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Xenorhabdus nematophila (Enterobacteriaceae) Secretes a Cation-selective Calcium-independent Porin Which Causes Vacuolation of the Rough Endoplasmic Reticulum and Cell Lysis*

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Xenorhabdus nematophila and *Photorhabdus luminescens* are two related enterobacteriaceae studied for their use in biological control and for synthesis of original virulence factors and new kinds of antibiotics. *X. nematophila* broth growth exhibits different cytotoxic activities on insect (*Spodoptera littoralis*, lepidoptera) immunocytes (hemocytes). Here we report the purification of the flhDC-dependent cytotoxin, a 10,790-Da peptide we have called α -Xenorhabdolysin (α X). We show that plasma membrane of insect hemocytes and of mammal red blood cells is the first target of this toxin. Electrophysiological and pharmacological approaches indicate that the initial effect of α X on macrophage plasma membrane is an increase of monovalent cation permeability, sensitive to potassium channel blockers. As a consequence, several events can occur intracellularly, such as selective vacuolation of the endoplasmic reticulum, cell swelling, and cell death by colloid-osmotic lysis. These effects, inhibited by potassium channel blockers, are totally independent of Ca^{2+} . However, the size of the pores created by α X on macrophage or red blood cell plasma membrane increases with toxin concentration, which leads to a rapid cell lysis.

Enterobacteriaceae of the genus *Xenorhabdus* and *Photorhabdus* are potent pathogens of various insect species (1), some strains of which are toxic for immunocompromised humans (2, 3). The basis of this infectivity is still poorly understood, although *Photorhabdus luminescens* was shown to have an oral insecticidal activity (4) because of entomotoxic proteins (5, 6). Other insecticidal toxins active after injection are also produced by *P. luminescens* (7). Genes coding for similar entomotoxins were cloned in *Xenorhabdus nematophila* (8), and various cytotoxic factors were identified in bacterial broth growth of this species (9, 10). Some of these factors are cytotoxic *in vitro* for insect immunocompetent cells and also have hemolytic activity on mammal red blood cells. Interestingly, all these

cytotoxic and hemolytic activities are absent in *P. luminescens* broth growth (10), suggesting differences in the mode of virulence of these two related entomopathogenic bacteria species. We know that these toxins have very little homology with known sequences and represent a new class of toxins (8). The first aim of this work was to study the mode of action of one of these new toxins on its cellular targets. In *X. nematophila* the existence of toxins active both on red blood cells and on hemocytes allows us to study the mode of action of these molecules on mammal cells and on insect cells. Two different hemolytic activities were identified that appear sequentially in the course of bacterial growth (10). The earliest hemolytic activity (activity C1) appears in broth growth when bacteria culture reaches the stationary phase. It lyses sheep red blood cells (SRBC)¹ but is inactive on rabbit red blood cells (RRBC) and is heat sensitive (30 min at 60 °C). It is under the control of *flhDC*, the flagellar master operon of *X. nematophila* (11). The second hemolytic activity (activity C2) appears late in the stationary phase, is heat resistant (one hour at 100 °C), active on RRBC, inactive on SRBC, and is not under the control of *flhDC*. Insertional inactivation of the *flhDC* gene in *X. nematophila* leads both to loss of C1 activity (C2 is maintained) and to a very attenuated virulence phenotype.

Because *X. nematophila* septicemia arises in the insect body, it is obvious that this bacteria is able to escape defense reactions and especially phagocytosis. The means by which entomopathogenic bacteria escape the defense reactions of insects is totally unknown. Hemocytes, the free cells in hemolymph, are the major immunocompetent cells in insects. Phagocytosis is mainly achieved by macrophage-like cells that belong to the morphotype "granular hemocyte 1" (GH1) (12). As GH1 are one of the targets of the cytotoxic activities evidenced in *X. nematophila* broth growth (10), these activities appear to be good candidates for supporting, at least in part, the immunosuppressive effect. In the present work we report the purification of the flhDC-controlled lysin. The mode of action of this lysin was studied on SRBC and on insect hemocytes. We show that insect macrophages (GH1) were the most sensitive hemocytes to the lysin and that this hemolysin was not recycled to react with multiple target cells but works as a porin. Finally, the swollen

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¹ The abbreviations used are: SRBC, sheep red blood cells; α X, α -Xenorhabdolysin; ER, endoplasmic reticulum; GH1, granular hemocyte 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HU, hemolysis unit; OD, optical density; PBFI, potassium-binding benzofuran isophthalate; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RRBC, rabbit red blood cells; TBA, tetrabutylammonium; TEA, tetraethylammonium; Vcmd, voltage clamp mode; WCR, whole cell recording; AM, acetoxymethyl ester.

appearance of hemocytes incubated with flhDC-controlled lysin prompted us to check for activity of this lysin on cell membrane permeability. We provide evidence that monovalent cation channels and larger pores are opened in the plasma membrane of the insect macrophages, leading to plasma membrane depolarization and cell death through colloid-osmotic lysis independent of Ca^{2+} movements.

EXPERIMENTAL PROCEDURES

Bacterial Strain, Production and Isolation of Hemolysin—*X. nematophila* (strain F1, phase variant I, laboratory collection) were grown in Luria Bertani broth at 28 °C. In these conditions the maximum production of flhD-dependent cytotoxic activity (C1) (10) was reached in 20-h-old cultures. A purification of the factor responsible for this activity was achieved from this C1 culture supernatant. Broth growth was centrifuged (30 min at 12,000 × g). The supernatant was immediately precipitated in 50% ammonium sulfate and centrifuged, and the supernatant was precipitated in 70% ammonium sulfate. The 70% pellet was dissolved and dialyzed against water, concentrated by lyophilization, and dissolved in low ionic strength buffer (phosphate buffer 10 mM, pH 7.8). This solution was submitted to chromatography on HiTrap Q column (Amersham Biosciences), and elution was achieved with NaCl. Positive fractions, as assayed by measuring SRBC hemolysis and insect hemocyte lysis, eluted at 150 mM NaCl were pooled, lyophilized, dissolved in water, and applied on a C18 reverse phase HPLC column. Active fractions, collected in a single and isolated peak, were immediately lyophilized. Before use, they were dissolved in phosphate-buffered saline (PBS) or HEPES buffer. In this study PBS contained 1 mM CaCl_2 and 2 mM MgCl_2 , unless otherwise stated. The titer of the lysin solution was evaluated by its hemolytic activity on SRBC (see below). Heat resistance was measured by incubating for 30 min at 60 °C prior to testing for cytotoxic and hemolytic activities. For trypsin resistance, fractions were incubated for 1 h at 37 °C with 30 units of trypsin (Sigma).

Insects, Hemocyte Monolayer Preparation, and Test for Cytotoxic Activity—Larvae of the common cutworm *Spodoptera littoralis* (lepidoptera) were reared with a photoperiod of 12 h on artificial diet at 24 °C. Three-day-old sixth instar larvae were selected and surface-sterilized with 70% (v/v) ethanol prior to collection of hemolymph in test tubes filled with anticoagulant buffer (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate, 26 mM citric acid; see Ref. 13) at 4 °C. After centrifugation, the hemocyte pellet was rinsed in PBS and resuspended in the same saline. 20 μl of hemocyte suspension were layered on glass coverslips. Hemocytes were allowed to adhere on glass for 15 min in a moist chamber at 23 °C and then gently rinsed with PBS before being used as monolayers.

Cytotoxic activity was tested on monolayers. Excess PBS was pipetted off the coverslip, replaced by 20 μl of the solution of lysin in PBS, and monolayers were incubated in a moist chamber at 23 °C. Hemocyte mortality was checked by adding 2 μl of trypan blue dye (0.04% final in PBS) and 5 min more of incubation. In a preliminary experiment, results were expressed as a percentage of mortality in total hemocyte population. In the other experiments, cytotoxic activity was expressed as percentage of dead cells among the GH1 population.

