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Escherichia coli haemolysin forms voltage-dependent ion channels in lipid membranes

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The action of the 107 kDa hemolysin from *Escherichia coli* on planar lipid membranes was investigated. We report that a single toxin molecule can form a cation-selective, ion-permeable channel of large conductance in a planar phospholipid bilayer membrane. The conductance of the pore is proportional to that of the bulk solution, indicating that the channel is filled with water. A pore diameter of about 2 nm can be evaluated. The pore formation mechanism is voltage-dependent and essentially resembles that of pore-forming colicins; this implies that opening of the channel is dependent on transfer of an electrical charge through the membrane. We propose that the physiological effects of *E. coli* hemolysin result from its ability to form ion channels in the membrane of attacked cells, and show that there is quantitative agreement between the effects of this toxin on model membranes and its hemolytic properties.

Introduction

The hemolysin of *Escherichia coli* is regarded as a significant factor of bacterial pathogenicity [1–6]. Several hemolysin determinants have now been cloned and their genetic organisation has been established [7–12]. However, little is known about how the toxin damages cells.

Jørgensen et al. [13] have previously suggested that *E. coli* hemolysin acts in a manner similar to a calcium ionophore. In contrast, we have proposed that the toxin acts as a more nonspecific pore-former which permits passage of uncharged molecules with diameters up to 2–3 nm across the lipid bilayer [14]. We have now directly studied the effects of purified 107 kDa hemolysin on planar lipid bilayers, and here present data indicating that monomers of this toxin generate water-filled transmembrane pores in planar lipid bilayers through a voltage-dependent process. The properties of these pores quantitatively account for the effects of the toxin on erythrocytes. The mechanism of hemolysin action closely resembles that of pore-forming colicins [15–18], but differs from pore-formation by toxins from Gram-positive organisms [19–21], complement [22–25] and cytotoxic lymphocytes [26–28]. This is the first detailed study on the action of a cytolysin from a Gram-negative organism that forms pores in mammalian target membranes.

Experimental procedures

Conductance measurements

Planar phospholipid bilayer membranes were prepared by the apposition of two monolayers by

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the Montal technique [29] using reduced egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (both from P-L Biochemicals, more than 99% pure) mixed in a ratio PC : PE = 5 : 1 on a molar basis. In some experiments the synthetic lipid 1,2-palmitoyloleoylglycerophosphocholine (POPC, by Avanti Polar Lipids) was used as the only component. Monolayers were spread from a 5 mg/ml solution of these lipids in *n*-hexane and, after evaporation of the solvent, membranes were formed on a hole in a 12 μm thick Teflon septum separating two buffered salt solutions. The hole had a diameter of about 0.2 mm and was pretreated with a 1 : 20 solution of hexadecane in hexane. Bathing soluitons, 4 ml on each side, contained various amounts of NaCl or KCl (as specified in the text), 1 mM EDTA (Merck) and 5 mM Hepes (Calbiochem) (pH 7.0). The conductivity of each buffer solution was measured with a Philips PW9509 digital conductimeter equipped with a PW9514 cell (cell constant, 1 cm^{-1}). The transmembrane potential was clamped to the desired value with two Ag|AgCl electrodes and the current sent to an $I-V$ converter built around a virtual grounded operational amplifier (Burr Brown OPA 104 C) with feedback resistors ranging from 10^6 to 10^8 Ω. The cis compartment was connected to the virtual ground and voltage signs are referred to it; current is defined positive when cations flow into this compartment. Baseline conductance of the membranes did not exceed 50 pS. Experiments were performed at room temperature.

Stock solutions of *E. coli* hemolysin prepared as in Ref. 14 were stored in small vials at $-75\,^{\circ}C$. For each experiment, the content of one vial was thawed and diluted to the desired concentration in the bathing solution of a preformed, stable bilayer. The activity of the thawed toxin (generally 1000 HU/ml) was determined by lysis of rabbit erythrocytes. *E. coli* hemolysin stored in this fashion was stable for at least 2 months; when left at room temperature, the toxin lost all activity both on erythrocytes and on planar lipid bilayes within 1 day. Unless otherwise stated, hemolysin was added only to the cis compartment, which was held at virtual ground, and addition was followed by a vigorous mixing of the chamber by magnetic bars.

