

THE NEMATOCYST EXTRACT OF *HYDRA ATTENUATA* CAUSES SINGLE CHANNEL EVENTS IN LIPID BILAYERS

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J. WEBER, T. SCHÜRHOLZ and E. NEUMANN. The nematocyst extract of *Hydra attenuata* causes single channel events in lipid bilayers. *Toxicon*, **28**, 403-409, 1990.—The nematocyst extract of *Hydra attenuata* causes single conductance events in reconstituted planar lipid membranes as well as in inside-out patches derived from liposomes. The smallest single channel conductance level of the toxins is 110 pS. The conductance levels increase stepwise with time up to 2000 pS. These large conductance jumps indicate channel cooperativity. If the membrane-voltage is changed from positive to negative values, the single channel events become undefined and noisy, indicating major reorganizations of the proteins which form the channels. The molecular properties of the ionophoric component(s) of the nematocyst extract may help explain the observed macroscopic effects, such as hemolysis of human erythrocytes, after addition of the nematocyst extract.

INTRODUCTION

NEMATOCYSTS are specialized organelles found exclusively in the fishing tentacles of the coelenterata. One of the non-marine coelenterata is *Hydra*, a freshwater polyp. When triggered by prey or predators, the nematocysts eject their contents within msec, thereby paralyzing and finally killing the target organisms (TARDENT and HOLSTEIN, 1982; HOLSTEIN and TARDENT, 1984).

The *Hydra* nematocysts contain large amounts of the divalent ions Ca^{2+} and Mg^{2+} . The divalent ion concentration inside the capsule is about 1 mole per liter (WEBER *et al.*, 1985). Approximately 75% of the nematocyst dry weight consists of soluble proteins; 80% of the protein weight is of low molecular mass ($M_r < 30,000$) and highly anionic, presumably serving as binding sites for $\text{Ca}^{2+}/\text{Mg}^{2+}$. The other 20% includes 30 different proteins, among them toxins and enzymes, but there is very low protease and no chitinase activity detectable. The extract displays a rather low divalent cation-dependent phospholipase activity on egg yolk (WEBER *et al.*, 1987). The nematocysts' soluble components are lethal for *Drosophila* flies ($\text{LD}_{50} = 2 \text{ ng/fly}$). Injection of these soluble components or a direct tentacle contact lead to the contraction of *Drosophila melanogaster* larvae. Depending on the amount injected, the larvae are partially or totally paralyzed. Unidirectional, wave-like contraction of individual muscles can be observed after injection. The number and speed

of these contractions gradually decrease in the course of time. The extract is hemolytic to human erythrocytes in the absence of divalent cations (KLUG *et al.*, 1985).

The toxins of related animals such as those of *Chrysaora* or *Chironex*, change the ionic permeability of biological membranes and of lipid bilayer membranes (BURNETT and CALTON, 1977). The addition of 5 mg sea nettle lethal factor from *Chrysaora* to black lipid membranes (BLM) lead initially to a conductance event of 31 pS at 100 mM NaCl. The conductance increases to higher multiples of 31 pS. Substitution of NaCl by 50 mM CaCl₂ or MgCl₂ abolishes the conductance events (COBBS *et al.*, 1983). The application of *Chrysaora* toxin to the nodal membrane of *Rana*, causes single conductance events of 760 ± 40 pS (DUBOIS *et al.*, 1983). SHRYOCK and BIANCHI (1983) measured a conductance of 185 pS for a single channel in BLM (Ringers solution at an ionic strength of 126 mM). A single conductance level of 30 pS is found after addition of the toxins from *Chironex* to BLM. At higher toxin concentrations (3 mg protein), which are usually lethal, the conductance levels increase in multiples of 30 pS and no Na⁺/K⁺ selectivity is detected (OLSON *et al.*, 1984). The toxins of *Stoichactis* applied to BLM (500 mM KCl) cause conductance levels which are different for a voltage of +100 mV (180 pS) and -100 mV (100 pS) (MICHAELS, 1979). VARANDA and FINKELSTEIN (1980) report a conductance value of 200 pS at 100 mM KCl for the same toxin (100 mM KCl).

The present study shows that the nematocyst extract contains at least one ionophoric component which drastically changes the cation permeability of the target membranes.

MATERIALS AND METHODS

The nematocyst extract was a gift from Dr J. WEBER (Zürich), who developed a new method for isolating undischarged nematocysts of *Hydra* (WEBER *et al.*, 1987). Lipid bilayer vesicles were prepared from 20 mg acetone-washed soybean phospholipids (Avanti, 20% PC) and 1.5 mg cholesterol (Sigma) dissolved in chloroform. The vesicles were formed after adding buffer to the nitrogen-dried lipid/cholesterol film in the round bottom flask. For the planar bilayer experiments, 20 ml buffer A (300 mM NaCl, KCl, NH₄Cl or Tris(hydroxymethyl)-aminomethan-hydrochloride (Tris-HCl), 1 mM CaCl₂, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes), pH 7) was added. In the patch-clamp experiments, 10 ml buffer B (300 mM NaCl or KCl, 1 mM CaCl₂, 10 mM Hepes, pH 7) was used. Glass beads increase vesicle formation during agitation of the bottle. The protein content of the nematocyst extract used in liposomes was 10 ng protein/mg lipid.