When potassium channel inhibitors tetraethylammonium (TEA) and tetrabutylammonium (TBA) were used, they were added to a solution of the lysin in HEPES buffer (pH 7.2) at concentrations of 50–300 mM. Results were expressed as percentage of dead cells in the GH1 population. Osmolarity was measured in an automatic micro-osmometer (H. Roebing, Messtechnik, Berlin).

The role of Ca^{2+} ions was tested after extensive dialysis of lysin against PBS without Ca-Mg. Monolayers were prepared in the same buffer after rinsing hemocytes several times in anticoagulant buffer (deprived of Ca-Mg and with EDTA).

Hemolytic Activity on SRBC and Titration of Lysin—Sheep red blood cells were provided by BioMérieux (Lyon, France) at 50% suspension. Before use, SRBC were extensively washed in PBS and adjusted to 5% suspension in this buffer. Tests were performed by using 50 μl of SRBC suspension to which 100 μl of the lysin solution were added. Incubation lasted 2 h at 37 °C. Then the suspension was centrifuged at 3,000 × g for 5 min. 130 μl of the supernatant were added to 770 μl of pure water, and absorbance was determined at 540 nm. One unit of hemolytic activity (1 HU) was defined as the OD measured after total hemolysis of 50 μl of a 5% SRBC suspension in 900 μl of distilled water. Hemolytic titer of a solution was calculated using the formula deduced from numerous absorbance determinations with serial dilutions of lysin: Titer (HU) = $2^{10(\text{OD}-0.72)}$. In some experiments, TBA (final concentra-

tions 100–300 mM) was added to red blood cell suspensions in HEPES buffer before incubations with hemolysin.

Production of Red Blood Cell Ghosts—Red blood cell ghosts were obtained after 5 min of incubation of one volume of a 5% red blood cell suspension in PBS and 5 volumes of distilled water. Lysed red blood cells were centrifuged (10,000 × g, 5 min), rinsed two times in distilled water, and then four times in PBS. For inhibition of hemolytic activity, lysin solutions were incubated with large amounts of ghost suspensions for 2 h at 37 °C and then centrifuged and tested for hemolytic activity.

Effect of Incubation Time and Increasing Target Cell Concentration on Hemolytic Activity—In a first series of experiments, 100 μl of different concentrations of lysin in PBS (see “Results”) were incubated with 50 μl of a 5% suspension of SRBC at 37 °C; absorbance of supernatant was determined from 0.5–24 h. In a second series of experiments, a constant amount of lysin was incubated for 2 h with increasing SRBC concentrations (5%, 10, and 20%), and the percentage of hemolysis was determined for each red blood cell concentration.

Neutral Red Uptake—Using the procedure described by Szabo *et al.* (14), hemocyte monolayers were prepared in 24-well tissue culture plates (10^6 cells per well) and incubated in PBS for 30 min at 24 °C with or without lysin at a titer that allowed vacuolation of the cells (max. 0.02 HU) but gave a low percentage of lysis in time of the experiment (30 min). Data were expressed as percentage of neutral red uptake values obtained in controls (no lysin treatment).

Measurement of Cytosolic-free Ca^{2+} Concentration—Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) was measured with fluorescent indicator fura-2 (15). For this purpose, insect hemocyte monolayer was prepared on either rectangular (20 × 7 mm) or square (10 × 10 mm) glass coverslips. After plating, cells were loaded with fura-2 after incubation for 30 min at room temperature with the extracellular solution: 124 mM NaCl, 3 mM KCl, 26 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 1.5 mM CaCl_2 , 1 mM MgSO_4 , 10 mM D-glucose (bubbled with O_2/CO_2 , 95:5) containing 5 μM of fura-2-AM and 0.02% Pluronic. $[\text{Ca}^{2+}]_i$ was monitored either by spectrofluorimetry or videomicroscopy. After rinsing, a rectangular coverslip was inserted in the quartz cuvette of a Aminco-Bowman 2 spectrofluorimeter (SLM Instruments) with an angle of 45° relative to the excitation beam. The toxin was applied directly in the quartz cuvette containing the extracellular solution, magnetically stirred, and thermostated at 25 °C. Fura-2 fluorescence was obtained by excitation of the preparation alternatively at 340 and 380 nm and by monitoring emissions (F_{340} and F_{380}) at 510 nm. The ratio of emissions at 510 nm (F_{340}/F_{380}) was recorded every 0.5 s. Alternatively, a square coverslip was transferred to the recording chamber mounted on an inverted microscope (Leica, DMIRB). Fura-2 emission was obtained by exciting alternatively at 340 and 380 nm with a rotating filter wheel (Sutter Instruments). Fluorescent signals were collected with a CCD camera (Hamamatsu), digitized, and analyzed with an image analysis software (“Acquacosmos,” Hamamatsu).

Measurement of Intracellular K^+ Concentration—To record K^+ efflux from insect hemocyte, intracellular K^+ concentration was measured with fluorescent K^+ -binding benzofuran isophthalate dye (or PBFI). For this purpose, cells were plated on a square (10 × 10 mm) glass coverslip and incubated for 30 min at room temperature with 5 μM of PBFI-AM and 0.02% Pluronic diluted in the extracellular solution. After rinsing, the coverslip was transferred to the stage of an inverted microscope (Leica DMIRB). PBFI fluorescence was obtained by exciting the preparation at 380 nm and was collected at 510 nm. Analysis and digitization were performed as described above.

Electrophysiology—For electrophysiological recordings, insect hemocyte monolayers were prepared on square (10 × 10 mm) glass coverslips. After plating, a coverslip was transferred to the recording chamber of an inverted microscope (IMT2, Olympus), continuously superfused (flow rate: 5 ml/min) with the extracellular solution described above and containing 10 mM HEPES (pH 7.4) at room temperature. Patch-clamp experiments were performed in the cell-attached and the inside-out configurations with glass microelectrodes (4–5 M Ω resistance). According to the experiment, electrodes were filled with various solutions: the extracellular solution (as described above), potassium-rich solutions comprising 150 mM potassium gluconate and 50 mM HEPES (pH 7.4) or 150 mM potassium chloride and 50 mM HEPES (pH 7.4), a TEA-based solution containing 150 mM TEA chloride and 50 mM HEPES, and a TBA-based solution composed of 150 mM TBA chloride and 50 mM HEPES (pH 7.4). For both cell-attached and inside-out experiments, the toxin αX was used at a concentration of 0.062 HU either diluted in the intra-electrode solution or bath-applied, respectively. Recordings were performed in the voltage clamp mode (Vcmd); command voltage refers to the voltage applied in the recording electrode. Transmembrane voltage (V_m) recorded in the cell-attached con-

TABLE I

Purification of α -Xenorhabdolyisin from bacteria culture medium

Pptn-dialysis: precipitation at 50–70% ammonium sulfate, pellet dissolved in water and dialyzed against 10 mM phosphate buffer. HiTrap Q: anion exchange column (Amersham Biosciences). C18: HPLC phase reverse chromatography. Act.: activity (hemolysis).

Purification steps	Vol. (ml)	Act./100 μ l	Total act.	Yield
Crude extract	620	0.5	3100	/
Pptn-dialysis	19	10.4	1984	64%
HiTrap Q	7	18.1	1271	41%
C18	0.9	16	144	4.6%

TABLE II

Insect hemocyte cytolysis and red blood cell hemolysis

Insect hemocyte cytolysis and red blood cells hemolysis induced by purified α X are compared to *X. nematophila* culture supernatant with C1 activity (10). Lysin concentration was lower in tests for hemocytes (0.2 HU) than in tests for red blood cells (0.85 HU). NT, not tested.

