inside the cell. Consequently, the cell swells and eventually ruptures. According to the colloid osmotic mechanism, permeation of small ions or molecules precedes the leakage of the large molecules.

* Corresponding author.

Figure 1 shows tetanolysin-induced leakage of hemoglobin and K^+ in the same cell population. The rates of leakage of hemoglobin and K^+ appear to be similar. Apparently, tetanolysin forms perturbations in the membrane large enough to allow penetration of a molecule as large as hemoglobin. Treatment of erythrocytes with streptolysin O also causes the simultaneous release of ^{86}Rb and hemoglobin (8). In those experiments it was shown that extracellular bovine serum albumin does not retard these losses (8), indicating a lesion larger than either hemoglobin or bovine serum albumin. Thus, studies with tetanolysin and streptolysin argue against a colloid osmotic lytic process.

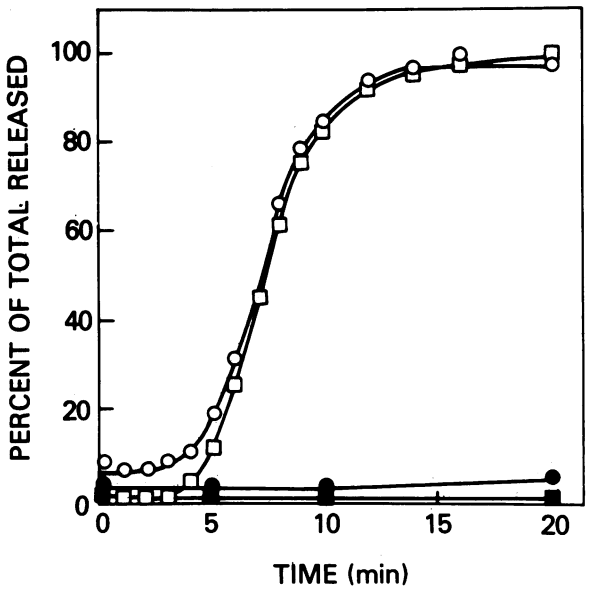

FIG. 1. Tetanolysin-induced release of K^+ and hemoglobin from erythrocytes. Tetanolysin (0.5 ml; 7,500 HU) was added to 50 ml of 0.85% NaCl–2 mM dithiothreitol–10 mM Tris-chloride (pH 7.0) containing a 1% (vol/vol) suspension of sheep erythrocytes which had been washed four times in saline. At the times shown, samples of 1.2 ml were withdrawn and centrifuged for 30 s in a Beckman Microfuge. The supernatant solutions were analyzed for hemoglobin by absorbance at 570 nm and for K^+ by using an atomic absorption spectrophotometer (Jarrell Ash; model 810). Experimental values were normalized as a percentage of the maximum value obtained after lysis in distilled water. Open symbols, with hemolysin; solid symbols, control; circles, K^+; squares, hemoglobin.

