Black Widow Spider Venom: Effect of Purified Toxin on Lipid Bilayer Membranes

Abstract. *A purified toxin (the B_5 fraction) from black widow spider venom added to the solution on one side of a lipid bilayer membrane interacts irreversibly with the membrane to produce a continuous, linear rise of membrane conductance with time. Conductances greater than 10^{-4} reciprocal ohm per square centimeter can eventually be attained without any loss of membrane stability. Membranes treated with toxin are ideally selective for alkali cations over anions and are substantially permeable to calcium ion. These effects of the toxin result from the formation of permanent channels in the membrane of uniform conductance, 3.6×10^{-10} reciprocal ohm (in 0.1 molar potassium chloride), that remain open almost all the time. Both the divalent cation permeability and the smaller conductances at low pH of toxin-treated membranes suggest that there is negative charge (possibly from carboxyl groups) associated with the channels. We discuss the possible relation of the action of this toxin on lipid bilayer membranes to its ability to stimulate massive transmitter release at the neuromuscular junction and to produce profound morphological changes on tissue cultured neurons.*

Black widow spider venom (BWSV) provokes an enormous increase in the frequency of spontaneous miniature endplate potentials at the neuromuscular junction, ultimately leading to the depletion of cholinergic vesicles from the presynaptic nerve terminal (*1*, *2*). Thus, BWSV stimulates the events leading to transmitter release (fusion of acetylcholine-containing vesicles with the membrane of the nerve terminal followed by exocytosis) and possibly inhibits the subsequent re-formation of synaptic vesicles from the axolemma. It also induces profound morphological changes in tissue cultured neurons (*3*). These morphological changes are restricted to neurons, and particularly to regions of the neuron with low intramembranous particle density. The latter observation suggests that the venom may interact directly with membrane lipid. To examine this possibility, we tested a purified toxin from BWSV on artificial lipid bilayer membranes. We report here that this toxin interacts irreversibly with lipid bilayer membranes to form cation selective channels.

Membranes were formed at room temperature by the brush technique (*4*) across a hole in a Teflon partition separating two Lucite chambers containing identical salt solutions. After the membranes were completely black, a small volume of an aqueous solution of toxin (0.05 to 0.1 mg/ml) was stirred into one chamber to a final concentration of 0.1 to 1.0 μg/ml. Membranes were formed from either bacterial phosphatidylethanolamine (PE) (2.5 percent solution in *n*-decane) or egg lecithin plus cholesterol (2 percent lecithin plus 2 percent cholesterol in *n*-decane). Membrane conductance (g) was measured by applying a step of current (ΔI) across the membrane through a pair of electrodes (calomel electrodes coupled to the solutions through saturated KCl junctions) and recording the resulting steady transmembrane potential difference (ΔV) through the same electrodes. The conductance was then given by $\Delta I/\Delta V$. The single channel data in Fig. 1A were obtained under voltage clamp conditions.

The BWSV is a complex mixture of many proteins with varying modes of action. We used the so-called B_5 fraction (molecular weight, 130,000; isoelectric point ranging from *p*H 5.2 to 5.5), which is the active agent at the neuromuscular junction and on tissue cultured neurons (*5*). This toxin is stable at −80°C in 50 m*M* sodium borate (*p*H 8) for several months (*5*). Prior to use, a small portion of the toxin was thawed and diluted to 0.05 to 0.1 mg/ml with either distilled water or 0.1*M* KCl, containing 5 m*M* tris (*p*H 7.5). The latter solution of toxin when kept at 4°C retained activity for several days; the former suffered significant loss of activity in 18 hours (*6*).

After addition of the toxin to a lipid bilayer membrane, the conductance increases linearly with time more or less indefinitely (Fig. 1B). (We have observed such responses for more than 2 hours.) Conductances greater than 10^{-4} mho/cm^2 (that is, more than 10,000-fold higher than that of unmodified bilayer) have been achieved. The rate of the conductance increase of a given membrane is roughly directly proportional to the toxin concentration, although there is considerable variation in activity from membrane to membrane. Most of this variation is probably caused by the stability of the toxin. A freshly diluted sample appears to be more active than one that has sat for a day or two at 4°C. The PE membranes appear to be more sensitive to toxin than lecithin-cholesterol membranes, but the variability mentioned above permits only tentative conclusions. There was no indication that membrane stability was affected by the toxin.