The planar bilayer was derived from vesicle suspensions at 20°C using the septum-supported, vesicle-derived bilayer (SVB) technique (SCHINDLER, 1980; SCHÜRHOLOZ and SCHINDLER, 1983). The nematocyst extract was added either together with the buffer to the dried lipid film in a round bottom flask, or to the aqueous phase of the compartment (*cis*-side) of the planar bilayer cell, after the vesicle suspension was replaced by buffer solution.

The potential difference across the planar bilayer was controlled by a voltage clamp circuit. The solution on the *trans*-side was maintained at zero potential. In both methods (patch-clamp, SVB) Ag/AgCl electrodes were used; positive membrane current corresponds to cation flow from the *cis*-side (toxin application) to the *trans*-side of the membrane.

For the liposome patch-clamp experiments (HAMILL *et al.*, 1981) 10 ml of the vesicle-suspension was added to the bottom of a culture dish (20 mm diameter) and then filled with buffer B (as described above). After a seal of > 10 GΩ had formed, the pipette was withdrawn from the vesicle and exposed to air, to ensure the formation of an inside-out patch (TANK *et al.*, 1982). The diameter of the vesicles used for patching was > 5 μm. Positive holding voltage was defined as a positive potential (cation-flow out) on the pipette interior of an EPC7 amplifier (LIST, Germany). The output signals were filtered at 3 kHz in both methods and recorded on a video tape recorder after digitization by a pulse code modulator, modified to accept DC signals (PCM 501, Sony).

RESULTS

The nematocysts extract of *Hydra* induced ion channels when incorporated into vesicle-derived planar bilayers and in inside-out patches of liposomes. At an extract concentration of 10 ng protein/mg lipid, conductance events were identified in about every fifth membrane patch. The area of the patch was about 0.2 μm².

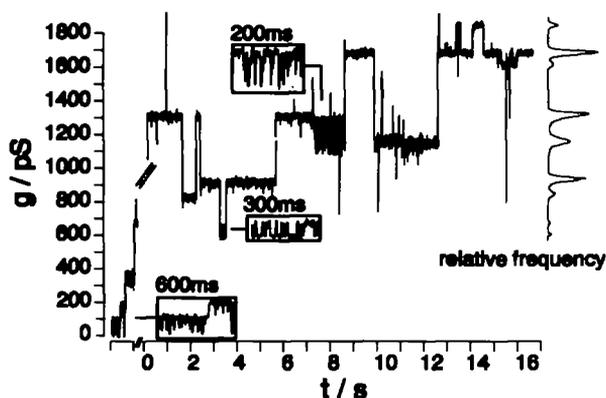


FIG. 1. SINGLE CHANNEL CONDUCTANCE (g) EVENTS CAUSED BY THE NEMATOCYST EXTRACT OF *Hydra attenuata* IN A PLANAR LIPID BILAYER, DISPLAYED ON THE TIME (t) SCALE.

Buffer in both the *cis* and the *trans* compartments of the bilayer sample cell; 0.3 M NaCl, 1 mM CaCl₂, 10 mM Na-pipes, pH 7.0, 22°C. Clamp voltage: +100 mV (*cis*-side). First conductance events are recorded 5 min after bilayer formation. At $t > 0$: typical channel closings and openings on relatively high conductance levels. Inserts: 200 msec, 300 msec and 600 msec expansions, respectively, of channel flickering. Amplitude histogram (relative frequency of conductance levels) of the time interval 0–17 sec.

When the extract was added to a vesicle-containing compartment of the planar bilayer, cell channel activity was not detected in the first 20 min after addition. The addition of the extract to the aqueous phase (after vesicle removal) led to the conductance events after 5–15 min. Interestingly, the time until channels appeared was not shorter when the planar bilayer is formed from vesicles preequilibrated with the toxin extract (time measured from membrane formation). At [Ca²⁺] = 10 mM, the time until channels appeared was 10–30 min compared with 5–15 min for solutions without Ca²⁺.