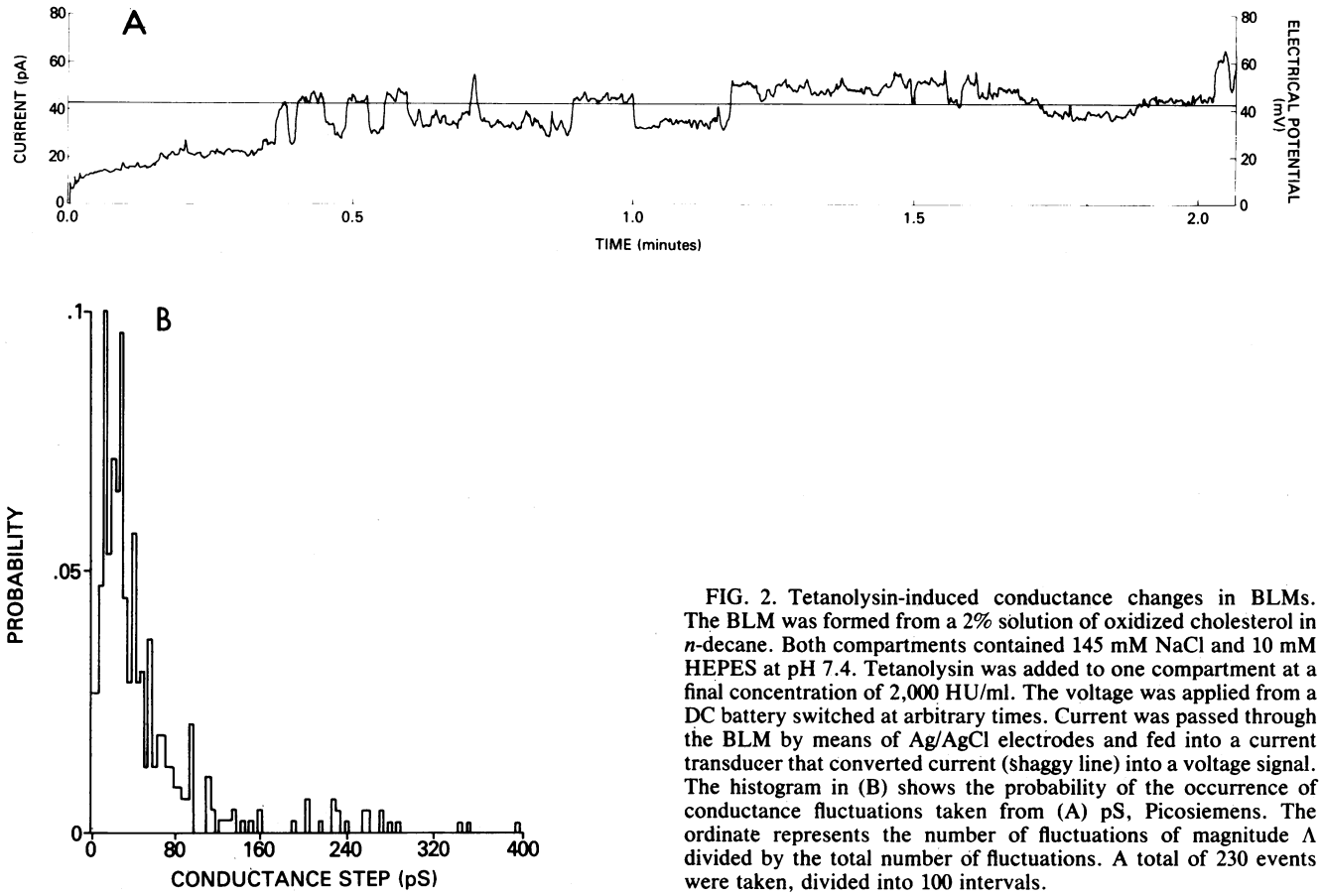

FIG. 2. Tetanolysin-induced conductance changes in BLMs. The BLM was formed from a 2% solution of oxidized cholesterol in *n*-decane. Both compartments contained 145 mM NaCl and 10 mM HEPES at pH 7.4. Tetanolysin was added to one compartment at a final concentration of 2,000 HU/ml. The voltage was applied from a DC battery switched at arbitrary times. Current was passed through the BLM by means of Ag/AgCl electrodes and fed into a current transducer that converted current (shaggy line) into a voltage signal. The histogram in (B) shows the probability of the occurrence of conductance fluctuations taken from (A) pS, Picosiemens. The ordinate represents the number of fluctuations of magnitude Λ divided by the total number of fluctuations. A total of 230 events were taken, divided into 100 intervals.

To characterize the nature of the lesion in more detail, we turned to studies with black lipid membranes (BLMs). The BLM has been used as an exquisitely sensitive assay system to study transport of ions mediated by transport entities such as carriers and channels. Changes in membrane conductance of the BLM will also occur when certain molecules perturb its structure by as yet undefined mechanisms, which have been called lipid perturbations (5). Based on an analysis of the pattern of conductance fluctuations, it is possible to distinguish between channel formation and lipid perturbations. Using that criterion we show that tetanolysin induces conductance changes by perturbing lipid bilayer structure.

BLMs were formed by spreading the membrane-forming solution under water across a circular hole (1-mm diameter) in a teflon cup (15). Each chamber contained 145 mM NaCl and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at pH 7.4. Measurements were made in a voltage clamp BLM apparatus. The membrane-forming solutions were either oxidized cholesterol (20) or a mixture of egg phosphatidylcholine (Avanti Biochemicals) and cholesterol. A portion of the tetanolysin stock solution, which contained 10^6 HU/ml, was added to one chamber of the BLM apparatus, and changes in current were measured with a very sensitive (picoamperes) current amplifier.