Addition of gangliosides to the same

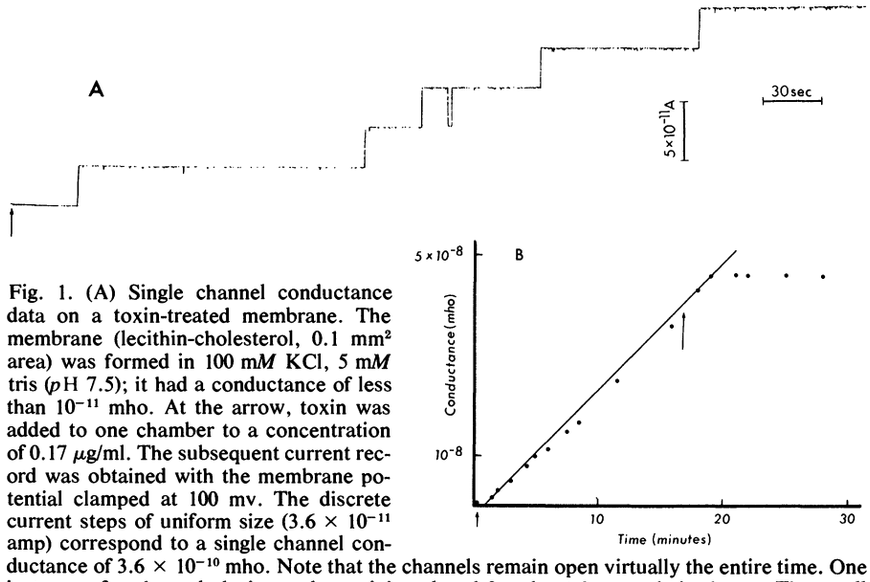

Fig. 1. (A) Single channel conductance data on a toxin-treated membrane. The membrane (lecithin-cholesterol, 0.1 mm^2 area) was formed in 100 m*M* KCl, 5 m*M* tris (*p*H 7.5); it had a conductance of less than 10^{-11} mho. At the arrow, toxin was added to one chamber to a concentration of 0.17 μg/ml. The subsequent current record was obtained with the membrane potential clamped at 100 mv. The discrete current steps of uniform size (3.6×10^{-11} amp) correspond to a single channel conductance of 3.6×10^{-10} mho. Note that the channels remain open virtually the entire time. One instance of a channel closing and remaining closed for about 3 seconds is shown. The small downward blips (that are absent before the first channel appears and become more frequent as more channels enter the membrane) may represent channel closures of too short a duration (< 300 msec) to be completely recorded. (B) The effect of purified toxin from BWSV on the conductance of lipid bilayer membranes. The membrane (PE, 1 mm^2 area) was formed in a solution containing 50 m*M* KCl, 50 m*M* NaCl, 3.3 m*M* $CaCl_2$, and 3.3 m*M* tris (*p*H 7.5); it had a conductance of less than 10^{-10} mho. At the first arrow, toxin was added to one chamber to a concentration of 0.33 μg/ml. The conductance is seen to increase linearly with time. At the second arrow, gangliosides were added to the same chamber to a concentration of 170 μg/ml. The conductance ceases to rise shortly thereafter.

chamber to which the toxin was added stopped the conductance rise, but did not reverse the response (Fig. 1B). Membranes formed with PE plus gangliosides are somewhat less sensitive to the toxin than are plain PE membranes. It thus appears that gangliosides can bind toxin but are not required (and indeed may be inhibitory) in the bilayer for toxin activity. A similar binding effect of gangliosides had previously been shown for the biological action of the toxin (*7*).

The continuous rise of membrane conductance for more than 2 hours after toxin was added to the medium implies that the toxin interacts more or less irreversibly with the membrane to form conductance pathways (Fig. 1A). The discrete current (conductance) steps of uniform size indicate formation of individual channels. The magnitude of the conductance of each of these steps (3.6×10^{-10} mho) shows that the toxin is functioning as a channel-former and not as a carrier. These channels remain "on" almost always; the occasional turning "off" or closing of a channel is of very brief duration.

