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Permeability Increase Induced by *Escherichia coli* Hemolysin A in Human Macrophages is Due to the Formation of Ionic Pores: A Patch Clamp Characterization

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Abstract. *Escherichia coli* hemolysin is known to cause hemolysis of red blood cells by forming hydrophilic pores in their cell membrane. Hemolysin-induced pores have been directly visualized in model systems such as planar lipid membranes and unilamellar vesicles. However this hemolysin, like all the members of a related family of toxins called Repeat Toxins, is a potent leukotoxin. To investigate whether the formation of channels is involved also in its leukotoxic activity, we used patch-clamped human macrophages as targets. Indeed, when exposed to the hemolysin, these cells developed additional pores into their membrane. Such exogenous pores had properties very different from the endogenous channels already present in the cell membrane (primarily K^+ channels), but very similar to the pores formed by the toxin in purely lipidic model membranes. Observed properties were: large single channel conductance, cation over anion selectivity but weak discrimination among different cations, quasilinear current-voltage characteristic and the existence of a flickering pre-open state of small conductance. The selectivity properties of the toxin channels appearing in phospholipid vesicles were also investigated, using a specially adapted polarization/depolarization assay, and were found to be completely consistent with that of the current fluctuations observed in excised macrophage patches.

Key words: HlyA — RTX toxins — Leukotoxicity — Pore Formation — Pore properties — Human macrophages — Patch clamp

Introduction

Escherichia coli hemolysin (HlyA) is a major virulence factor for some *E. coli* strains involved in human extraintestinal diseases like urinary tract infections, peritonitis, meningitis, and septicemia [9, 12, 60].

HlyA belongs to a family of related exoproteins elaborated by gram-negative organisms including *E. coli, Proteus, Morganella* [39], *Pasteurella* [56], *Actinobacillus* [40, 42], and *Bordetella* [27] species. These toxins share several peculiar features, conferred by a common genetic structure comprising four linked genes necessary to toxicity. They encode four proteins termed ABCD. A is the cytotoxin, which is secreted through a peculiar secretion pathway involving the two cellular proteins B and D [32], and activated via a post-translational acylation by C [28, 36, 55]. The cytotoxin always contains a remarkable motif, a repeated Ca^{++}-binding glycine-rich nonapeptide, which originated the family name of Repeat ToXins (RTX) [16, 43, 59].

Many RTX toxins are hemolytic [6, 20, 52], but all are leukotoxic, and a few very specifically [13, 14, 17, 45]. HlyA itself can be cytolytic for neutrophils [5], monocytes [7, 23] and granulocytes [22]. A common mechanism has been implicated in both hemolytic and leukotoxic activity: the formation of toxin-mediated pores in the membrane of the attacked cell [5, 6, 15, 37]. However, this concept was derived from indirect evidences such as increased cell permeability, release of soluble molecules and osmotic protection from macroscopic cell damage.

Formation of ion channels was directly demonstrated using planar lipid membranes (PLM) for HlyA [4, 49] and later for other RTX toxins [2, 3, 42, 57] including two very specific leukotoxins [50]. These studies have provided a detailed description of the channel properties. However, it was not clear to what extent these findings were relevant to the cellular mechanism of leukocytes killing by RTX toxins.

To approach this problem, we have applied the patch clamp technique [29] to human monocyte-derived mac-

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rophages exposed to HlyA. These cells were chosen because: (i) human monocytes represent a major target of the toxin during infection [7, 23]; (ii) monocyte-derived macrophages are readily accessible by patch clamp [24, 25, 46]. We found that HlyA induces in these cells the formation of exogenous channels very similar to those previously observed in PLM, suggesting this mechanism might indeed be implicated in HlyA cytotoxic action.

Materials and Methods

Toxin

Lyophilyzed *E. coli* hemolysin used in these experiments was kindly donated by Sucharit Bhakdi. Hemolytic activity was titrated against human erythrocytes as described earlier [11, 18] with a final RBC concentration in the wells around 5×10^6 cell/ml. Titers were calculated as the reciprocal of the dilution giving ≈50% hemolysis after 1 hr and expressed in HU/ml. Under normal conditions, we found that the relation between hemolytic activity and molar concentration of HlyA (calculated from its UV absorption using an extinction coefficient ϵ_{280} of 73960 M^{-1} cm^{-1} derived from the amino acid composition) was 1 HU/ml $= 2 \times 10^{-9}$ M.

Isolation and In Vitro Culture of Monocyte-derived Human Macrophages

Human blood samples (20–40 ml) were drawn in Heparin (5 U/ml final concentration) from healthy donors. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) at a density of 1.007 g/ml according to the method of Bϕyum [10]. Heparinized blood was diluted with two volumes of Dulbecco's phosphate buffered saline (DPBS, Ca^{2+} and Mg^{2+} free) and 10 ml aliquots applied to 5 ml Ficoll-Hypaque in sterile plastic tubes. After 30 min centrifugation at 400 g (20°C), the interface layer containing mononuclear cells was collected, washed three times with RPMI 1640 modified medium (supplemented with 10% FCS, 4mM L-glutamine and 200 μg/ml gentamicin) at 300 g for 10 min (20°C) and resuspended in 1 ml of medium. Cells were counted under a phase contrast microscope using a haemocytometer. Cell viability, checked with 0.4% trypan blue [34], was close to 100%. 200 μl aliquots of cell suspension were applied either in 96-well culture plates (for the viability experiments) or on glass cover slips on the bottom of Petri dishes (for the patch clamp experiments). Monocytes were separated from lymphocytes by adherence, i.e., after 2 hr at 37°C (7.5% CO_2) cells were washed three times with medium to remove all nonadherent elements. Thereafter cells were cultured in RPMI 1640 modified medium in 7.5% CO_2 humidified air at 37°C and, at various culture days (from 7 to 20), monocytes-derived macrophages were used. Medium was changed every 5–7 days.

In the viability experiments the percent of permeabilization of HMDM was determined by the trypan blue uptake assay [34]. 1.5×10^3 cell/ml (cell numbers were approximated by counting 3 fields per well with a microscope) were incubated for 1 hr at 37°C in DPBS with the indicated amount of toxin, and then stained with 0.4% trypan blue for four minutes. Nonviable, stained cells were counted under the microscope. Alternatively the degree of swelling was evaluated visually.