Results and Discussion

Formation of ion channels in planar lipid bilayers by E. coli hemolysin

Within a few minutes following addition of *E. coli* hemolysin to one side of a voltage-clamped phospholipid membrane, current steps of homogeneous size were observed (Fig. 1), indicating the formation of ion channels in the bilayer. The flow rate through the conduction events of Fig. 1 is indeed roughly $3 \cdot 10^7$ ions/s, i.e., 3 to 4 orders of magnitude larger than for a carrier mechanism. Channels opened at negative 'trans' voltages at a rate that was dependent on the magnitude of the potentials (Fig. 2). Traces in Fig. 2a show that at negative voltages the current increased at a rate related to the magnitude of the applied potential.

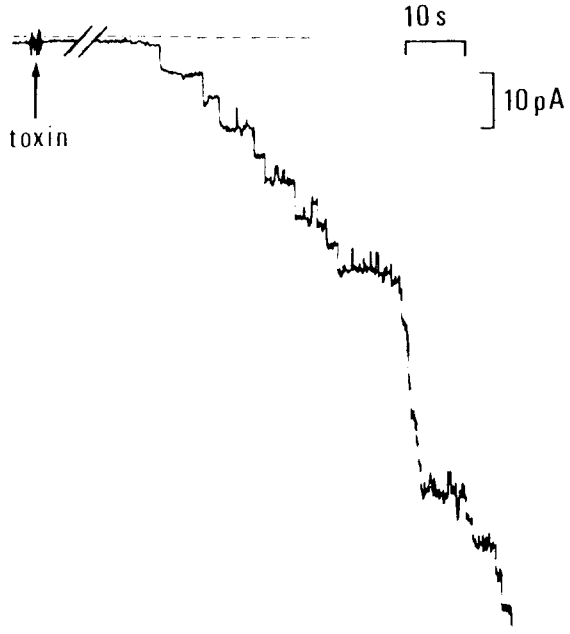

Fig. 1. Single-channel effects of *E. coli* hemolysin on planar lipid bilayers. PC/PE planar bilayers were formed in 100 mM NaCl/1 mM EDTA/5 mM Hepes (pH 7) (buffer A), and clamped at -20 mV (negative sign was on the 'trans' side). 5 HU/ml of hemolysin were introduced into the aqueous phase of the cis compartment, which was then stirred intensively. Note the downward deflections of current in steps of uniform size which are due to opening of single ion channels in the bilayer. A dashed line in this and the following figures indicates zero current; a double bar indicates an interruption of 3 min.

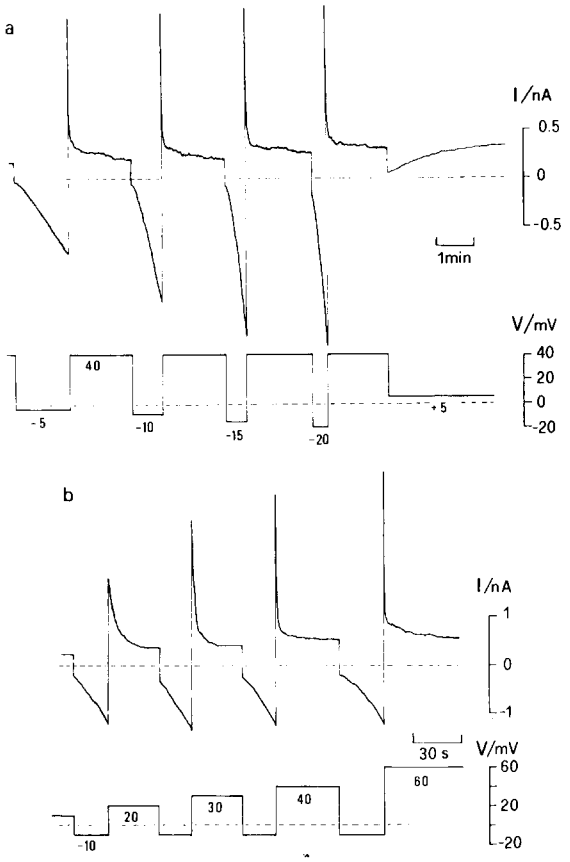

Fig. 2. Macroscopic current response of *E. coli* hemolysin-treated membranes to voltage. (a, b) The upper trace of each pair is current (I in nA) and the lower trace is the applied voltage (V in mV). 12.5 HU/ml of *E. coli* hemolysin were added to a stable membrane prepared in symmetrical buffer A. When negative voltages were applied, the current increased linearly with time following a short initial lag; the current increases corresponded to the opening of channels in the bilayer. At positive voltages applied to the trans side, the current decreased to very low values as the channels closed. The effects of increasingly negative or positive voltages are shown in parts (a) and (b), respectively.