	<i>S. littoralis</i> hemocytes	SRBC	RRBC
	percent of lysis ^a	OD ^b	OD ^b
Control (Ca-Mg)	11.5 \pm 5.7	0.01 \pm 0.02	0.030 \pm 0.001
PBS (with Ca-Mg)	C1	89.3 \pm 3.7	0.710 \pm 0.01
	α X	76.2 \pm 4.9	0.696 \pm 0.062
Temperature (60 °C, 30 min)	C1	15.9 \pm 4.4	0.033 \pm 0.02
	α X	12.9 \pm 4.3	0.054 \pm 0.01
Trypsin (30 units, 1 h)	C1	13.2 \pm 3.7	0.041 \pm 0.01
	α X	14.7 \pm 7.2	0.034 \pm 0.009
PBS (w/o Ca-Mg)	C1	90.6 \pm 7.2	0.700 \pm 0.1
	α X	77.6 \pm 5.1	0.684 \pm 0.057
SRBC ghosts	C1	12.8 \pm 3.0	0.031 \pm 0.06
	α X	10.5 \pm 2.2	0.026 \pm 0.007

^a Means \pm S.E., on 12 \times 2 monolayers from 6 \times 2 larvae.

^b Means \pm S.E., on 4 measurements from 6 different extracts. For SRBC total hemolysis gave an OD of 0.72.

figuration equals $V_i - V_{cmd}$, where V_i is the voltage of the inner face of the patch (16). On graphs, only V_{cmd} is given because V_i is not known. The voltage-dependence of the currents recorded in the presence of α X in the cell-attached mode was studied by stepping V_{cmd} from -80 to $+80$ mV with an increment of 20 mV. In this protocol, each voltage step lasted 2 s. Single channel currents were recorded with a patch-clamp amplifier (Axopatch 200 B, Axon Instruments) and digitized (Digidata 1200 Interface, Axon Instruments). Signals were filtered at 1 kHz and sampled at 10 kHz. Continuous recording and analysis of the currents were performed with John Dempster's softwares 'WinCDR' and 'WinWCP.'

Osmotic Protection—Possible osmotic protection of insect hemocytes and SRBC was tested with protectants of different sizes: polyethylene glycol 6,000 and 4,000 and dextran 1,000, all at 30 mM, and raffinose (MW 504) and sucrose (MW 342), both at 50 mM. These protectants were added to the lysin solutions, and hemocytes or red blood cells were incubated as described above. In a series of experiments, after incubation and measurement of the optical density of the supernatant (hemolysis), the red blood cell pellet was resuspended and incubated 5 min more in PBS and measured again for hemolysis. For cytolysis, two series of monolayers were incubated with lysin and protectant. In one series, the percentage of macrophage lysis was determined at the end of incubation. In the other series, at the end of incubation monolayers were washed and incubated 5 min more in PBS. Then the percentage of lysis was determined and compared with that obtained without rinsing the cells.

Electron Microscopy—Hemocyte monolayers were incubated for 0.5 h with lysin diluted in PBS (titer 0.02 HU) or in PBS for control, fixed in 5% glutaraldehyde, then in 1% osmium tetroxide, and embedded in Epon. Ultra-thin sections were stained according to Reynolds (17).

RESULTS

Purification of the Lysin—Purification of the lysin was achieved as described under "Experimental Procedures" and is summarized in Table I. Matrix-assisted laser desorption ioni-

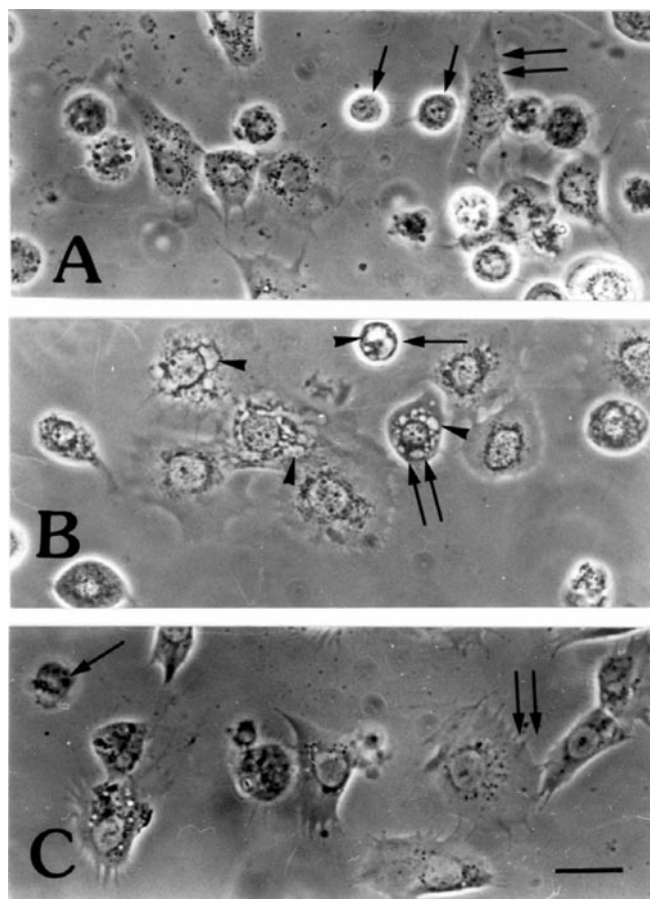


FIG. 1. **Vacuolation of insect hemocytes by α X and inhibition by TBA.** Hemocytes monolayers were incubated for 30 min in PBS without toxin (A), in the presence of 0.02 HU of α X (B), or with 0.02 HU of α X and 50 mM TBA (C). Arrowheads, vacuoles in macrophages (arrow) or in plasmatocytes (double arrow). Bar = 10 μ m.

zation time-of-flight analysis of the C18 active fraction gives only one peak with a MW of 10,790. The profile mass fingerprint (PMF) after trypsin digestion of this molecule was determined (thanks to N. Galeotti, P. Marin, and E. Demay from Centre CNRS INSERM de Pharmacologie et Endocrinologie, Montpellier, France). This PMF was used to search protein data bases, but the analysis did not yield any protein identification. We called this lysin α -Xenorhabdolyisin (α X). It was hemolytic for SRBC but not for RRBC (Table II).

Vacuolation and Lysis of Hemocytes—The effects of different dilutions of α X on *S. littoralis* hemocytes were compared with the effects of culture supernatant with C1 activity (10) under the same experimental conditions (Table II). With α X solutions of 0.02 HU or more, death of the hemocytes occurred by necrosis, as tested by trypan blue uptake, in less than one hour. The main hemocyte types, which are plasmatocytes (Pl) and Granular Hemocytes 1 (GH1, insect macrophages), were unequally sensitive, with GH1 showing a higher percentage of lysis than Pl did for the same α X titer (not shown). Therefore, most of the numerations reported in this study were achieved on GH1 alone. Before lysis, Pl and GH1 exhibited extensive vacuolation (Fig. 1, A and B), suggesting a modification of plasma membrane permeability by the toxin. Neutral red uptake quantification did not show any significant difference between vacuolated cells and untreated hemocytes (not shown). Transmission electron micrograph of α X-treated cells showed the presence of numerous ribosomes on the cytoplasmic side of the vacuole membrane. These vacuoles were dilated endoplasmic reticulum cisternae and perinuclear cisterna (Fig. 2). Other cellular or-

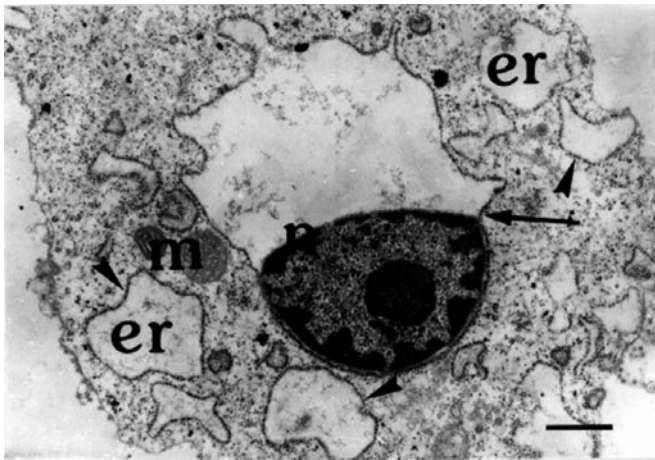


FIG. 2. α X triggers endoplasmic reticulum vacuolation in insect hemocyte (0.02 HU for 30 min). Vacuoles are dilated vesicles of rough endoplasmic reticulum (er) or nuclear cisterna (arrow). Mitochondria, m. Nucleus, n. Ribosomes, arrowheads. Bar = 1 μ m.

ganelles, especially mitochondria, Golgi apparatus, or lysosomes appeared only very slightly altered, if any.