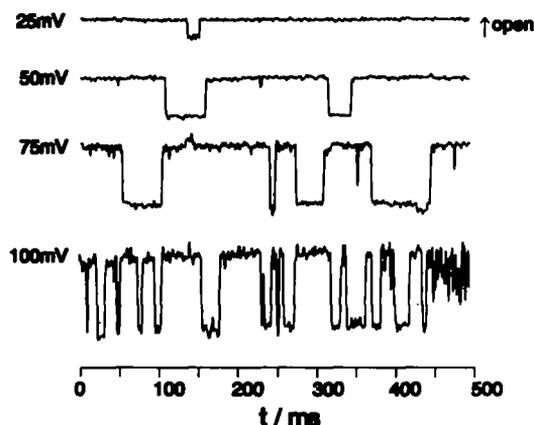


FIG. 2. CHANNEL TRACES OF IONOPHORIC COMPONENT(S) OF THE NEMATOCYST EXTRACT INCORPORATED IN LIPID LIPOSOMES; 0.15 M NaCl, 1 nM CaCl₂, 10 mM Na-PIPES, pH 7, 22°C, 10 MIN AFTER THE FIRST EVENTS OCCURRED.

The most frequent conductance event is 240 pS; opening and closing on top of a 1000 pS conductance level. Channel activity is recorded in the inside-out patch clamp mode at +25, 50, 75 and +100 mV (pipette inside positive).

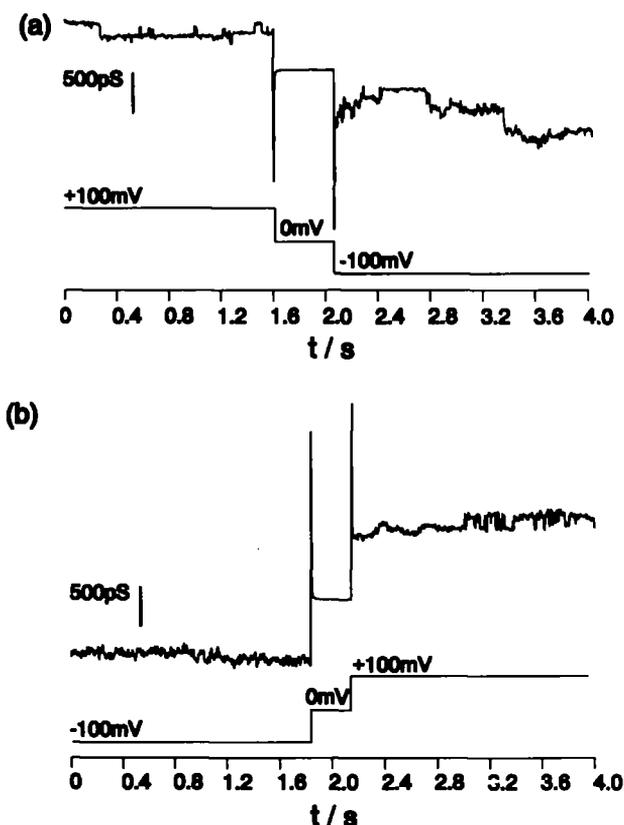


FIG. 3. CHANGE IN THE CHANNEL PATTERN OF THE IONOPHORIC COMPONENT(S) OF THE EXTRACT IN A PLANAR LIPID BILAYER AFTER CHANGING THE VOLTAGE POLARITY.

(a) From +100 mV (*cis*-side, toxin addition) to -100 mV; 0.3 N NaCl, 1 mM CaCl₂, 10 mM Napipes, pH 7.0, 22°C. (b) Return to +100 mV (reappearance of discrete channel pattern).

A typical trace of Hydra extract-induced conductance events is shown in Fig. 1. Two channel properties are readily obvious: switching between different long-lasting conductance levels as well as a frequent switching between apparently only two states (flickering; see the inserts in Fig. 1 and Fig. 2). The flickering events can last for about 5 min and occur over the whole range (about 2000 pS) of the longer lasting (open times \gg open times of flickering) conductance levels.

The smallest channel conductance is $110 \text{ pS} \pm 20 \text{ pS}$ at 300 mM NaCl or KCl. The most abundant channel amplitude was in the range of $220 \pm 25 \text{ pS}$. After extract application, only small conductance steps are seen; with time, gradually larger steps occurred, going up to a level of, e.g. 2000 pS. The ion channels, once formed, are open most of the time. Therefore, when the mean conductance has reached a larger constant value, the smaller conductance changes, especially the flickering, appear as single channels on top of a larger constant conductance level.

In the range of 0 to +100 mV the channels show an ohmic current-voltage behaviour (Fig. 2). When the voltage was reversed to a negative value (pipette interior), the clear channel events vanished within 1 sec and turned into undefined conductance changes.

TABLE 1. OPEN (τ_o) AND CLOSED (τ_c) TIMES, RESPECTIVELY, OF FLICKERING AT VARIOUS HOLDING VOLTAGES (V_h)

V_h (mV)	τ_o (msec)	τ_c (msec)
50	170 \pm 35*	19 \pm 4
75	30 \pm 5	13 \pm 3
100	19 \pm 4	6 \pm 2

*The open and closed times are mean values \pm S.D. of 6 traces. The values were computed for each holding potential and each trace from at least 200 single channel events.