In some control experiments, a mouse macrophage-like cell line (RAW 264.7, obtained from ATCC) was maintained in monolayer culture in RPMI 1640 modified medium (supplemented with 10% FCS, 1 mM L-glutamine, 50 μg/ml vancomycin and 10 μg/ml gentamicin) and incubated under 7.5% CO_2 at 37°C. Medium was changed every 5–7 days. At least 24 hr before the experiment these cells were scraped with a rubber policeman and replated at the concentration of 2×10^6 cell/ml on glass cover slips on the bottom of Petri dishes where cells were allowed to readhere.

Patch Clamp of Mature Macrophages

Glass cover slips with adherent mature HMDM, were transferred to a 1 ml recording Petri dish. Membrane currents mediated by endogenous channels, or induced by the addition of the hemolysin, were recorded by the patch clamp technique [29]. The standard pipette solution contained (in mM): KF 110, KCl 5, $MgCl_2$ 2, $CaCl_2$ 1.2, EGTA 11, Hepes 10, sucrose 35, buffered to pH 7.3 by KOH. The standard external buffer solution consisted of (in mM): NaCl 150, KCl 5, $CaCl_2$ 2, Glucose 20, Hepes 10, buffered to pH 7.3 by NaOH. Where indicated, 10 mM tetraethylammoniumchloride (TEA-Cl) was added to the external solution, or the main salt (NaCl 150 mM) was replaced by 150 mM of one of the following salts: KCl, Gdn · Cl, TEA · Cl or TBA · Cl. Either the whole-cell or the outside-out excised-patch configurations were used.

Application of Toxin

HlyA was added to the external solution (bathing the cell or patch) either manually, by an oxford pipette, or via a fast superfusion procedure. In this last case two pipettes (one containing the bath-control solution and the other the same solution plus the toxin) were alternatively positioned in front of the cell, while the solution of the Petri dish was continuously renewed at a speed of 1.3 ml/min with toxin-free external buffer via a peristaltic pump. This procedure allowed us to exchange the cell-bathing solution in a few seconds. When the manual procedure was used, excess toxin was eventually washed away from the bath solution by a peristaltic pump (same speed as above). Experiments were performed at room temperature. The permeability induced by HlyA was always studied in the presence of 10 mM TEA^+ to reduce the endogenous outward-rectifying potassium currents.

Current records were obtained using an Axon 200 amplifier in the voltage-clamp mode. Signals were digitally sampled (maximum sampling interval: 200 μs per point) with a 4 Mega Atari ST personal computer equipped with a 16 bits A/D/A board (Instrutech, Elmont, N.Y.) and directly stored on the computer hard disk. The resistance of the patch pipettes was in the range of 2 to 5 MΩ. Patches with a seal resistance ⩾1 GΩ were considered in the whole-cell configuration, whereas only excised patches with a seal resistance around 100 GΩ were used to study HlyA single channel properties. Analysis of the current records was performed both on the Atari PC, by the commercial software provided by Instrutech or, by transferring the data to a MacIntosh personal computer. Endogenous potassium currents were analyzed only after the capacitive and leakage current were subtracted by the P/4 procedure. As HlyA-induced currents are almost linear over the analyzed voltage range, the P/4 procedure was not utilized in that case. Representative experiments were reported to exemplify the effects of the toxin, however each experiment was repeated at least two to three times on different cells with similar results.

Creation and Dissipation of Transmembrane Potential in Lipid Vesicles

Large unilamellar lipid vesicles (LUVET) comprised of egg PC (from Avanti Polar Lipids) or asolectin (from Fluka), were prepared by ex-

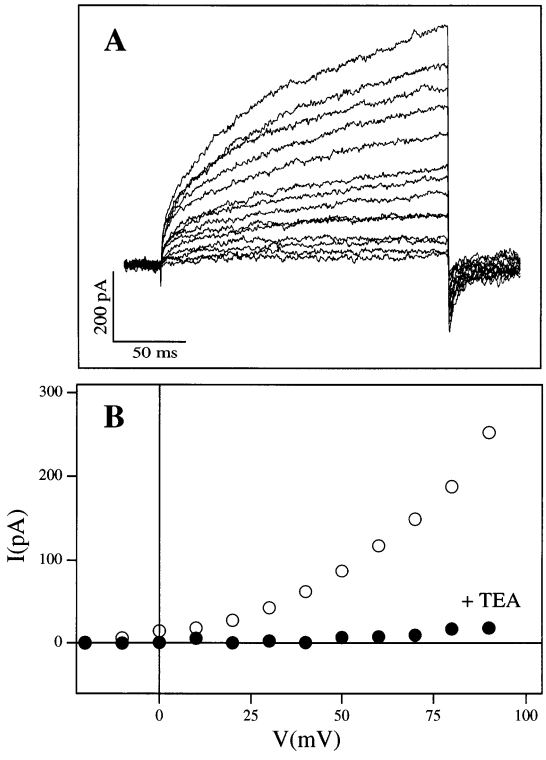

Fig. 1. Typical human macrophage delayed rectifier potassium currents recorded in the whole-cell configuration. (*A*) Family of outward potassium currents evoked by 200 msec depolarizing pulses from −20 to 110 mV (in 10 mV increments), stepping from a holding potential of −60 mV. Tail potentials to −100 mV. Capacitive and leakage current were subtracted using a standard P/4 correction procedure. (*B*) Potassium current-voltage characteristics (empty circles), obtained by plotting the maximum current from at least three different data files, as a function of the step potential. 10 mM TEA inhibits the potassium current (filled symbols). The effect of TEA was reversible (*not shown*).

trusion of freeze-thawed multilamellar liposomes [33, 44]. The starting lipid concentration was 10 mg/ml. Extrusion was performed by 31 passages in a LiposoFast Basic unit (from Avestin, Ottawa, Canada) equipped with two stacked polycarbonate filters bearing holes with an average diameter of 100 nm (Nucleopore). The buffers used for these experiments, all at pH 7.0, contained 5 mM Hepes and 200 mM of one of the following cations (in a chloride salt form): Li^+, Na^+, K^+, Rb^+, Cs^+, Gdn^+, TEA^+ or TBA^+. In the rest, these solutions will be indicated simply by the name of the cation. To create a negative inner potential, vesicles prepared in the K^+ buffer were suspended at a final lipid concentration of 137 μg/ml into a quartz cuvette (1-cm path length, stirred and thermostatted to 23°C) containing a different cation buffer. Valinomycin was then added, from a stock ethanolic solution, to a final concentration of 28 nM. To create a positive inner potential, vesicles were prepared in TEA^+ buffer and thereafter treated as above. The formation of an inner potential was detected by adding 6 μM 8-anilino-1-naphtalensulfonate (ANS) [53, 54]. Development of a negative inner potential decreases the number of ANS molecules absorbed into the bilayer through a repulsive interaction and thus reduces the total fluorescence. Under these conditions, the depolarizing effect of a toxin results in an increase of the observed fluorescence. Correspondingly, a decrease is seen when vesicles bearing a positive inner potential are depolarized.