Cytolytic and hemolytic activities were lost after incubation of α X or C1 broth growth at 60 °C for 30 min or incubation in the presence of trypsin (Table II). The lytic effects on hemocytes and on SRBC were still observed in a non-added calcium medium (Table II). The absence of Ca^{2+} did not alter the difference in sensitivity between plasmatocytes and granular hemocytes 1 to α X (not shown).

α X Molecules Are Not Recycled—To test for the possible recycling of α X after a first exposure to cells, we conducted three kinds of experiments. In the first series, a suspension of SRBC was incubated with different dilutions of α X (Fig. 3). Incubations lasted up to 24 h with a measurement of OD at different incubation times. In these experiments we observed an increase of hemolysis up to 2 h, and then the OD reached a plateau value. Such long term incubations were not performed with insect hemocytes.

In the second series of experiments, increasing SRBC concentrations (5–20%) were incubated for 2 h in 0.2 or 0.05 HU of α X. Fig. 4 shows that the percentage of hemolysis elaborated decreased with increasing target cell concentration.

In the last series of experiments, different dilutions of α X were first incubated with SRBC ghosts, and then the supernatant was further incubated with a 5% suspension of SRBC or with *S. littoralis* hemocyte monolayers. Neither hemolytic nor cytolytic activity was detected (Table II).

Effects of α X on Hemocyte Ca^{2+} and K^+ Ions Concentrations—We have investigated whether potential toxin-mediated modifications of membrane permeability could result in a change in hemocyte Ca^{2+} and K^+ cytosolic concentrations. To record $[\text{Ca}^{2+}]_i$ changes, fura-2-loaded cells were exposed to increasing concentrations of α X (0.032–0.25 HU), and the fluorescence ratio 340/380 of the hemocyte monolayer was recorded at 510 nm in a spectrofluorimeter. Toxin at all these concentrations led to cell death. At a concentration of 0.25 HU, a transient rise in $[\text{Ca}^{2+}]_i$ could be recorded that was apparently regulated quickly. Lower concentrations of toxin, ranging from 0.062 to 0.032 HU, led to a dose- and time-dependent increase in $[\text{Ca}^{2+}]_i$ (Fig. 5A). In a Ca^{2+} -free external medium (no added Ca^{2+}), the toxin at a concentration of 0.25 HU had almost no detectable effects. Therefore, this suggests that the toxin-induced $[\text{Ca}^{2+}]_i$ rise results from a Ca^{2+} influx from the external medium (Fig. 5A). The analysis of the fura-2 emissions at 510 nm, obtained by exciting at 340 (F340) and 380 nm

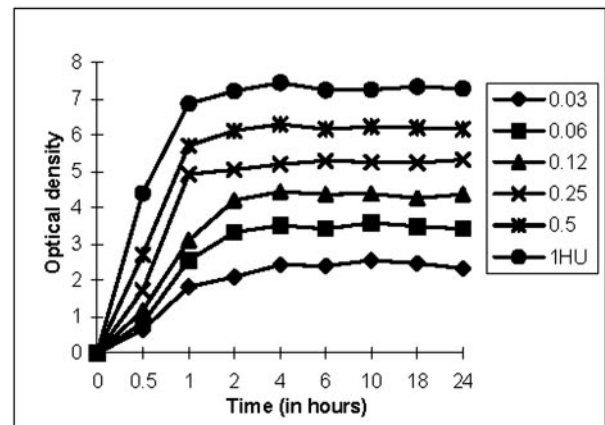


FIG. 3. Time course of α X-induced hemolysis of SRBC. 5% SRBC were incubated with 0.03–1 HU of α X for 0.5–24 h and centrifuged; optical density of supernatant was then determined. This result is representative of three distinct experiments.

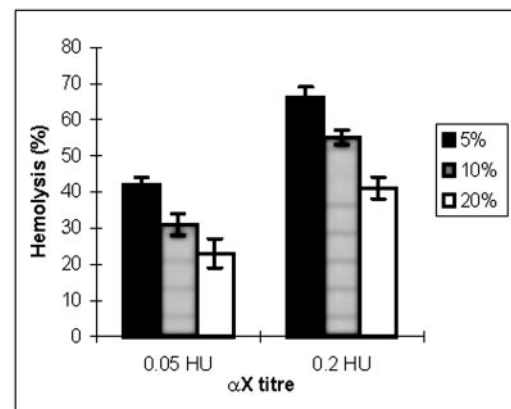


FIG. 4. Decrease in percent hemolysis with increase in SRBC concentration. 5, 10, and 20% SRBC in PBS were incubated for 1 h with 0.05 or 0.2 HU of α X. Results were expressed as a percentage of total hemolysis. Data are means of three distinct experiments \pm S.E.

(F380), indicates that although the initial increase in fluorescence ratio in the presence of α X was effectively because of an increase in $[\text{Ca}^{2+}]_i$ as indicated by the variations of fura-2 emissions in opposite directions (Fig. 5B), the decrease of fluorescence ratio more likely reflects leakage and dilution of the probe in the medium because both F340 and F380 decrease in parallel. The toxin at 0.062 HU elicits rapid and unregulated increases in $[\text{Ca}^{2+}]_i$ in visually identified GH1 and plasmatocytes, as observed using videomicroscopy (Fig. 5C). Observation in phase contrast microscopy of these cells during the experiment confirms that the cells were lysed at the time of $[\text{Ca}^{2+}]_i$ rise (Fig. 5D). Because the toxin is able to induce cell death in the absence of external calcium, it can be postulated that the increase in $[\text{Ca}^{2+}]_i$ detected upon exposure of cells to the toxin is not responsible for its toxicity but more likely reflects ionic disturbance across cell membrane and cell lysis.

Cytoplasmic concentration of K^+ was monitored in PBFI-loaded hemocytes. At each α X (0.02 HU) application to the medium, an immediate loss of K^+ could be recorded that was in part regulated up to the cell lysis. This lysis was evidenced by a large decrease in PBFI fluorescence, revealing leakage of the probe in the medium as illustrated in three visually identified GH1 (Fig. 5E).