TABLE 1. Characteristics of tetanolysin-induced conductance in BLM

Parameter	Tetanolysin	Gramicidin
Conductance step Λ with highest frequency	28 pS	4 pS[a,b]
Histogram width ($\Delta\Lambda/\Lambda$)	2.25	0.09[a]
Concentration dependence	Nonlinear	Proportional[c]
Voltage dependence	None	None[d]
Selectivity[e]	0.8	1[f]

[a] See reference 3. pS, Picosiemens.
[b] Extrapolated to 0.15 M
[c] Log (conductance) proportional to log (concentration) (11).
[d] Some voltage dependence due to membrane thinning (2).
[e] Transference number for positive monovalent cations.
[f] See reference 16.

In our first BLM experiments using phosphatidylcholine and cholesterol we found that tetanolysin exhibited the same cholesterol specificity as in liposomes (1, 17) and cells (18): below 30% cholesterol there was no effect of tetanolysin on bilayer conductance, and above 30% the BLM ruptured (data not shown). Although BLM rupture indicated that tetanolysin grossly perturbed its structure, the phosphatidylcholine-cholesterol BLM was not amenable to the study of tetanolysin-lipid interaction, since we only observed all-or-none lysis. We therefore decided to use oxidized cholesterol BLMs, which are known to be more resistant to disruption.

Figure 2A shows the effect of tetanolysin on the conductance of an oxidized cholesterol BLM. Conductance changed in the form of a very heterogeneous pattern of fluctuations in step size, rather than in the discrete, uniform steps characteristic of channels (10). This impression is confirmed quantitatively by examining a histogram of the distribution of conductance fluctuations (Fig. 2B). Histograms of conductance steps produced by channels (e.g.,

gramicidin and porin) exhibit narrow variations in conductance step size around the mean conductance of the channel. This variation is defined as $\Delta\Lambda/\Lambda$, where Λ is the value of the conductance step size at the peak of the histogram (with the highest probability of occurrence), and $\Delta\Lambda$ is the interval covering 75% of the events around Λ. Structural channel formers such as gramicidin typically give values of less than 0.1 (Table 1). An analysis of the histogram in Fig. 2B yields a value of $\Delta\Lambda/\Lambda = 2.25$ for tetanolysin-induced conductance. Such an analysis leads us to conclude that the conductance seen with tetanolysin represents lipid perturbations rather than true channel formation.

We had seen similar heterogenous fluctuation patterns with the hepatic asialoglycoprotein receptor (6) and with melittin (12). However, in contrast to those proteins, the conductance induced by tetanolysin was not voltage dependent (data not shown).

Another indication that tetanolysin-induced conductance is mediated by lipid perturbations is its concentration dependence. For a number of agents the logarithm of the conductance has been shown to be a linear function of the logarithm of the concentration of the channel former in the medium neighboring the BLM. The concentration dependence of tetanolysin-induced conductance was quite nonlinear and complex: below a certain concentration range (approximately 2,000 HU/ml), there was essentially no conductance increase. At a threshold concentration of 2,000 HU/ml there was a steep rise in tetanolysin-induced conductance. Above 2,500 HU/ml the conductance increase was virtually independent of protein concentration.

We measured the ion selectivity of tetanolysin-induced conductance by measuring the membrane potential of the BLM as a function of salt gradient. This measurement yielded a slope of -40 mv/log decade concentration gradient for NaCl, indicating that the tetanolysin-induced conductance is cation selective, with a transference number of 0.83. This is consistent with the fact that tetanolysin is negatively charged at neutral pH. This negative charge at the mouth of the perturbation presumably permits cations to pass, whereas anions are somewhat restricted. The properties of tetanolysin-induced conductance are summarized in Table 1, and compared with the properties of a structural channel, gramicidin.