Table. Effects of *E. coli* HlyA on the viability of human monocyte-derived macrophages

[HlyA][a] (HU/ml)	1000	100	50	10	5	1	0.01
Nonviable[b] cells [%]	100	100	100	100	23 ± 6	0	0
Swelling[c]	+	+	*ND*	+/−	*ND*	−	−

[a] Toxin titer was represented by its hemolytic activity on human erythrocytes; 1 HU/ml means ≈ 50% of RBC are lysed within 1 hr.
[b] Percent of nonviable (i.e., permeabilized) adherent HMDM as determined by the trypan-blue assay.
[c] Appearance of macroscopic swelling by inspection under the microscope is indicated by +; normal shape by −; a mixed population by +/−. *ND:* not determined.

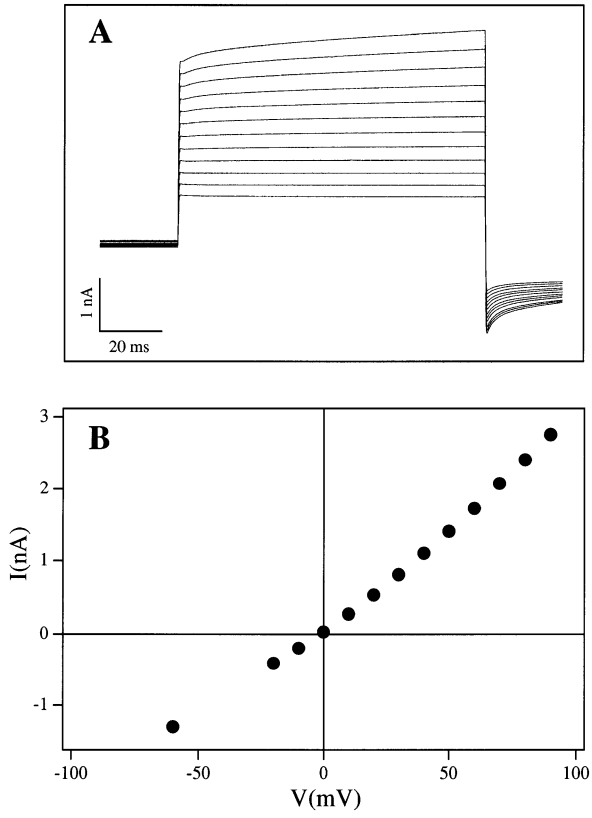

Fig. 2. Effects of HlyA on ion currents through HMDM in the whole cell configuration. (*A*) The exposition of the cell to the perfusion pipette containing a concentration of 10 HU/ml of HlyA induced a rapid increase of the whole-cell current. Bath solution contained 10 mM TEA which drastically decreases the endogenous potassium currents (*see* Fig. 1). Depolarizing pulses from −20 to 90 mV (in 10 mV increments) were applied. Holding potential was −60 mV, tail potential −100 mV. (*B*) Steady-state current-voltage characteristics of the records shown in panel *A*. The current leakage before the exposition to the toxin was in the order of 100 pA at 80 mV. No P/4 correction was used in this case.

Alternatively, the ability of HlyA to create a selective permeability for the different cations was tested directly by using it in place of valinomycin, under otherwise identical conditions. Fluorescence was measured with a spectrofluorometer, either Jasco FP550 or Spex Flu-

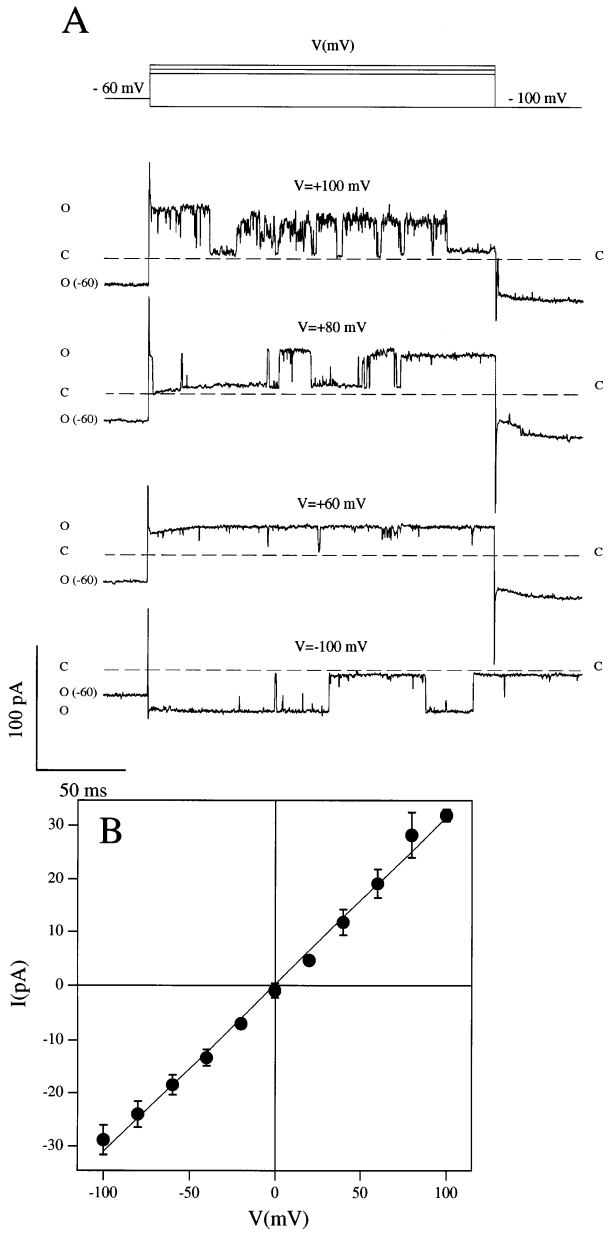

Fig. 3. Effects of HlyA on ion currents through excised HMDM patches in the outside-out configuration. (*A*) Typical single channel openings recorded in an HMDM patch, after the addition of HlyA at concentrations ranging from 0.5 to 10 HU/ml. Voltage protocol is shown in the upper panel. O and C indicate open and closed level. O(−60) indicates open level at −60 mV. (*B*) Single channel current amplitudes plotted as a function of the transmembrane potential. Mean values (± SEM) were derived averaging current transitions from three different experiments. The straight line, corresponding to a channel conductance of 312 ± 7 pS (mean ± SD), is the best fit.

oromax. We used an excitation wavelength of 380 nm (band pass 5 nm) and an emission wavelength of 490 nm (band pass 5 nm).