Channels Opened on Insect Macrophage Membrane by α X Are Monovalent Cation-selective—From the videomicroscopy data, one can hypothesize that α X mediates to an ionic imbalance, leading to cell death. This imbalance could initially be

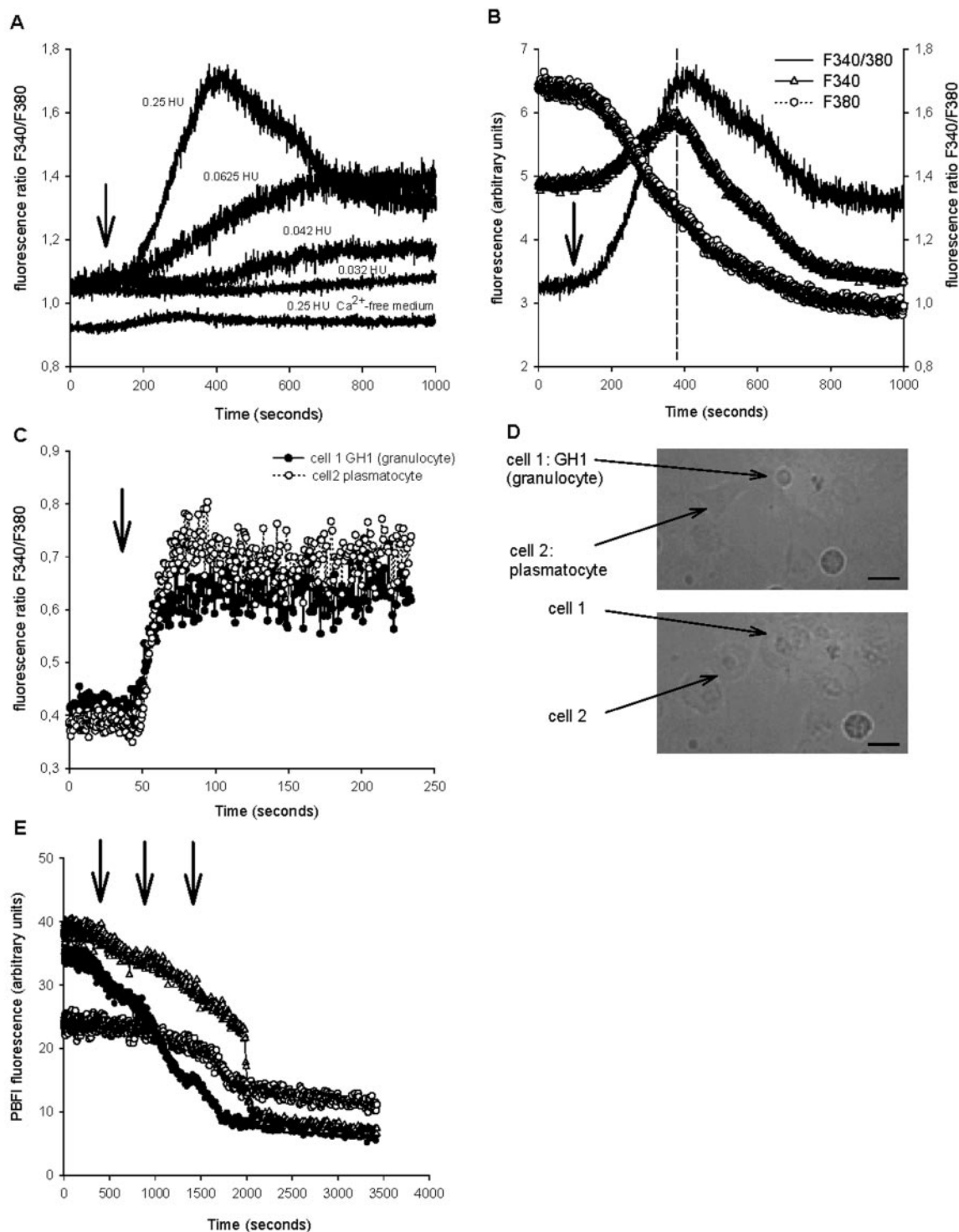
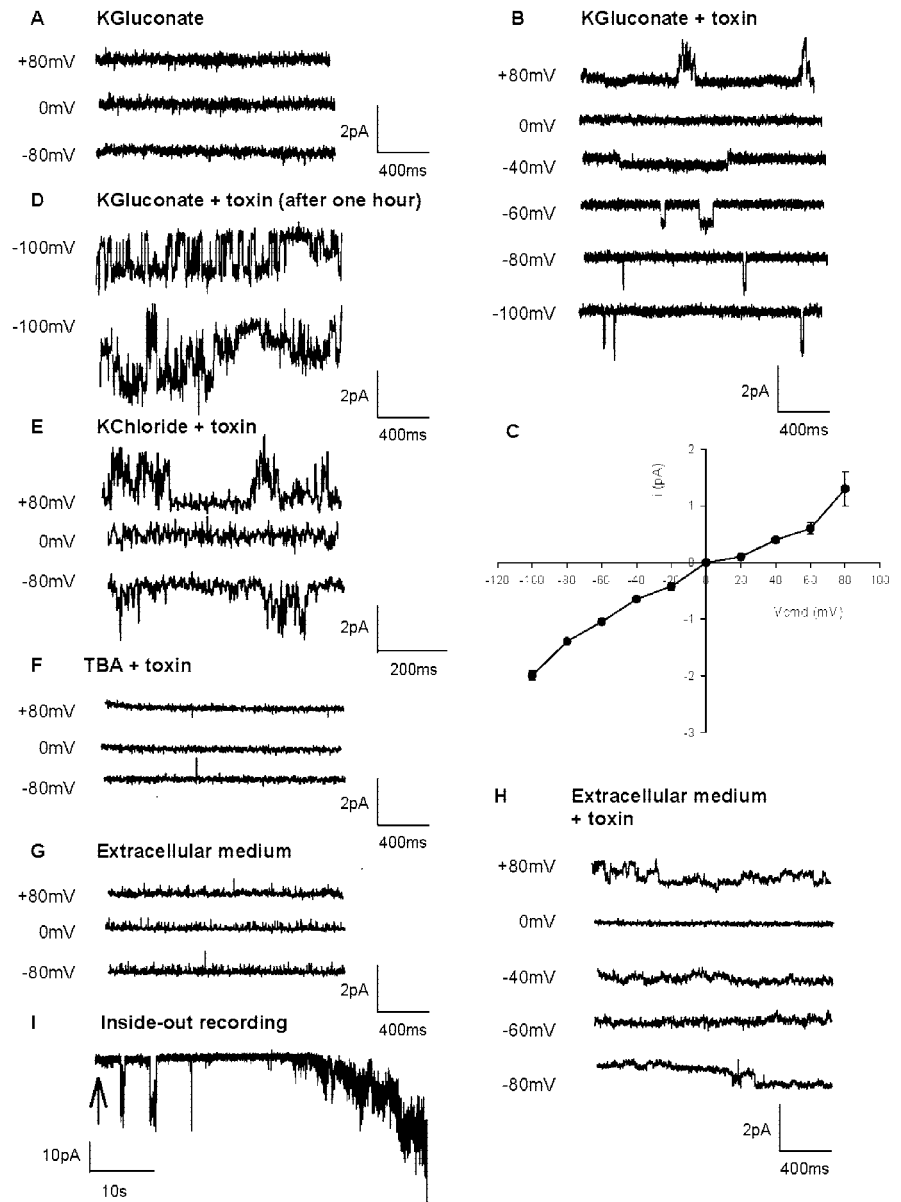


FIG. 5. Effect of αX on cytosolic Ca^{2+} and K^+ concentrations. *A* and *B*, after loading with fura-2 (30 min, room temperature), hemocyte preparation plated on rectangular coverslips was transferred to the recording chamber of a spectrofluorimeter. Fluorescence ratio was collected every 0.5 s. *A*, concentration-dependence (from 0.032 to 0.25 HU) of αX effect on $[Ca^{2+}]_i$. *B*, analysis of respective emissions recorded at 510 nm by excitation at 340 and 380 nm after the application of αX at 0.25 HU. *C* and *D*, effect of αX (0.062 HU) in visually identified GH1 and plasmatocyte. *C*, time course of $[Ca^{2+}]_i$ increase after application of the toxin in a GH1 and a plasmatocyte. *D*, phase contrast microphotographs obtained (*upper panel*) prior to the application of αX and (*lower panel*) at the time of $[Ca^{2+}]_i$ peak in these two cells. Scale bar represents 20 μm . *E*, effect of αX on $[K^+]_i$ in visually identified GH1. Hemocyte monolayer was loaded with PBFI (30 min, room temperature) and then used for videomicroscopy. Potassium leakage was measured in identified GH1 after successive applications of αX (0.02 HU). On all graphs, vertical arrows indicate the time of application of αX . Traces are representative of at least three distinct determinations.