In contrast, the rate of closure at +40 mV did not depend on the negative voltage previously applied. Even at small positive voltages (+5 mV), the channels still turned on and the current eventually leveled off to reach a steady state. Attempts to reach a steady state at large (more than −30 mV) negative potentials always led to membrane breakdown. Traces in Fig. 2b show that the rate of closure of channels increased with the magnitude of the positive voltage applied, whilst the rate of opening at −10 mV was not dependent on the positive voltage previously applied.

The voltage-dependence of ion-channel formation closely resembled that reported for colicin K, Ib, E1, A and Ia [15,30–33]. In contrast to colicins, however, *E. coli* hemolysin did not require the presence of negative lipids in the membrane and was active on neutral phospholipid bilayers of phosphatidylcholine and phosphatidylethanolamine, and also on bilayers of the synthetic lipid, POPC (data not shown). Furthermore, the hemolysin still created pores at small positive voltages (less than 10 mV), whereas colicins always require large negative voltages such as those present in polarized cells [15,30–33]. This fact can explain why the toxin is active on erythrocytes, which have a very low inner potential, and why it does not lose its activity upon depolarization of the cell, which ensues quickly after the escape of a few ions from the cytoplasma and well before any osmotic imbalance is created.

As in the case of colicins, we think that the channels do not detach from the membrane when positive 'closing' potentials are applied. This is based on the fact that the activity of the hemolysin, measured by the rate of pore opening at a fixed negative potential after closing at a fixed positive potential, continously increases during the first stages of the experiment, as more toxin molecules become attached to the membrane, and eventually levels off to reach a steady-state value. It never decreases, despite the fact that the activity of the toxin in the bathing solution is decreased either by natural inactivation, which always takes place, or even by perfusion of the cis compartment, which we have performed with 8 vol. of toxin-free solution and which we have tested to be more than 98% efficient in removing a control dye (data not shown).

Under the steady-state conditions indicated above the rate of pore-opening increased exponentially with applied voltage: an e-fold increase was registered for every 10.5 mV in 100 mM NaCl, and for every 10 mV in 500 mM NaCl or KCl. A slight saturation was observed only for rates larger than 1000 pore openings/min (Fig. 3a). Assuming a two-state equilibrium between an open and a