Abbreviations

HlyA: *Escherichia coli* hemolysin A; RTX: Repeat ToXins; PLM: planar lipid membranes; HMDM: human monocyte-derived macrophages; HRBC: human red blood cells; ATCC: American Type Culture Collection; ANS: 8-anilino-1-naphtalensulfonate; TEA-Cl: tetraethylammonium chloride; TBA-Cl: tetrabuthylammonium chloride; Gdn-Cl: guanidinium chloride; EGTA: ethylen-glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid; PC: phosphatidylcholine; LUVET: large unilamellar extruded lipid vesicles

Results

Whole-Cell Recording

Human macrophages, adherent to Petri dishes, were patch-clamped in the whole-cell configuration. They exhibited typical potassium currents (Fig. 1*A*), as indicated by their reversal potential ($V_{rev} \approx -30$mV) and by the fact that they were blocked by TEA (Fig. 1*B*). Previous studies on macrophages have reported the presence of two major classes of endogenous channels, both of them selective for potassium: the delayed rectifier and the calcium activated channel [24, 25, 46]. The channels we observed are of the first type, as clearly indicated by their voltage dependence. As a matter of fact, Ca^{++}-activated K^+ channels were kept silent under our experimental conditions by the use of an internal buffer which chelates all calcium present.

These cells were quite sensitive to the action of HlyA, only about five times less than washed HRBC, as determined by dye exclusion (Table). At the toxin concentration used for patch clamp (about 10 HU/ml), cells around the area of toxin application appeared swollen after some time, whereas the one under observation remained flat. This indicated that perfusion of internal proteins through the patch pipette prevented the development of an osmotic pressure. Concomitantly, a large permeability increase developed which could be characterized (Fig. 2) using the same voltage protocol as in Fig. 1. This new conductance appeared to be constantly activated and poorly selective, V_{rev} being close to zero (Fig. 2), as it would be expected for channels formed by the toxin itself [49].

Similar results were obtained in control experiments using a murine macrophage cell line (RAW 264.7). This demonstrates that our findings do not depend upon the protocol of preparation of peripheral blood macrophages, and it confirms also that HlyA, at variance with some other member of the RTX family [14], is not species specific.

Outside-out Excised-Patch Experiments

To detect the discrete events which gave rise to the new conductance, we used the outside-out excised-patch configuration. In this way, single channel signals were elicited (Fig. 3) whose conductance and kinetic properties were very similar to those observed exposing artificial PLM to the same toxin [4, 49, 51]. In particular, as in the

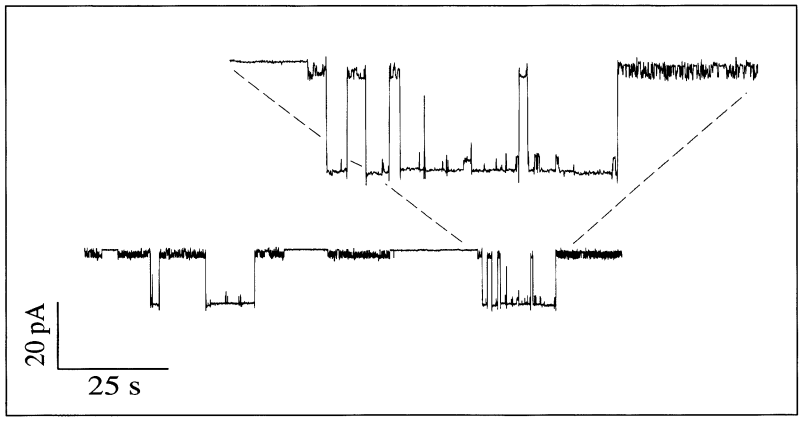

Fig. 4. HlyA induces the opening of long-lasting channels.Inset shows the section of the record delimited by the dotted lines ata higher time and current resolution. Note that the rapidly flickering minichannel systematically disappears on the opening of the standardchannel, thus suggesting that it is a substrate of the large channel. Applied potential was −60 mV. Other conditions as in Fig. 3.

model membranes, the pore appeared to fluctuate between an open and a closed state (although some intermediate states were also observed), the *I–V* curve of the open channel was almost linear, the derived conductance was quite high, 312 ± 7 pS in 150 mM external NaCl (Fig. 3B), and V_{rev} virtually zero indicating almost no selectivity between K^+ and Na^+. A low conductance open state, well described in PLM [4, 51], was also detected (Fig. 4).

The number of channels appearing in the patch was dependent on the duration of the exposure to the toxin (Fig. 5). However, in some cases, after removal of the toxin from the bath, the number of open channels slowly decreased with time, possibly because of their migration into the omega region (i.e., the inner contact between the patch and the pipette) or by degradation of the toxin.

The selectivity of these newly formed channels was next studied by varying the composition of the external medium (Fig. 6). We found that the current at positive voltages (cations moving outwardly) was nearly constant under the different conditions, whereas that at negative voltages (cations moving inwardly) was dependent on the type of cation present and was inversely related to its size. Small cations like Na^+, K^+, and Gdn^+ had a high conductance, whereas large cations, like TEA^+ and TBA^+ in particular mode, had a reduced mobility through the pore (Figs. 6 and 8). Furthermore, the reversal voltage appeared to be negative with TEA^+ and even more with TBA^+, demonstrating that these cations are less permeant than K^+. These results are consistent with a pore large in size and cation selective as the one demonstrated in PLM [49, 51].