because of a K^+ efflux. Therefore, using patch-clamp recordings we have examined whether αX could alter K^+ ion permeability through native GH1 membranes. In a first attempt to study membrane modifications elicited by the toxin, conventional patch-clamp whole cell recordings (WCRs) were undertaken in

visually identified GH1. It must be mentioned that experiments were conducted only within 90 min following hemocyte plating. Indeed, the hemocytes would naturally deteriorate after 90 min and give unreliable data. Stable WCRs were almost impossible to get from GH1 because these cells sealed

FIG. 6. Patch-clamp recordings obtained from GH1. *A–H*, cell-attached recordings performed in the presence of αX applied directly in the recording electrode at 0.062 HU. *I*, inside-out recording obtained from a patch of GH1 membrane and exposed to αX at 0.062 HU. *A*, recording obtained with K gluconate-based (150 mM K gluconate, 50 mM HEPES, pH 7.4) filling solution without the toxin. *B* and *D*, recordings obtained with K gluconate-based (150 mM K gluconate, 50 mM HEPES, pH 7.4) filling solution in the presence of the toxin. *D*, the recording obtained 60 min after getting the seal. *C*, averaged current-voltage relationships obtained from four distinct experiments performed with K gluconate-based filling solution. Data are presented as means \pm S.E., *graph*. *E*, recording obtained with KCl-based (150 mM KCl, 50 mM HEPES, pH 7.4) filling solution with the toxin. *F*, recording obtained with TBACl-based (150 mM TBACl, 50 mM HEPES, pH 7.4) filling solution with the toxin. *G* and *H*, recordings obtained with extracellular filling solution without (*G*) and with the toxin (*H*). *I*, inside-out recording was performed under symmetrical conditions achieved with extracellular medium and at a holding voltage of -60 mV. The *arrow* indicates the time of application of αX (0.062 HU). *Traces* are representative of at least three distinct determinations.



almost immediately after obtaining a gigaohm seal and ruptured the patch by applying a negative pressure ($n = 20$). By contrast, plasmotocytes gave easy access to WCRs ($n = 5$). This discrepancy between the two cell populations could be attributed to specific membrane properties. Indeed, GH1 have a rough plasma membrane with invaginations and pseudopods, whereas plasmotocytes have a smoother plasma membrane that allows easier access to microelectrodes (not shown). This is the main reason why cell-attached recordings were preferred to WCRs. In addition, to examine the pore-forming activity of the αX and to avoid a lethal exposure of the cells to this toxin, αX was applied directly within the recording electrode. For this purpose, a fraction of the toxin was diluted in the internal medium of the electrode and, just as in the perforated patch technique using pore-forming antibiotics (16, 18), toxin-induced modifications could be recorded underneath the electrode. It must be emphasized that a concentration of 0.062 HU was chosen because it gave a good activity without damaging the seal. Indeed, in the presence of higher concentrations of the toxin, tight seals could not be established. In a first set of experiments, we therefore examined the selectivity of these αX pores for K^+ ions. For this purpose, recordings were performed

under symmetrical concentrations of K^+ obtained by filling electrodes with K^+ -based solutions (K gluconate and KCl). In this respect, we first tested K gluconate-containing solutions. In the absence of the toxin, no current could be recorded when stepping V_{cmd} from -80 to $+80$ mV (Fig. 6A). In the presence of the toxin, channel formation could be observed after 3 to 20 min of contact ($n = 5$) (Fig. 6B). These currents reversed when $V_{cmd} = 0$ mV and displayed a linear voltage dependence. The conductance of αX -generated channels was evaluated at 21 ± 2 pS ($n = 4$) from the current-voltage relationship (Fig. 6C). The effect of the toxin was time-dependent because after 30–45 min of contact ($n = 4$), longer openings could be observed prior to the rupture of the seal (Fig. 6D). Similar data were obtained when recording electrodes were filled with KCl-based intracellular solution (Fig. 6E), which suggests that αX generates pores permeant to K^+ ions. To confirm this possibility, conventional K^+ conductance blockers, *i.e.* tetrasubstituted ammonium derivatives, tetraethylammonium chloride (TEACl), and tetrabutylammonium chloride (TBACl), were further tested for their ability to block currents recorded in the presence of αX . When K^+ was substituted for TBA^+ ions, no currents could be recorded from the patches when αX was applied ($n = 4$) (Fig. 6F).

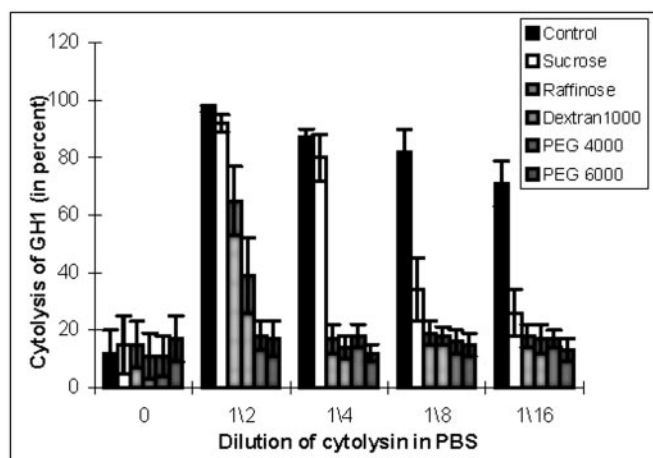


FIG. 7. Effect of protectants on α X-induced GH1 lysis. The columns represent the percentages of GH1 lysis (trypan blue staining) after 1 h of incubation of hemocyte monolayers in PBS (0) or in different dilutions of α X supplemented with various protectants (PEG and dextran, 30 mM; raffinose and sucrose, 50 mM). Data are means of three independent experiments \pm S.E.

A similar result was obtained with TEA⁺ ions (data not shown). Therefore, ammonium derivatives block α X-generated conductances. In addition, because the counter anion of these compounds was Cl⁻ ion in both cases and because similar data were obtained with both KCl- and K gluconate-filled electrodes, one can suggest that α X-formed pores are not permeant for Cl⁻ ions.

Under symmetrical K⁺ conditions, equilibrium potential (E_K^+) = 0 mV as calculated with the Nernst equation. Under these conditions, currents mediated by K⁺ fluxes are expected to reverse when $V_m = E_K^+ = 0$ mV. Here, reversal of the currents obtained in the presence of α X was observed when $V_{cmd} = 0$ mV. Therefore, this suggests that $V_m = V_{cmd}$ in the presence of the toxin in the recording electrode.

In a second set of experiments, cell-attached recordings were performed with electrodes filled with the extracellular solution. In the absence of toxin, voltage-dependent ionic channels could not be evidenced in GH1 because no microscopic currents could be recorded by stepping V_{cmd} from -80 to +80 mV (Fig. 6G). In the presence of the toxin, channel-like openings could be recorded at extreme membrane potentials, i.e. -80 and +80 mV. No current could be detected when $V_{cmd} = 0$ mV (Fig. 6H). This tends to indicate that cations flowing through α X-generated pores are not selective. Indeed, currents flowing through non-selective cationic channels are expected to reverse when $V_m = 0$ mV.

We next examined whether the effect of the toxin was selective for the outside domain of the GH1 membrane. For this purpose, patch-clamp recordings were performed in the inside-out configuration. Patches of GH1 were held at -60 mV under symmetrical conditions (extracellular medium in the electrode and in the bath) and allowed to equilibrate for 5 min after excision. Exposing the inner face of the GH1 membrane to α X (0.062 HU, $n = 4$) resulted in the occurrence of large inward currents followed by a rapid loss of the seal. This indicates that α X may have an effect on both sides of the plasma membrane of GH1 (Fig. 6I).