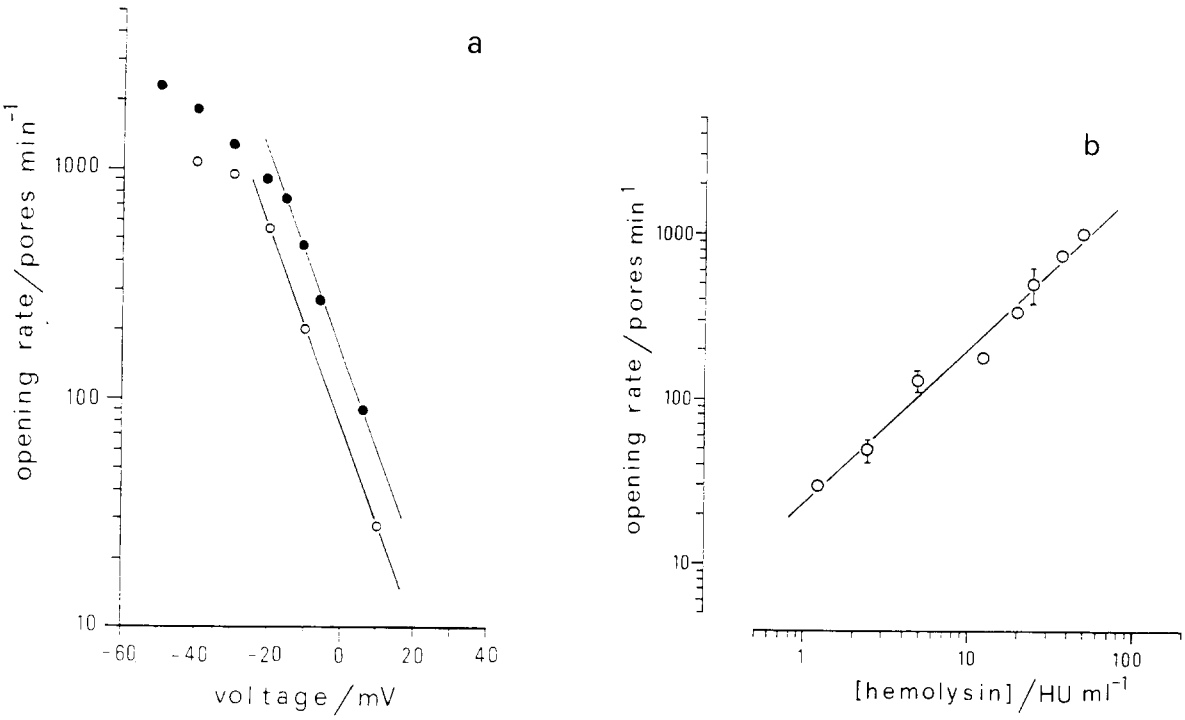

Fig. 3. (a) Voltage-dependence of the pore formation rate was obtained using the linear current increase at negative voltages divided by the single-channel current, evaluated as in Fig. 1. Membranes were prepared either in buffer A (full symbols) or in 500 mM KCl/1 mM EDTA/5 mM Hepes (pH 7) (open symbols; 25 and 12.5 HU/ml of hemolysin were added, respectively. Points are mean values of experiments done in triplicate. The change in pore formation rate extrapolated from the linear part of the half-logarithmic plot is e-fold/10.5 mV in NaCl and e-fold/10 mV in KCl. (b) Concentration dependence of the pore formation rate at a given voltage in a double-logarithmic plot. Each point is the mean of at least three independent determinations on different membranes taken when a steady state had been reached. Clamp voltage was -20 mV and membranes were prepared in buffer A. The slope of the regression line is 0.94.

closed configuration of the channel, we can write:

$$C \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} O \tag{1}$$

$$\frac{dO}{dt} = k_1 C - k_{-1} O \tag{2}$$

and

$$C + O = N \tag{3}$$

where O and C are the number of open and closed channels respectively, N is the total number of pores in the membrane in either configuration, k_1 and k_{-1} are the rate constnats for the opening and the closing reaction, respectively, and dO/dt is the pore-opening rate. When channels reopen after being closed at a positive voltage (as in Fig. 2a), the initial condition that O is nil may be applied and hence from Eqns. 2 and 3 we obtain:

$$\left.\frac{dO}{dt}\right|_{in} = k_1 N \tag{4}$$

with $dO/dt|_{in}$ indicating the initial pore-opening rate. Provided that N is fixed under steady-state conditions, the exponential dependence of Fig. 3a implies that:

$$k_1 = k_1^0 \exp(-\alpha V) \tag{5}$$

where k_1^0 and α are constants. Since the opening rate constant is generally expected to depend exponentially on the difference in energy between the closed state and the activated state which precedes the opening, Eqn. 5 indicates that the opening reaction requires the movement of a gating charge through the applied electric field (for a

comprehensive discussion see Ref. 34). From the slope of the linear portion of the plot (Fig. 3a), we can estimate that the voltage-driven opening of channels requires the movement of a minimum of 2.5 elementary charges across the bilayer. This estimate is a lower limit, because it assumes that the voltage dependence of the gating mechanism resides completely in the forward 'opening' reaction and is nil in the backward 'closing' reaction and that the gating particle actually experiences all of the applied voltage. If either of these conditions is not fulfilled, the real moving charge is larger than what we have indicated [34]. The sign of the charge is also not defined unambigously: it can be either a positive charge moving from the cis to the trans side of the membrane or a negative charge going the opposite way.