Lipid Vesicles Transmembrane Potential Experiments

To establish whether these new channels were the same that are induced by HlyA in model membranes, the selectivity of the ionic pores formed by this toxin in lipid vesicles was evaluated. Vesicles with a transmembrane gradient of K^+ develop an internal potential when exposed to a K^+ carrier like valinomycin, if the other cations present are either not transported or transported at a lower rate. This derives from the selectivity of the carrier and the developed internal potential, detected with ANS, is either positive, for an inward K^+ gradient, or negative in the opposite case. We used this property to study whether the channels formed by HlyA would dissipate such a potential or not. Dissipation implies that the channel is not selective, whereas maintenance of the potential would indicate that HlyA is at least as selective as valinomycin. We found different behavior of HlyA depending on the ionic species used (Fig. 7*A*). For example, with the couple K^+_{in}/TBA^+_{out} there was virtually no dissipation, indicating that the selectivity was the same as with valinomycin (i.e., both TBA and chloride are impermeant). With smaller cations, e.g., the couple K^+_{in}/Gdn^+_{out}, dissipation was observed. By this technique we cannot determine either the permeability of K^+, because there is no gradient in this case, or that of Na^+, because its rate of transport by valinomycin is so high that the potential developed upon addition of the carrier, albeit observable, dissipates within a few minutes. We determined the following sequence for the permeability (P) through the HlyA channel: $P_{Li+} \approx P_{Gdn+} > P_{TEA+} > P_{TBA+}$ (Fig. 7*A* lower traces). In these experiments, we used similar buffer solutions as in the cell experiments, i.e., the K^+ buffer was internal. However, exchange of the external for the internal solution (i.e., TEA^+_{in}/K^+_{out}), gave qualitatively the same result, with a toxin-induced decrease in ANS signal (Fig. 7*A* upper trace). This rules out the possibility that the variation of ANS signal upon addition of HlyA is due to a direct adsorption of the dye to the toxin, because in such case an increase should always be observed. Furthermore, it implies that the selectivity of the channel was the same both for inwardly and for outwardly directed currents.

Finally, we tried to direct determination of the selectivity of the HlyA pore by simply omitting valinomycin from the protocol (Fig. 7*B*). Asolectin vesicles, loaded with either TEA^+ or K^+, were transferred to a solution containing a different cation and ANS. There-

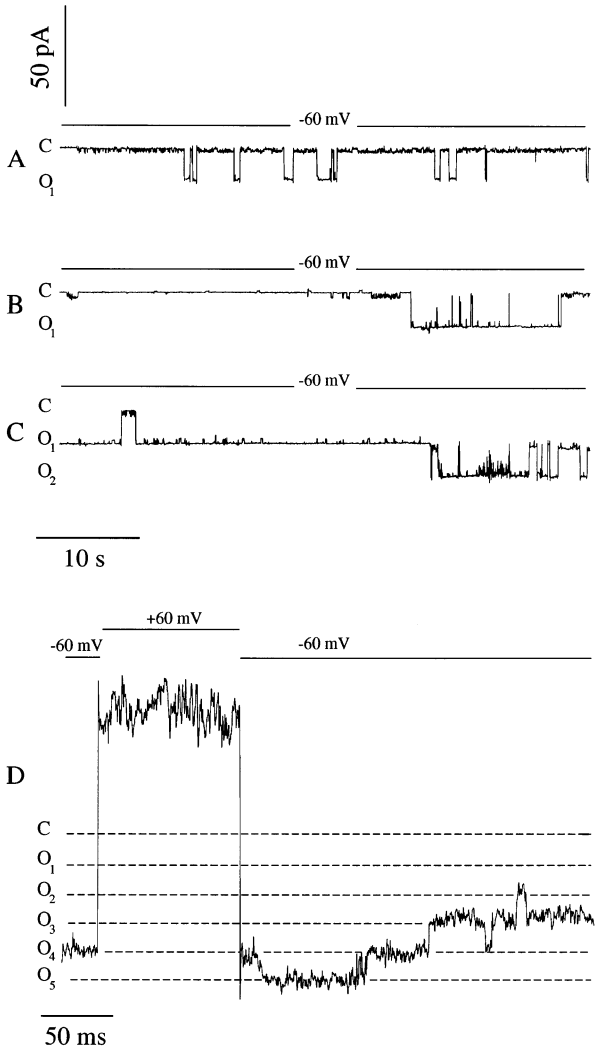

Fig. 5. The number of HlyA channels incorporated into the membrane patch depends on the time elapsed from the exposition to the toxin. Trace *A* was recorded 10 sec after the exposition of the patch to HlyA (10 HU/ml), applied by the fast perfusion procedure. Thereafter the toxin was removed from the bath. Trace *B* was recorded 40 min later and shows that no more channels were incorporated. Trace *C* and *D* were recorded at 100 sec and 4 min after a new, continuous, exposition to the same concentration of HlyA. They show the simultaneous presence of two (trace *C*) and at least 5 (trace *D*) channels. Note the different potential protocol used in trace *D* (with respect to the first three records), which allows to distinguish the overlapping openings of the five channels.

after, they were exposed to HlyA. Depending on the ion couple used, we either observed an increase of the fluorescence (indicating the development of a positive inner potential) when the external cation was more permeant than the internal, or a decrease, when it was less permeant. Thus, the slope of the fluorescence change could be used as an estimate of the relative permeability of a cation. This parameter is reported in Fig. 8 as a function

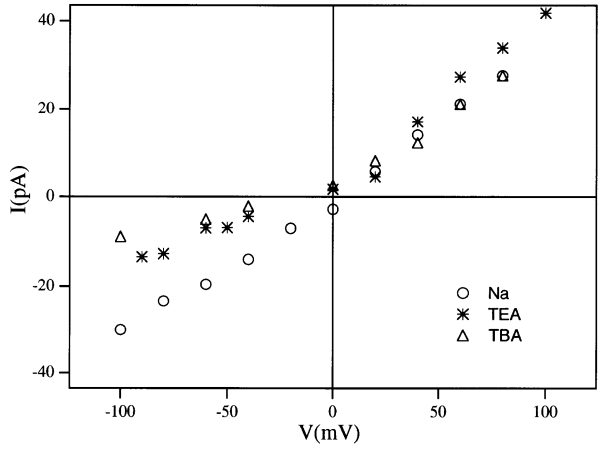

Fig. 6. Current-voltage characteristics of HlyA single channels recorded in cell-free excised outside-out patches. KCl was used on one side (standard pipette solution) and 150 mM of either NaCl (standard bath solution), or TEA-Cl, or TBA-Cl on the other side. The outward cation current (positive voltages) remains fairly constant whereas the inward current (negative voltages) decreases when the size of the external cation increases. Note that the reversal voltage (intersection of the curve with the x-axis) becomes negative with TEA^+ and even more with TBA^+. Other conditions as in Fig. 3.

of the m.w. of the external cation (for the two kinds of vesicles) and compared to the single channel conductance that was measured at −60 mV in the excised-patch experiments. A remarkable agreement was observed. This is, to our knowledge, the first example of a direct and simple determination of the selectivity of the channels formed by a pore-forming protein in a lipid vesicle system.