Osmotic Protection—Osmotic protection of insect hemocytes and of SRBC was tested with potential protectants of different sizes. Polyethylene glycols (PEG) 4,000 and 6,000 and dextran 1,000 were used at a final concentration of 30 mM and raffinose and sucrose at a final concentration of 50 mM. Protection of hemocyte cytolysis (Fig. 7) and of SRBC hemolysis (not shown) was obtained with all these protectants, depending on the

TABLE III

Dissociation of binding and cytolytic activity

Monolayers were set up and incubated for 45 min in the absence of α X (control 1), in α X solution (0.2 HU) (control 2), or α X solution in PEG 4,000. In the first series of monolayers, percentage of GH1 lysis was determined at the end of 45 min of incubation. In the second series, monolayers were first incubated for 45 min and then rinsed in PBS, incubated for 5 min more in this saline, and GH1 lysis was determined. Data are presented as means \pm S.E. of independent experiments where each experimental value is determined in duplicate.

	Percentage of lysed GH1		
	No lysis (control 1)	Lysin in PBS (control 2)	Lysin in PBS + PEG 4,000
First series of monolayers	12.0 \pm 1.5	98.1 \pm 1.2	23.2 \pm 11.7
Second series of monolayers	15.4 \pm 2.6	97.3 \pm 1.1	98.5 \pm 1.1

concentration of α X solution. For the same α X concentration, protectants were more efficient against cytolysis of insect macrophages than against hemolysis of SRBC. Although a total inhibition of cytolysis was obtained with PEG 4,000 at the highest toxin concentration (1 HU), hemolysis was only reduced by one half by PEG 4,000 at this concentration. Total hemolysis inhibition was observed with PEG 6,000. No change in OD was recorded when the different protectants were added to the supernatant obtained after incubation of red blood cells with the toxin. This shows that there was no direct effect of the protectants on hemoglobin absorbance.

We next examined whether protectants were either inhibiting the insertion of the toxin in the plasma membrane or were blocking pores formed by the toxin. For this purpose the following protocols with insect hemocyte monolayers were designed. In a first series of experiments, the percentage of GH1 lysis was determined in monolayers incubated for 45 min in PBS (control 1), in PBS containing lysin (0.2 HU) (control 2), and in PBS containing lysin (0.2 HU) and PEG 4,000 as protectant. In a second series of experiments, monolayers were incubated under the same conditions; then hemocytes were rinsed in PBS and incubated 5 min more in PBS. The percentage of GH1 lysis was determined. The percentage of cell lysis obtained after washing monolayers incubated with lysin and protectant was the same as the percentage determined after incubation with lysin in the absence of protectant (Table III).

In the last series of experiments, after incubation of SRBC with lysin solution (0.5 HU) and protectant the optical density of supernatant (hemolysis) was measured, and the red blood cell pellet was resuspended in PBS. After 5 min more of incubation, red blood cells were pelleted again and the OD of the supernatant measured. The sum of optical densities of the two supernatants was close to the OD of the supernatant of SRBC incubated with α X in the absence of protectant (Fig. 8). Because cytolysis and hemolysis were restored after washing off the protectant, we conclude that the observed inhibitions were not because of direct inactivation of α X by PEG.

Effect of Potassium Channel Blockers on Hemocyte Cytolysis and Red Blood Cell Lysis—TEA and TBA were tested on cytotoxic and TBA on hemolytic activities triggered by α X. In a first series of experiments, they were added to solutions of α X giving almost 50% (TEA) or 80% (TBA) GH1 mortality (from 0.04 to 0.2 HU). The cytolytic activity on hemocytes was almost totally inhibited with 100 mM TEA or 50 mM TBA in the time of experiments (not shown). In experiments conducted with lower toxin concentration (0.01–0.02 HU) and a shorter incubation time, vacuolation of hemocytes was also extremely reduced when TEA (100 mM) or TBA (50 mM) was added to the incubation medium (Fig. 1C for TBA). The best protection achieved with TBA over TEA was attributed to the larger size of the TBA molecule, which allows a better blockade of the K⁺ channels (19).

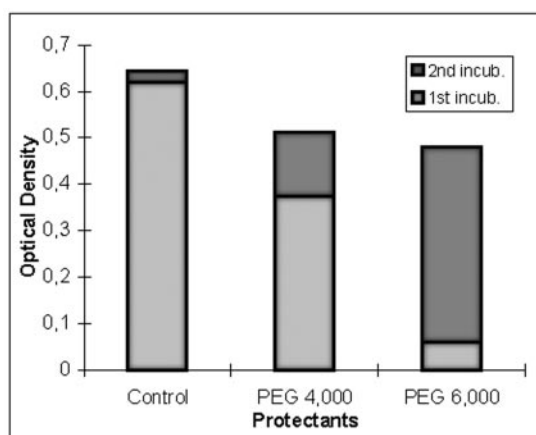


FIG. 8. **Dissociation of toxin binding and hemolytic activity.** 5% SRBC were incubated for 45 min with α X (0.5 HU) supplemented or not (control) with PEG and then centrifuged; OD of supernatant was determined (1st incubation). The red blood cell pellet was suspended and incubated for 5 min more in PBS with neither toxin nor PEG added, centrifuged again, and OD of the supernatant determined (2nd incubation). There was an actual osmotic protection in the first incubation. Removal of the first supernatant washed off the protectant but not the lysin that was bound to the red blood cell membrane. In these conditions hemolysis was recovered. Experiments were carried out in quadruplicate, and similar results were obtained.

In a second series of experiments, SRBC were incubated with different dilutions of α X with 100–300 mM TBA. TBA solutions were able to inhibit the hemolytic activity depending on the α X and TBA ratio, with the lower lysin concentration providing the better protection (not shown).

DISCUSSION

Two different cytotoxic activities (C1 and -2) on insect hemocytes were evidenced in the culture medium of the entomopathogenic bacteria *X. nematophila* (Ref. 10, this work, and Table II), with C1 being under the control of *flhDC*, the flagellar master operon (11). This *flhDC*-dependent activity was also hemolytic for SRBC but did not lyse RRBC. SRBC hemolysis and insect hemocyte cytotoxicity were equally sensitive to heat or trypsin treatments of the culture medium and were independent of Ca^{2+} ions. In the present work the α X, a 10.8-kDa toxin responsible for this *flhDC*-dependent activity, was purified from *X. nematophila* broth growth with C1 activity. Culture medium and the present purified toxin had the same heat and trypsin sensitivity and the same range of specificity for insect hemocytes and hemolytic activity on SRBC; RRBC was insensitive. After incubation of broth growth or purified lysin solution with SRBC ghosts, hemolysis as well as hemocyte cytotoxicity was lost. We conclude that the purified lysin is responsible for both SRBC hemolysis and insect hemocyte cytotoxicity. It is also responsible for the C1 activity previously evidenced in *X. nematophila* culture medium. Titration curves show there was no linear relationship between lysin concentration and hemoglobin release (optical density). This suggests that α X has a concentration-dependent binding affinity to its target, as shown for α -toxin of *Staphylococcus aureus* (20). Alternatively, possible interactions between lysin molecules could occur as degrees of polymerization, depending on toxin concentration. To our knowledge, an hemolysin secreted by an entomopathogenic bacteria, which is also active on insect hemocytes, is purified here for the first time.

The total disappearance of cytotoxic activity after incubation with SRBC ghosts and the results of osmotic protection and patch-clamp experiments show that the plasma membrane is a target of α X. Furthermore, these results are consistent with data obtained with long term incubations (up to 24 h) of α X

with SRBC and with data obtained with increasing target cell concentration. In these last experiments, the amount of hemolysis did not increase later than 2 h of incubation, and the percentage of hemolysis decreased when target cell concentration was increased. We conclude that once fixed on a plasma membrane site, α X is not recycled to react with multiple target cells, at least with red blood cells. According to Rowe and Welch (21), these data show that α X looks like a pore-forming toxin rather than a lysin with an enzymatic activity. Results of microspectrofluorimetry, of patch-clamp studies, and of osmotic protection experiments are consistent with such pore-forming activity.