Although the internal diameter of hemolysin rings (20 to 30 nm) would be adequate to allow passage of macromolecules (7, 9, 14, 17), such structures do not appear to form holes through the membrane when examined by electron microscopy after freeze-fracture of erythrocytes (7, 14). This is consistent with our observed frequency distribution of conductance steps, indicating that the permeability pathway induced by tetanolysin is caused by lipid perturbations rather than by formation of structural channels.

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LITERATURE CITED

1. **Alving, C. R., W. H. Habig, K. A. Urban, and M. C. Hardegree.** 1979. Cholesterol-dependent tetanolysin damage to liposomes. Biochim. Biophys. Acta **551**:224–228.
2. **Bamberg, E., and P. Lauger.** 1973. Channel formation kinetics of gramicidin A in lipid bilayer membranes. J. Membrane Biol. **11**:177–194.
3. **Bamberg, E., K. Noda, E. Gross, and P. Lauger.** 1976. Single channel parameters of gramicidin A, B and C. Biochim. Biophys. Acta **419**:223–228.
4. **Bernheimer, A. W.** 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344**:27–50.
5. **Blumenthal, R., and R. D. Klausner.** 1982. The interaction of proteins with black lipid membranes. Cell Surface Rev. **8**:43–82.
6. **Blumenthal, R., R. D. Klausner, and J. N. Weinstein.** 1980. Voltage-dependent translocation of the asialoglycoprotein receptor across lipid membranes. Nature (London) **288**:333–338.
7. **Cowell, J. L., K.-S. Kim, and A. W. Bernheimer.** 1978. Alteration by cereolysin of the structure of cholesterol-containing membranes. Biochim. Biophys. Acta **507**:230–241.
8. **Duncan, J. L.** 1974. Characteristics of streptolysin O hemolysis: Kinetics of hemoglobin and ^{86}rubidium release. Infect. Immun. **9**:1022–1027.
9. **Duncan, J. L., and R. Schlegel.** 1975. Effect of streptolysin O on erythrocyte membranes, liposomes and lipid dispersions. J. Cell Biol. **67**:160–173.
10. **Ehrenstein, G., and H. Lecar.** 1977. Electrically gated ionic channels in lipid bilayers. Quart. Rev. Biophys. **10**:1–34.
11. **Goodall, M. C.** 1970. Structural effects in the action of antibiotics on the ion permeability of lipid bilayers. III. Gramicidins "A" and "S" and lipid specificity. Biochim. Biophys. Acta **219**:471–478.
12. **Kempf, C., R. D. Klausner, J. N. Weinstein, J. Van Renswoude, M. Pincus, and R. Blumenthal.** 1982. Voltage dependent *trans*-bilayer orientation of mellitin. J. Biol. Chem. **257**:2469–2476.
13. **Mitsui, K., N. Mitsui, and J. Hase.** 1980. Purification and some properties of tetanolysin. Microbiol. Immunol. **24**:575–584.
14. **Mitsui, K., T. Sekiya, Y. Nozawa, and J. Hase.** 1979. Alteration of human erythrocyte plasma membrane by perfringolysin O as revealed by freeze-fracture electron microscopy. Biochim. Biophys. Acta **554**:68–75.
15. **Mueller, P., D. O. Rudin, H. T. Tien, and W. C. Wescott.** 1962. Reconstitution of cell membrane structure *in vitro* and its transformation into an excitable system. Nature (London) **194**:979–980.
16. **Myers, V. B., and D. A. Haydon.** 1972. Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. Biochim. Biophys. Acta **274**:313–322
17. **Rottem, S., R. Cole, W. H. Habig, M. F. Barile, and M. C. Hardegree.** 1982. Structural characteristics of tetanolysin and its binding to lipid vesicles. J. Bacteriol. **152**:888–892.
18. **Rottem, S., M. C. Hardegree, M. W. Grabowski, R. Fornwald, and M. F. Barile.** 1976. Interaction between tetanolysin and mycoplasma cell membrane. Biochim. Biophy. Acta **455**:876–888.
19. **Seeman, P.** 1974. Ultrastructure of membrane lesions in immune lysis, osmotic lysis and drug-induced lysis. Fed. Proc. **33**:2116–2124.
20. **Tien, H. T., S. Carbone, and E. A. Dawidowicz.** 1966. Formation of "black" lipid membranes by oxidation products of cholesterol. Nature (London) **212**:718–719.