Discussion

Many bacterial toxins have been shown to be able to form pores in model lipid membranes [48]. Among these are both membrane damaging toxins (e.g., besides HlyA, *Staphylococcus aureus* α-toxin [47], *Aeromonas hydrophila* aerolysin [61], *Vibrio cholera* hemolysin [41] and *Pseudomonas aeruginosa* cytotoxin [58]) as well as membrane translocating toxins (e.g., *Corynebacterium diphtheriae* diphtheria toxin [19], clostridial tetanus and botulinum toxins [31], *Bacillus anthracis* anthrax toxins [8] and *Pseudomonas aeruginosa exotoxin* A [26]). The physiological role of this pore-forming ability has been discussed thoroughly. In the case of membrane-damaging toxins, it was proposed that this mechanism might serve to inflict a lethal damage and to extract nutrients from the attacked cell. In the case of toxins with intracellular target, it might arrange a tunnel through which the enzymic part of the molecule crosses the membrane. However, as a first step to investigate their physiological role, these channels have to be dem-

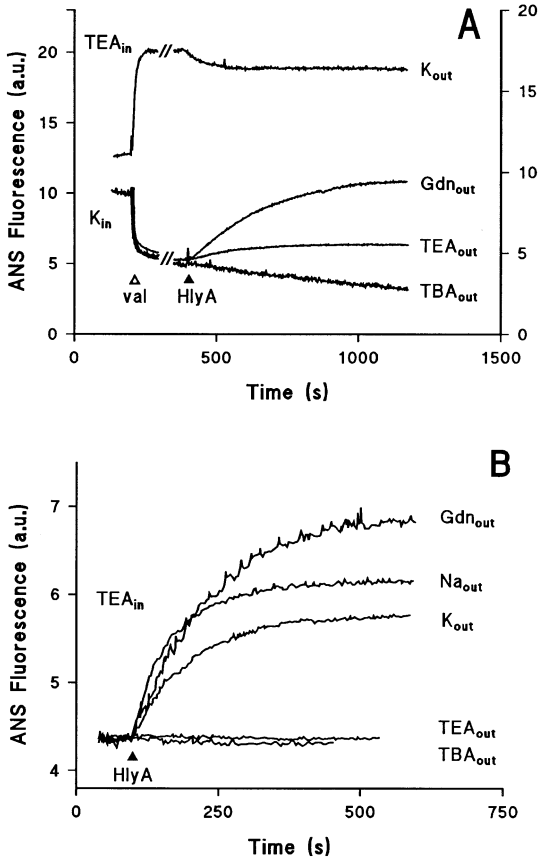

Fig. 7. Creation and dissipation of transmembrane potential in lipid vesicles by HlyA. (*A*) PC vesicles prepared in K^+ buffer were transferred to a solution containing a different cation and ANS (lower traces). Thereafter, first valinomycin and then HlyA were added (as indicated). Valinomycin caused the formation of an inner negative potential, as reported by the decrease of ANS fluorescence. After addition of HlyA, the fluorescence remained constant with TBA^+, indicating that all ionic species, except K^+, were impermeant. In the other cases it increased, indicating that the second cation species was also permeant. In the upper trace (right scale) the protocol was similar, but TEA^+-loaded vesicles were transferred to a K^+ solution. In this case a positive inner potential developed after valinomycin, indicated by the increase in ANS fluorescence, whereas HlyA again produced a reduction of the transmembrane potential. Final concentration of lipid vesicles, ANS, valinomycin and HlyA were 137 μg/ml, 6 μM, 28 nM, and 20 HU/ml respectively. Emitted fluorescence was measured at 490 nm, excitation was at 380 nm and both slits were set at 5 nm. (*B*) Asolectin vesicles loaded with TEA^+ were transferred to a solution containing the indicated cation and ANS. After addition of HlyA (indicated by an arrowhead) the fluorescence usually increased, indicating the development of a positive inner potential implying that the external cation was more permeant than TEA^+. A decrease was observed only with TBA^+ confirming that this cation was less permeant than TEA^+. Experimental conditions as in *A* except that valinomycin was omitted.

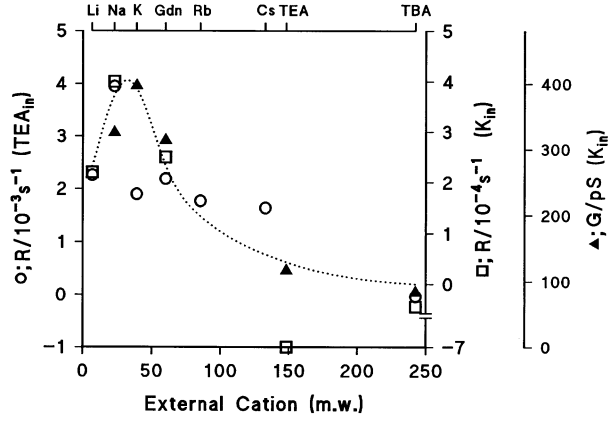

Fig. 8. Size dependence of the cation permeability through HlyA pores. The slope of the percent fluorescence change (*R*) observed under the conditions of Fig. 7*B*, is reported *vs.* the m.w. of the cation (open circles). Open squares are for similar experiments, but with K^+-containing vesicles. Note that a different scale was used (right) and that both TEA^+ and TBA^+ gave negative values in this case, proving they were less permeant than K^+. The single channel conductance at −60 mV, determined from an *I–V* characteristic such as that in Fig. 6, is also reported for comparison (closed triangles and rightmost ordinate scale). Dotted line was drawn by eye.

onstrated in the respective target cells. Such studies are best performed using the patch clamp technique, and are only now beginning to appear [1, 21, 35, 38].

Our experiments indicate that *E. coli* hemolysin can form ion channels in the membrane of attacked macrophages. This could have profound physiological implications since these cells are in charge of the first stages of the immunological response of the host against the bacteria. Such pores are very similar to those which were described in PLM. For example, they exhibit a complex gating mechanism, fluctuating between a fully open state, a low conductance state and a closed state [4, 51]. Only the fully open and the closed state have a long lifetime, whereas the low conductance state is populated very shortly. These substrates possibly correspond to different configurations of the protein. The channel, in the open state, has a large conductance and displays cation selectivity, i.e., anions are much less permanent than cations. However, up to the size of K^+, cations flow through the channel according to the mobility they have in solution [30], suggesting the pore is lined with water [4, 49]. With larger cations the permeability decreases, probably because of the steric hindrances encountered while passing through the pore. The selectivity properties of the permeability induced by the toxin in a purely lipidic membrane was found to be completely consistent. This validates the use of such a simple system to get information on the mode of action of bacterial toxins. Furthermore, to derive such data from lipid vesicles we have developed a new and simple assay which might prove of general interest and application.