At a fixed voltage, the rate of channel-opening is linearly related to the concentration of the toxin in the bathing solution (Fig. 3b). Comparing this result to the predictions of Eqn. 4 one simply finds that the total number of bound molecules N is linearly proportional to the concentration of the toxin added to the solution, which is in agreement with the concept that the toxin is active as a monomer. This is consistent with the single-hit kinetics of the lytic process [6,13], and with the finding that only monomers are detectable in detergent-solubilized membranes [14]. The data cannot exclude the possibility that pore-formation is due to insertion of preformed, detergent-labile oligomers into the bilayer. Simple calculations indicate a fairly good agreement between the pore formation rate in planar bilayers at -20 mV in the presence of 1 HU/ml of toxin, which is roughly $4 \cdot 10^8$ pores per min per m^2 of bilayer, and the rate of hemolysis of rabbit erythrocytes, which is $1 \cdot 10^8$ lethal hits per min per m^2 of cell surface, with the same amount of toxin; this last number is actually a lower limit, since multiple hits on the same cell are not detected in the test. At low toxin concentrations, or during the early stages in each experiment, discrete current transitions corresponding to opening and closing of channels can be recorded (Fig. 4). The voltage-dependence of this phenomenon closely matches that of membranes containing many channels, confirming that bulk current responses like those in Fig. 2 actually result from the sum of the contributions of many identical and independent single-channel events.

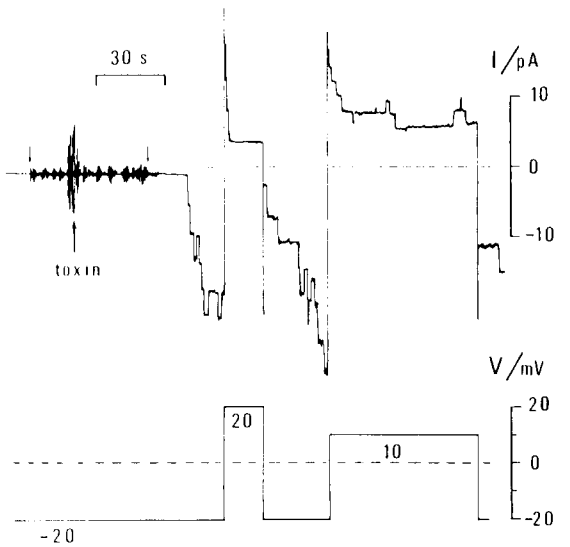

Fig. 4. Microscopic behaviour of *E. coli* hemolysin-treated planar lipid membranes. Upper trace: current (in pA) flowing through a membrane, prepared in symmetrical buffer A, containing few channels; lower trace: applied voltage (in mV). The turn-on of single channels at negative voltages (downward deflections) and the turn-off at positive voltages are resolved as discrete current steps. 5 HU/ml of hemolysin were added where indicated. The large noise in the region between the two small arrows was due to intense stirring of the cis solution.

The current/voltage (I/V) curve of the single channel appears to be ohmic (Fig. 5a), and the derived conductance is roughly linearly related to the conductivity of solutions containing differing amounts of NaCl (Fig. 5b), indicating that the pore is filled with water. Assuming each pore to be a cylinder filled with aqueous solution, its conductivity, G, is given by:

$$G = (\pi r^2/l) \cdot \sigma \qquad (6)$$

σ being the bulk solution conductivity and r and l representing radius and length of the pore, respectively. If $l \approx 5$–7 nm, a pore diameter of 1.1–1.3 nm can be evaluated. This is compatible with the value of 2–3 nm derived from marker release studies in erythrocytes [14]. The lower value found here probably stems in part from the strong cation selectivity of the channel (Fig. 5a), which puts some limitations on the applicability of the G/σ relationship used above. In fact, a diameter 1.6-