The conductance of a membrane treated with the toxin is directly proportional to the salt concentration. For example, symmetrically raising the KCl concentration 7.7-fold (from 0.01*M* to 0.077*M*) produced an immediate 6.4-fold increase in conductance. The subsequent rate of rise of conductance, however, increased 28-fold rather than only 6.4-fold. It therefore appears not only that the conductance of preexisting channels is directly proportional to KCl concentration, but also that the rate of formation of new channels increases with KCl concentration.

Toxin-treated membranes are ideally selective for alkali cations. A semilogarithmic plot of membrane potential as a function of the ratio of the KCl activities on the two sides of the membrane yields a straight line with a slope of 59 mv per decade. There is no significant discrimination between Na^+ and K^+. Toxin-treated membranes also appear to have substantial permeability to Ca^{2+}. Thus with 100 m*M* KCl (plus 5 m*M* tris, *p*H 7.5) on both sides of the membrane, the addition of 1 m*M* $CaCl_2$ to one side and 10 m*M* $CaCl_2$ to the other generates a transmembrane potential of 13.5 mv (1 m*M* $CaCl_2$-side positive). The channels are reversibly titratable by acid, with a *p*K of around 5.2; the membrane conductance at *p*H 3.9 is 1/20 the conductance at *p*H 7.7 (Fig. 2).

Many proteins can increase the conductance of lipid bilayer membranes by disrupting them in a nonspecific, detergent-like manner, producing wobbling of the conductance and eventual breakage of the membrane (*8*). The effects of the purified toxin from BWSV described above are decidedly not of this class. Conductance rises monotonically and continuously with time; membranes remain stable even at very high conductances; the membranes are ideally selective for alkali cations over anions, and

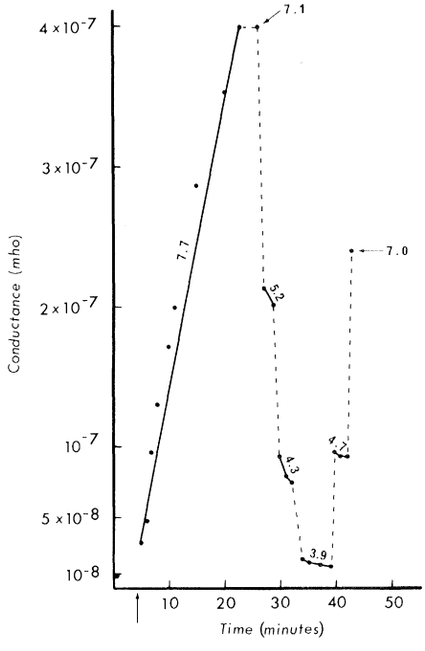

Fig. 2. The effect of *p*H on the conductance of a toxin-treated membrane. The membrane (lecithin-cholesterol, 1 mm^2 area) was formed in 100 m*M* KCl, 5 m*M* tris (*p*H 7.7); it had a conductance of less than 10^{-10} mho. Toxin from a 4-day-old stock solution was added to one chamber to a concentration of 0.17 μg/ml. After 20 minutes, the conductance had reached 9×10^{-9} mho, at which time (indicated by the vertical arrow) toxin diluted from a freshly thawed sample was added to the same chamber to a concentration of 0.33 μg/ml. (Concentration of fresh toxin plus 4-day-old toxin equals 0.5 μg/ml.) The conductance rose rapidly, and linearly, with time for the next 15 minutes, after which the *p*H was first lowered by additions of HCl to the two chambers and then raised by additions of NaOH. The dashed lines indicate the periods during which the *p*H was altered by additions of HCl or NaOH; the numbers on the graph indicate the *p*H at which the given conductances were obtained. With each reduction in *p*H there is an "immediate" fall in conductance. (After this precipitous drop there is a subsequent further slow decline of the conductance. We did not wait for a new steady conductance value to be reached at each *p*H, however, but proceeded with the *p*H changes.) Furthermore, with each elevation of *p*H there is an "immediate" rise in conductance. The *p*H effects are for the most part reversible, although there appears from the record to be some irreversible loss of toxin activity at low *p*H's.

the macroscopic conductance results from the summation of well-defined individual channel conductances. These effects are distinctive.