After returning to positive voltage, the clear and distinct channels were again seen within 1 sec (Fig. 3).

The frequency of flickering increased with voltage (Fig. 2). In Table 1 the time constants of the open (τ_o) and the closed state (τ_c) of a flickering trace are listed as a function of voltage. Between 50 and 100 mV, (τ_o) decreased by a factor of about 10, (τ_c) by a factor of 3. The numerical values of the time constants are derived from fittings of the time open-/closed time distributions with one exponential-function $y = A \cdot e^{-t/\tau}$, where τ is either the mean open or the mean closed time.

Channel formation was measured in four different 300 mM salt solutions: NaCl, KCl, NH_4 and Tris-HCl (pH 6.8). The single conductances of the smallest steps are 110 pS (NaCl), 110 pS (KCl), 130 pS (NH_4Cl) and 55 pS (Tris-HCl). A similar conductivity profile was attributed to that of a nonselective water filled pore (LEWIS *et al.*, 1983). When Ca^{2+} was added to the aqueous phases (*cis*- and *trans*-side), containing 300 mM NaCl, the conductivity did not change significantly between $[\text{Ca}^{2+}] = 1$ mM and 10 mM.

DISCUSSION

A characteristic feature of a variety of ionophoric cell toxins is the spontaneous incorporation into membranes from the aqueous phase. The ionophoric component(s) of the nematocyst extract of *Hydra attenuata* also go into the lipid-membranes and cause ion channel formation. Compared with other pore forming molecules like gramicidin and alamethicin, the *Hydra* nematocyst extract needs a relatively long incubation time until channels can be recorded electrically. When planar bilayers are formed from vesicles containing gramicidin, channels can be recorded instantaneously after bilayer formation (unpublished results).

The ionophoric component of the nematocyst extract of *Hydra* needs about 15 min to form channels and time is the same when the vesicles used to form planar bilayers are incubated with the extract or when the extract is added to the preformed planar lipid bilayer. Therefore, it seems that the time-consuming step is not the mere incorporation of the ionophoric component(s). Rather, the data suggest that the formation of the channels involves major reorganizations of the ionophoric molecules on or within the bilayer.

In the presence of Ca^{2+} the incubation time increased. If the channel formation depends on the diffusion-controlled aggregation of ionophoric molecules similar to that of alamethicin (SCHWARZ and SAVKO, 1982) the effect of Ca^{2+} can be rationalized in terms of an increase in lateral pressure of the bilayer and the concomitant decrease of the diffusion

coefficient of the toxins. It therefore appears that the channels are caused by aggregation of several ionophoric molecules as is the case with alamethicin. The aggregation model is also supported by the size distribution of the channels. The stepwise increase in the conductance levels is different from the cooperative opening of clusters of several single channel proteins such as the nicotinic acetylcholine receptor (SCHINDLER *et al.*, 1984; SCHÜRHOLZ *et al.*, 1988). The ionophoric component forms pores up to 2000 pS, which are open most of the time. This property can disturb the transmembrane-potential by facilitating the flux of ions and water across the membrane. The observed macroscopic effects of the nematocyst extract (KLUG *et al.*, 1985) may be attributed to the properties of the ionophoric component characterized here.

After reversing the voltage polarity, the channel behavior changes within one sec. Such a small time constant suggests rearrangements of larger aggregates rather than a reorientation of a single molecule. The time constants of the open and closed states decrease with increasing voltage thereby increasing the flickering frequency. When the time constant of the open state decreased to more than that of the closed state, the probability of the channel being in the open state also decreased. But the shift from 90% to 75% open appears too small to account for an obvious functional role of this channel behaviour; the flickering is only a small change on top of a larger constant conductance.

It is likely that in the nematocysts the ionophoric components are inhibited, perhaps complexed with inhibitors so that there is no effect on the nematocyst membrane. Since hemolysis of human erythrocytes is inhibited in the presence of Ca^{2+} and Mg^{2+} solutions, $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding appears to be involved in the inactivation of the ionophoric component of the extract. In the concentration range of 1–10 mM Ca^{2+} , which does not affect bilayer stability, there is no effect on the channel activity of the reconstituted toxins. Either this concentration range is too small compared with the natural divalent ion concentration (about 1 mole per liter) of the nematocyst or the Ca^{2+} -inhibition effect may be indirect; perhaps a further component of the extract binds Ca^{2+} and, as a Ca^{2+} -complex, may inhibit the channel-active toxins.

In summary, our channel data suggest the possibility that a microscopic membrane mechanism may be responsible for the dramatic macroscopic effects of the nematocyst extract: hemolysis of erythrocytes or larvae contraction may be caused by the pore formation after incorporation of ionophoric components of the nematocyst extract.

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