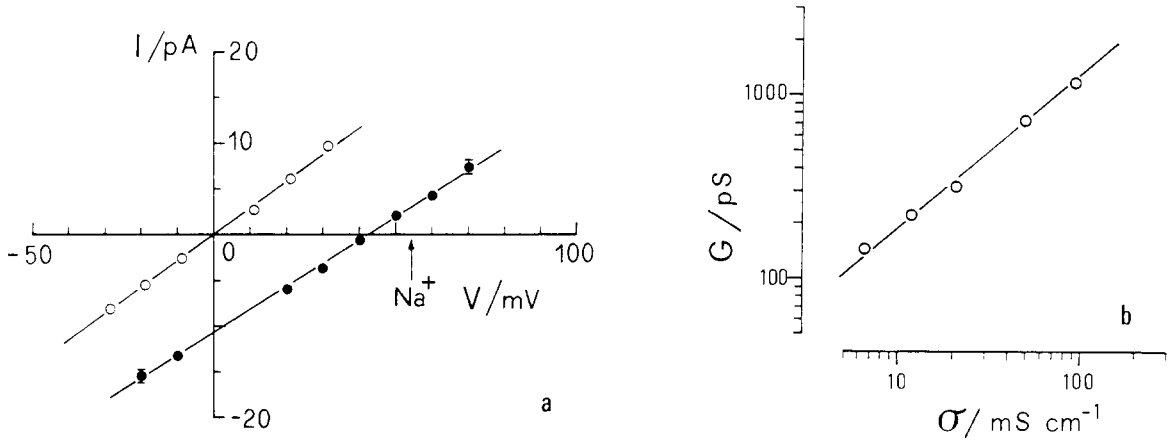

Fig. 5. (a) Single-channel current amplitude as a function of the applied voltage. Results derive from membrane separating two symmetrical 200 mM NaCl solutions (open circles), and from membrane separating two asymmetrical solutions containing either 500 mM (cis side) or 50 mM (trans side) NaCl (closed circles); all solutions contained 1 mM EDTA and 5 mM Hepes (pH 7). Each point is the average ± S.D. of at least 20 determinations during the same experiment. The current/voltage (I/V) curve is ohmic within resolution in this voltage range. The I/V curve extrapolates at a reversal voltage (V_{rev}) of +43 mV in the asymmetrical situation, indicating a substantial cation selectivity. Ideal cation selectively under these conditions is indicated by an arrow. (b) Double-logarithmic plot of single-channel conductance, G, related to conductivity of the aqueous (NaCl) solution. G was determined as the slope of an I/V curve as in Fig. 3b. All points are mean ± S.D. of at least three independence determinations on different membranes. All saline solutions contained 1 mM EDTA and 5 mM Hepes (pH 7). The slope of the regression line is 0.84.

times the calculated dimesion is obtained if one uses the equivalent conductivity of Na^+ alone instead of the bulk salt conductivity. The large conductance of the channel under physiological conditions, roughly 200 pS in 100 mM NaCl, perfectly accounts for the action of the toxin on erythrocytes. Thus, one can calculate that the total release of internal K^+ will occur within 1 min of the opening of the first channel in the erythrocyte membrane. Indeed, flux experiments have shown that efflux of cellular K^+ and influx of external Ca^{2+} are essentially complete within 1 min [14].

Ion selectivity of E. coli hemolysin channels

The ion selectivity of the channel was studied by determining the reversal voltage (V_{rev}) in a 10-fold salt gradient either by measuring the intersection of the single-channel I/V curve with the voltage axis (Fig. 5a), or by the method of 'tail currents' [35,36] on membranes containing many channels (Fig. 6). V_{rev} by the two methods always agreed within 1 mV and indicated a substantial cation selectivity with permeability ratios P_{K^+}/P_{Cl^-} of 14.8 ± 0.8 and P_{Na^+}/P_{Cl^-} of 15.3 ± 0.9, consistent with the earlier report that the hemolysin lesion in erythrocytes renders the cells permeable to cations (K^+ and Ca^{2+}) but not to PO_4^{3-} [13]. Colicin channels are also cation-selective, but to a slightly lesser extent [15,35]. The ratio between single-channel conductance in KCl and NaCl for *E. coli* hemolysin is 1.8 at 50 mM and 1.6 at 500 mM, similar to the ratio between the absolute mobilities of the two cations in water (about 1.5). This is a further indication that the pore is filled with water.