The permeability of the channels to divalent cation (Ca^{2+}) and their titration by acid suggest that there is a negative charge (or charges) associated with the channel. The *p*K of 5.2 is consistent with the charge being a carboxyl group.

As to the relation of these channels in lipid bilayer membranes to the effects of toxin on presynaptic terminals and tissue cultured neurons, there are at least two possibilities. (i) All of the biological effects of the toxin are sequelae to permeability changes produced by the channels; and (ii) the biological effects are more or less direct consequences of toxin interaction with the membrane. In both cases, the biological action of BWSV is triggered entirely by its interaction with the plasma membrane. Thus the toxin may not have to enter the cell or nerve terminal to have an effect.

With regard to the first possibility, the toxin might act at the neuromuscular junction by depolarizing the presynaptic terminals (*9*), thus opening the voltage-dependent calcium channels there and allowing entry of Ca^{2+} into the terminals with a subsequent release of transmitter (*10*). Alternatively, Ca^{2+} could enter the terminal through the toxin-induced channels themselves. The discrete volleys of spontaneous miniature end-plate potentials observed with BWSV in calcium-containing media (*11*) could result from Ca^{2+} entry into the presynaptic terminals by either of these means. The BWSV activity in calcium-free (EGTA-containing) media (*1*), in contrast, could result from Na^+ entry or K^+ loss (through the toxin-induced channels) triggering intracellular release of Ca^{2+} from bound pools in the terminals (*12*). The morphological changes produced by toxin in tissue cultured neurons could likewise result from changes in cytoplasmic ion concentrations (particularly Ca^{2+}) affecting membrane components or cytoskeletal components associated with the membrane (see below).

The second possibility is suggested by the experiments with tissue cultured neurons, which indicate that membrane lipids might be directly connected with toxin activity. Recent findings suggest that a redistribution of membrane components may be a crucial step in toxin action (*7*). This redistribution appears to be dependent on membrane mobility and to be modulated by cytoplasmic assemblies of microtubules and microfilaments. Thus, the conductance increases produced by

the toxin in lipid bilayer membranes may not represent the primary mechanism of its biological action. Rather, they are a manifestation that the toxin molecules can span the membrane, and hence may be capable of interacting directly with cytoplasmic filamentous systems. Such interactions in addition to producing the morphological changes in tissue cultured neurons could also affect both transmitter release and synaptic vesicle recycling in presynaptic terminals. Conceivably, the toxin has two modes of action—the permeability changes induced by the channels cause transmitter release, whereas toxin interactions with cytoplasmic filamentous systems both inhibit recovery of synaptic vesicles from the axolemma and produce morphological changes in tissue cultured neurons.

Our results with lipid bilayers might lead to the prediction of nonspecific toxin effects on biological membranes (*13*). Yet studies on tissue cultured cells demonstrate that, while toxin produces striking morphological changes in several types of neurons, it does not affect nonneuronal cells—including glial cells, fibroblasts, macrophages, and muscle cells (*7*). Therefore, specificity of toxin action must reside either in differential toxin binding by specific cell types or in differential responses of cells to uniformly bound toxin. A still unanswered question is whether the toxin binds to specific proteins in the nerve membrane or only to lipids.

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6. Bacterial PE was obtained from Supelco Inc.; egg lecithin from Sylvana Chemical Company; cholesterol from Eastman Kodak (recrystallized twice from ethanol); *n*-decane (99.9 percent) from Chemical Samples Company. A mixture of bovine brain gangliosides in physiological saline was obtained from Dr. Francesco de LaValle (Fidia Research Labs, Padova, Italy) and stored at 4°C.
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9. The toxin does not depolarize the crayfish stretch-receptor (*5*), but this does not preclude its depolarizing other membranes. Its ability to depolarize a given cell membrane depends on the number of toxin-induced channels formed and the original membrane conductance. If the former are too few or the latter too large (or both), the parallel conductance produced by the toxin-induced channels will not significantly shunt the membrane potential.
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13. The concentrations of toxin used on the bilayers are comparable with those used in the biological experiments.
14. This work was supported by NSF grant BMS 74-01139 to A.F., NIH grant NS 10883 to W. P. Hurlbut, and an NSF predoctoral fellowship to L.L.R.