Microspectrofluorimetry data suggest that the toxin-induced calcium rise after a latency period resulted from Ca^{2+} influx from the external medium. However, when the maximal increase of the ratio 340/380 nm has been obtained fura-2 emissions tend to display parallel decreases. The more likely explanation is that the toxin induced membrane disruption leading to leakage of fura-2 in the medium. Because the toxin was able to induce cell death in the absence of external calcium, it can be postulated that the increase in cytosolic Ca^{2+} detected upon exposure of the cells to the toxin was correlated neither with specific Ca^{2+} entry nor with mobilization of Ca^{2+} from internal stores. More likely it reflects cell lysis, as shown by light microscopic observation (Fig. 5D). On the other hand, α X induced an immediate loss of K^{+} , which could be temporarily regulated by the cell until lysis. These last observations are in accordance with the results of patch-clamp experiments.

Cell-attached patch-clamp experiments were designed to evaluate the pore-forming activity of α X in native GH1 membranes. It must be emphasized that no voltage-dependent channels could be detected in the absence of the toxin. However, this does not imply that GH1 are “electrically” silent. Indeed, the activity of Ca^{2+} -activated K^{+} channels or second messenger-operated channels, for instance, remains to be established in these cells. Electrophysiological recordings to study α X actions had to be adapted to the specific membrane properties of GH1 and to the very high sensitivity of these cells to α X. For this purpose, α X was directly applied in the recording electrode. This procedure enabled us to evaluate the pore-forming activity of this toxin. Interestingly, the bacterial toxin, α -toxin from *S. aureus*, has previously been used for its pore-forming activity in the perforated patch method (16). To observe microscopic currents due to channel formation, low concentrations of α X had to be applied. Indeed, as also evidenced for *Helicobacter pylori* (14), for instance, high concentrations of α X could prevent tight seal formation between the cell membrane and recording electrodes. In the presence of α X, the activity of cation-selective channels could be evidenced. These currents could be totally blocked by K^{+} channel antagonists (TEA^{+} and TBA^{+}). This tends to indicate that α X primarily alters membrane permeability by forming K^{+} permeable pores. Under physiological ionic concentrations, non-selective cationic currents could be recorded in the presence of the toxin. Under symmetrical K^{+} conditions, the conductance of α X-generated pores was rather small (21 ± 2 pS). This is probably because the toxin was applied at a low concentration and had a small area of contact with the membrane of GH1. However, the effect of the toxin on channel forming was time-dependent. Indeed, an increase in currents was observed with time. This could be because of an increase in the number of pores and/or the size of these pores, as demonstrated for other porins (22).

Taken together, these results show that the first effect of α X on the insect hemocyte membrane was an increase in ionic permeability, mainly for monovalent cations. Modification of ion permeability by bacterial toxins in eukaryotic cells is well

documented. Anion-selective channels are formed in SF-9 insect cells by the δ -endotoxin from *Bacillus thuringiensis* (23) and in HeLa cells by VacA from *H. pylori* (14). Channels with weak discrimination among different cations are formed in human macrophages by HlyA from *Escherichia coli* (24). Alpha-X from *X. nematophila* was more specific because it created channels rather selective for monovalent cations, as did the major cytolysin of *S. aureus* (25). In our experiments, inhibition of the current recorded in the presence of α X by the specific blockers of potassium channels, TEA and TBA, showed that the cation channels opened or created by α X could be rather specific for potassium. This specificity has been evidenced for aerolysin from *Aeromonas hydrophila* on baby hamster kidney (mammalian) cells (26). TEA and TBA also inhibited both ER vacuolation and cell lysis, showing that disturbance of potassium permeability induced by α X could be sufficient to lyse the target cells. Furthermore, as in experiments conducted with VacA toxin on HeLa cells (14), inhibition of insect macrophage lysis by blockers was more effective at the lowest α X doses. We cannot dismiss the possibility that α X activates unknown endogenous channels rather than forming new ones. However, there is evidence in favor of a pore-forming activity. First, the results of osmotic protection experiments are consistent with the formation of pores. Second, pore size increase with toxin concentration is well documented for pore-forming molecules such as complement (pore sizes ranging from 0.7 to 15 nm) (27) or *E. coli* toxin (0.6 to 1.3 nm) (28) and other RTX toxins (29). A total protection of insect macrophage cytolysis was obtained with PEG 4,000 (pore radius: 1.9 nm) (30) whatever the α X concentration, but protection of SRBC, at the highest α X concentrations, was only obtained with PEG 6,000 (pore radius: 2.9 nm). This suggests that the maximum size of the pores formed in red blood cell plasma membrane would be larger than that of pores made by the same lysin in insect macrophage plasma membrane. A larger size of pore created in red blood cells than pore in nucleated cells was already reported for ShlA from *Serratia marcescens* (31), but the reason for this variability is unknown. Finally, our results on osmotic protection led us to conclude that α X processes through colloid-osmotic lysis (32). In colloid-osmotic lysis, transmembrane channels allow only ions and small molecules to pass freely across the cell membrane. Therefore, the osmotic pressure generated by the high concentration of macromolecules inside the cell causes a water influx that leads to cell swelling and sometimes to cell burst. This colloid-osmotic process is consistent with our results on Ca^{2+} and K^{+} movements through hemocyte plasma membrane. Low doses of α X mediate a selective leakage of K^{+} (and possibly other monovalent cations) from hemocytes. However, low doses of α X do not enhance membrane permeability for Ca^{2+} or larger molecules. In this respect, intracellular Ca^{2+} entry was observed only upon cell lysis. Therefore, Ca^{2+} entry was not a cause but a consequence of cell lysis.

The cell burst was the most obvious effect of α X from *X. nematophila* on hemocytes, but prior to lysis, insect hemocytes showed extensive vacuolation of the cytoplasm. Vacuolation is a non-classic pathway of toxicity of bacterial toxins. However, it is achieved by cereulide from *Bacillus cereus* (33), VacA toxin from *H. pylori* (34), aerolysin from *A. hydrophila* (26), ShlA hemolysin from *S. marcescens* (31), and HlyA hemolysin from *Vibrio cholerae* (35) in different mammalian cell types *in vitro*. Among these toxins, only vacuolation triggered by ShlA and HlyA is followed by a lysis of cells in culture. Because all these toxins (cereulide, VacA, aerolysin, ShlA, and HlyA) triggered the vacuolation of different cellular organelles, we conducted two kinds of experiments to characterize which cell organelle

was subjected to vacuolation by α X. Neutral red is a supravital dye of the endosome/lysosome system (36). Data obtained with neutral red uptake experiments suggest that the vacuoles observed in hemocytes after α X incubation do not belong to this endosome/lysosome system. This conclusion is not in agreement with studies performed on mammal cells with VacA, ShlA, or HlyA. Examination of α X-treated hemocytes under electron microscopy lead us to conclude that the vacuoles are in fact dilated cisternae of the ER. Vacuolation of ER by a bacterial toxin is reported here for the first time in non-mammalian cells. Another case of ER vacuolation by bacterial toxin was reported by Abrami *et al.* (26) for aerolysin on baby hamster kidney cells. For aerolysin (37–39), as for α X (this study), the first target of the toxins is the plasma membrane, where they form channels selective for small cations before they trigger ER vacuolation. However, a fundamental difference between aerolysin and α X is their respective effects on intracellular Ca^{2+} concentration. Indeed, aerolysin induces Ca^{2+} release from intracellular stores as well as a Ca^{2+} influx (39), but α X does not. In this respect the activity of α X on its target cells appears different from the different actions already studied and described for all other bacterial toxins.

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***Xenorhabdus nematophila* (Enterobacteriaceae) Secretes a Cation-selective Calcium-independent Porin Which Causes Vacuolation of the Rough Endoplasmic Reticulum and Cell Lysis**

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