On the basis of osmotic protection it was proposed that HlyA induces red blood cells lysis via a colloid osmotic shock ensuing from the formation of hydrophilic pores on their plasma membrane [6]. In this paper, we

demonstrate that probably also its leukotoxic effects do in fact result from its ability to make pores in white cells.

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References

1. Beise, J., Hahnen, J., Andersen-Beckh, B., Dreyer F. 1994. Pore formation by tetanus toxin, its chain and fragments in neuronal membranes and evaluation of the underlying motifs in the structure of the toxin molecule. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **349:**66–73
2. Benz, R., Hardie, K. R., Hughes, C. 1994. Pore formation in artificial membranes by the secreted hemolysins of *Proteus vulgaris* and *Morganella morganii. Eur. J. Biochem.* **220:**339–347
3. Benz, R., Maier, E., Ladant, D., Ullmann, A., Sebo, P. 1994. Adenylate cyclase toxin (CyaA) of *Bordetella pertussis.* Evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli. J. Biol. Chem.* **269:**27231–27239
4. Benz, R., Schmid, A., Wagner, W., Goebel, W. 1989. Pore formation by the *Escherichia coli* haemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregates. *Infect. Immun.* **57:**887–895
5. Bhakdi, S., Greulich, S., Muhly, M., Eberspächer, B., Becker, H., Thiele, A., Hugo, F. 1989. Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J. Exp. Med.* **169:**737–754
6. Bhakdi, S., Mackman, N., Nicaud, J.-M., Holland, I. B. 1986. *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *Infect. Immun.* **52:**63–69
7. Bhakdi, S., Muhly, M., Korom, S., Schmidt, G. 1990. Effects of *Escherichia coli* hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release. *J. Clin. Invest.* **85:**1746–1753
8. Blaustein, R.O., Koehler, T.M., Collier, R.J., Finkelstein, A. 1989. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. *Proc. Natl. Acad. Sci. USA* **86:**2209–2213
9. Brooks, H.J.L., O Grady, F., McSherry, M.A., Cattell, W.R. 1980. Urophatogenic properties of *Escherichia coli* alpha-haemolysin. *J. Med. Microbiol.* **15:**11–21
10. Bϕyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21:**77–89
11. Cauci, S., Monte, R., Ropele, M., Missero, C., Not, T., Quadrifoglio, F., Menestrina, G. 1993. Pore-forming and hemolytic properties of the *Gardnerella vaginalis* cytolysin. *Mol. Microbiol.* **9:**1143–1155
12. Cavalieri, S.J., Bohach, G.A., Snyder, I.S. 1984. *Escherichia coli* alpha-haemolysin: characteristics and probable role in pathogenicity. *Microbiol. Rev.* **48:**326–346
13. Chang, Y.-F., Renshow, H.W., Augustine, J.L. 1985. Bovine pneumonic pasteurellosis: chemiluminescent response of bovine peripheral blood leukocytes to living and killed *Pasteurella hemolytica, Pasteurella multocida* and *Escherichia coli. Am. J. Vet. Res.* **46:**2266–2271
14. Clinkenbeard, K.D., Mosier, D.A., Confer, A.W. 1989. Effects of *Pasteurella haemolytica,* leukotoxin on isolated bovine neutrophils. *Toxicon* **27:**797–804
15. Clinkenbeard, K.D., Mosier, D.A., Confer, A.W. 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. *Infect. Immun.* **57:**420–425
16. Coote, J.G. 1992. Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiol. Rev.* **88:**137–162
17. Czyprynski, C.J., Noel, E.J. 1990. Influence of *Pasteurella haemolytica* A1 crude leukotoxin on bovine neutrophil chemiluminescence. *Infect. Immun.* **58:**1485–1487
18. Dalla Serra, M., Pederzolli, C., Menestrina, G. 1992. A fluorimetric assay for the effects of cytolytic toxins on the transport properties of resealed erythrocyte ghosts. *J. Biochem. Biophys. Meth.* **25:**83–94
19. Donovan, J.J., Simon, M.I., Draper, R.K., Montal, M. 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. USA* **78:**172–176
20. Ehrmann, I.E., Gray, M.C., Gordon, V.M., Gray, L.S., Hewlett, E.L. 1991. Hemolytic activity of adenylate cyclase toxin from *Bordetella pertussis. FEBS Lett.* **278:**79–83
21. Eriksen, S., Olsnes, S., Sandvig, K., Sand, O. 1994. Diphteria toxin at low pH depolarizes the membrane, increases the membrane conductance and induces a new type of ion channel in Vero cells. *EMBO J* **13:**4433–4439
22. Gadeberg, O.V., Orskov, I. 1984. In vitro cytotoxin effect of alpha-hemolytic *Escherichia coli* on human blood granulocytes. *Infect. Immun.* **45:**255–259
23. Gadeberg, O.V., Orskov, I., Rhodes, J.M. 1983. Cytotoxic effect of an alpha-hemolytic *Escherichia coli* strain on human blood monocytes and granulocytes in vitro. *Infect. Immun.* **41:**358–364
24. Gallin, E.K. 1986. Ionic channels in leukocytes. *J. Leuk. Biol.* **39:**241–254
25. Gallin, E.K., McKinney, L.C. 1988. Patch-clamp studies in human macrophages: single channel and whole cell characterization of two K^+ conductances. *J. Membrane Biol.* **103:**55–66
26. Gambale, F., Rauch, G., Belmonte, G., Menestrina, G. 1992. Properties of *Pseudomonas aeruginosa* exotoxin A ionic channel incorporated in planar lipid bilayers. *FEBS Lett.* **306:**41–45
27. Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A., Danchin, A. 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis:* cloning and expression in *Escherichia coli. Mol. Microbiol.* **2:**19–30
28. Hackett, M., Guo, L., Shabanowitz, J., MS, Hunt, D.F., Hewlett, E.L. 1994. Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis. Science* **266:**433–435
29. Hamill, O.P., Marty, A., Neher, E., Sakmann, B. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100
30. Hille, B. 1984. Ionic channels of excitable membranes. Sinauer Associates Publishers, Sunderland Massachussets
31. Hoch, D.H., Romero-Mira, M., Ehrlich, B.E., Finkelstein, A., DasGupta, B.R., Simpson, L.L. 1985. Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: relevance to translocation of proteins across membranes. *Proc. Natl. Acad. Sci. USA* **82:**1692–1696
32. Holland, I.B., Wang, R., Seror, S.J., Blight, M. 1989. Haemolysin secretion and other protein translocation mechanisms in gram-negative bacteria. *In:* Society for General Microbiology Symposium 44. M.Banmerg, I.Hunter and M.Rhodes, editors. pp. 219–254. Cambridge University Press, Cambridge
33. Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Char-

acterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* **812:**55–65
34. Hudson, L., Hay, C.F. 1980. Practical Immunology. Blackwell Scientific Publications, Oxford
35. Hurlbut, W.P., Chieregatti, E., Valtorta, F., Haimann, C. 1994. α-latrotoxin channels in neuroblastoma cells. *J. Membrane Biol.* **138:**91–102
36. Issartel, J.-P., Koronakis, V., Hughes, C. 1991. Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature* **351:**759–761
37. Iwase, M., Lally, E.T., Berthold, P., Korchak, H.M., Taichman, N.S. 1990. Effects of cations and osmotic protectants on cytolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. *Infect. Immun.* **58:**1782–1788
38. Korchev, Y.E., Alder, G.M., Bakhramov, A., Bashford, C.L., Joomun, B.S., Sviderskaya, E.V., Usherwood, P.N.R., Pasternak, C.A. 1995. *Staphylococcus aureus* alpha-toxin-induced pores: channel-like behavior in lipid bilayers and clamped cells. *J. Membrane Biol.* **143:**143–151
39. Koronakis, V., Cross, M., Senior, B., Koronakis, E., Hughes, C. 1987. The secreted haemolysins of *Proteus mirabilis, Proteus vulgaris,* and *Morganella morganii* are genetically related to each other and to the alpha-haemolysin of *Escherichia coli. J. Bacteriol.* **169:**1509–1515
40. Kraig, E., Dailey, T., Kolodrubetz, D. 1990. Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans:* homology to the alpha-hemolysin/leukotoxin gene family. *Infect. Immun.* **58:**920–929
41. Krasilnikov, O.V., Muratkhodjaev, J.N., Zitzer, A.O. 1992. The mode of action of *Vibrio cholerae* cytolysin. The influences on both erythrocytes and planar lipid bilayers. *Biochim. Biophys. Acta* **1111:**7–16
42. Lalonde, G., McDonald, T.V., Gardner, P., O'Hanley, P.D. 1989. Identification of a haemolysin from *Actinobacillus pleuropneumoniae* and characterization of its channel properties in planar phospholipid bilayers. *J. Biol. Chem.* **264:**13559–13564
43. Ludwig, A., Goebel, W. 1991. Genetic determinants of cytolytic toxins from gram-negative bacteria. *In:* Source Book of Bacterial Protein Toxins. J.E. Alouf and J.H. Freer, editors. pp. 147–186. Academic Press, London
44. MacDonald, R.C., MacDonald, R.I., Menco, B.P.M., Takeshita, K., Subbarao, N.K., Hu, L. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* **1061:**297–303
45. Mangan, D.F., Taichman, N.S., Lally, E.T., Wahl, S.M. 1991. Lethal Effects of *Actinobacillus actinomycetemcomitans* Leukotoxin on Human T Lymphocytes. *Infect. Immun.* **59:**3267–3272
46. McCann, F.V., Keller, T.M., Guyre, P.M. 1987. Ionic channels in human macrophages compared with the U-937 cell line. *J. Membrane Biol.* **96:**57–64
47. Menestrina, G. 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: voltage dependent inhibition by di- and trivalent cations. *J. Membrane Biol.* **90:**177–190
48. Menestrina, G. 1991. Electrophysiological methods for the study of toxin-membrane interaction. *In:* Sourcebook of Bacterial Protein Toxins. J.E. Alouf and J.H. Freer, editors. pp. 215–241. Academic Press, London
49. Menestrina, G., Mackman, N., Holland, I.B., Bhakdi, S. 1987. *Escherichia coli* haemolysin forms voltage-dependent channels in lipid membranes. *Biochim. Biophys. Acta* **905:**109–117
50. Menestrina, G., Moser, C., Pellett, S., Welch, R.A. 1994. Pore-formation by *Escherichia coli* hemolysin (HlyA) and other members of the RTX toxins family. *Toxicology* **87:**249–267
51. Ropele, M., Menestrina, G. 1989. Electrical properties and molecular architecture of the channel formed by *E. coli* hemolysin in planar lipid membranes. *Biochim. Biophys. Acta* **985:**9–18
52. Rosendal, S., Devenish, J., MacInnes, J.I., Lumsden, J.H., Watson, S., Xun, H. 1988. Evaluation of heat-sensitive, neutrophil-toxic, and hemolytic activity of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae. Am. J. Vet. Res.* **49:**1053–1058
53. Shiver, J.W., Donovan, J.J. 1987. Interaction of diphtheria toxin with lipid vesicles: determinants of ion channel formation. *Biochim. Biophys. Acta* **903:**48–55
54. Slavik, J. 1982. Anilinonaphtalene sulfonate as a probe of membrane composition and function. *Biochim. Biophys. Acta* **694:**1–25
55. Stanley, P., Packman, L.C., Koronakis, V., Hughes, C. 1994. Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science* **266:**1992–1996
56. Strathdee, C.A., Lo, R.Y. 1987. Extensive homology between the leukotoxin of *Pasteurella haemolytica* Al and the alpha-hemolysin of *Escherichia coli. Infect. Immun.* **55:**323–326
57. Szabo, G., Gray, M.C., Hewlett, E.L. 1994. Adenylate cyclase toxin from *Bordetella pertussis* produces ion conductance across artificial lipid bilayers in a calcium- and polarity-dependent manner. *J. Biol. Chem.* **269:**22496–22499
58. Weiner, R.N., Schneider, E., Haest, C.W.M., Deuticke, B., Benz, R., Frimmer, M. 1985. Properties of the leak permeability induced by a cytotoxic protein of Pseudomonas aeruginosa (PACT) in rat erythrocytes and black lipid membranes. *Biochim. Biophys. Acta* **820:**173–182
59. Welch, R.A. 1991. Pore-forming cytolysins of gram-negative bacteria. *Mol. Microbiol.* **5:**521–528
60. Welch, R.A., Dellinger, E.P., Minshew, B., Falkow, S. 1981. Haemolysin contributes to virulence of extra-intestinal *E. coli* infections. *Nature* **294:**665–667
61. Wilmsen, H.U., Pattus, F., Buckley, J.T. 1990. Aerolysin, a haemolysin from *Aeromonas hydrophila,* forms voltage gated channels in planar lipid bilayers. *J. Membrane Biol.* **115:**71–81