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Modulation of C3b Hemolytic Activity by a Plasma Protein Distinct from C3b Inactivator

Abstract. *A human plasma protein binds to cell-bound C3b, the major cleavage product of the third component of complement. Consequent upon this binding, C3b no longer functions in either the classical or alternative pathways. This C3b inhibitory activity is a property of a protein previously designated β1H on the basis of its electrophoretic mobility.*

During complement activation, C3 (*1*) is enzymatically cleaved by either the classical or alternative pathway convertases into C3a and C3b. Biological activities of C3b include the enhancement of immune adherence and phagocytosis, continuation of the cytolytic sequence by reaction with C5 to C9, and generation of additional alternative pathway convertase by complexing with factor B and properdin. The C3b inactivator, an enzyme present in plasma, blocks these activities of C3b by cleaving it into two fragments, C3c and C3d (*2*). We have described a plasma protein, the C3b inactivator accelerator (A·C3bINA), that increases the rate at which the C3b inactivator inactivates C3b (*3*). We report here that, in addition to potentiating the activity of C3b inactivator, A·C3bINA directly binds to C3b and blocks its activities in both classical and alternative pathways.

A·C3bINA was purified (*4*) from the euglobulin fraction (*p*H 6.5) of human serum by NaCl gradient chromatography on QAE Sephadex A-50 in 0.01*M* tris-HCl containing 0.07*M* NaCl and 0.002*M* EDTA. The C3b inactivator was passed through the column with the starting buffer, and A·C3bINA was eluted, with the gradient at a conductivity of 11.0 reciprocal ohms (0°C). During subsequent chromatography on Bio-Rex 70 in 0.02*M* sodium phosphate, *p*H 7.0, A·C3bINA eluted with the NaCl gradient at a conductivity of 11.0 reciprocal ohms. In the final gel filtration step through Sephadex G-200 in 0.05*M* barbital, 0.14*M* NaCl, *p*H 7.5, A·C3bINA appeared at a volume corresponding to a molecular weight of approximately 300,000. From 1155 ml of plasma, containing 62 g of protein, 2.0 mg of A·C3bINA was obtained (*4*). On 4 percent polyacrylamide gel electrophoresis in 0.1 percent sodium dodecyl sulfate (SDS), a single dense band and two fainter bands were observed. The dense band contained more than 93 percent of the protein, as determined by staining with Coomassie blue and densitometry; its appearance was not altered by prior reduction of the sample with 0.2*M* β-mercaptoethanol in 8.0*M* urea. On standard polyacrylamide disc gel electrophoresis at *p*H 9.2 in the absence of SDS, A·C3bINA failed to enter the gel; a diffuse zone was observed in the upper 1 cm of gel. Immunoelectrophoresis of A·C3bINA against antiserum to normal human serum yielded a single arc of β mobility. Immunization of rabbits with 100 μg of purified A·C3bINA in Freund's complete adjuvant produced an antiserum that formed a dense precipitin arc identical to that observed with antiserum to normal human serum. A second faint arc in the alpha region was detected when this antiserum was used in immunoelectrophoresis against normal human serum: this arc was removed by treating the antiserum with a small amount of insolubilized (*p*H 6.5) pseudoglobulin (*5*). As shown below, the absorbed antibody neutralized the activity of A·C3bINA. In double diffusion, the monospecific antiserum produced reactions of nonidentity with antiserums to C1q, C4, C3, C5, C6, C7, C8, properdin, factor B, $C\bar{1}$ inhibitor, C3b inactivator, or any of 13 other antiserums to plasma proteins. A reaction of identity was observed with antiserum to β1H globulin (*6*), a protein previously named on the basis of