Effects of proteolytic enzymes on membranes treated with E. coli hemolysin

As shown in Fig. 7, the permeabilizing effects of 25 HU/ml of hemolysin on the cis side were completely abolished by 9 U/ml of pronase on the same side, but the enzyme did not protect the membrane from the action of the same amount of toxin added in the trans compartment. Similarly, the conductance induced by 25 HU/ml of *E. coli* hemolysin added on the cis side of a bilayer was unaffected for more than 30 min after addition of 13 U/ml pronase to the trans side, but disappeared within a few minutes if the enzyme was added to the cis side (not shown). The same results were obtained with trypsin (0.65 U/ml) and α-chymotrypsin (56 U/ml).

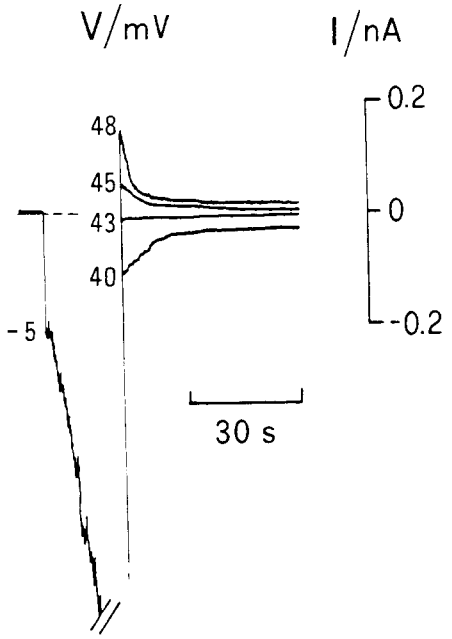

Fig. 6. Ion-selectivity in membranes containing many channels demonstrated by the method of tail currents [35,36]. A negative voltage (−5 mV) was applied to open the channels. Therefore, the potential was switched to different positive voltages and the potential yielding zero current was determined. Only the first part of one current trace during the opening prepulse is shown in the figure; the conductance at the end of the prepulse was constant in all cases. Traces shown have been obtained on a membrane separating two asymmetrical KCl solutions (500 mM cis and 50 mM trans) containing 1 mM EDTA/5 mM Hepes (pH 7), using 20 HU/ml of hemolysin. Extrapolated V_{rev} was 43.2 mV, indicating a P_{K^+}/P_{Cl^-} ratio of 16.

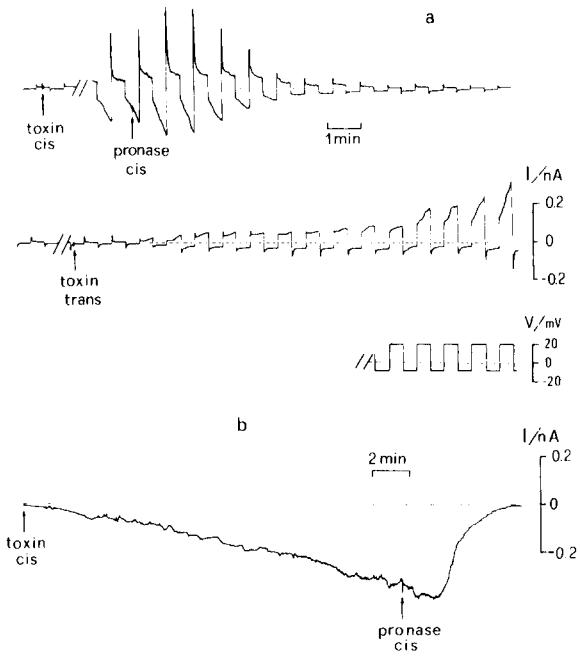

Fig. 7. Effects of proteolytic enzymes on the conductance induced by *E. coli* hemolysin in lipid bilayers. (a) *E. coli* hemolysin, 25 HU/ml, was added to a membrane prepared in buffer A and a continuous square wave was applied (voltage varied between −10 mV and +20 mV, duration was 50 s). A dynamic equilibrium was reached after a short period, channels turning on at the negative voltage with a constant rate and turning off at the positive voltage with a fixed time constant. Addition of pronase (9 U/ml) on the cis side (arrow) produced total disappearance of the toxin-induced conductance within 10 min. Subsequent addition of hemolysin to the trans side (arrow in lower trace) led to appearance of new channels. The lower trace is a direct continuation of the upper trace; upper and lower gaps represent 20 and 23 min interval, respectively. As expected, channel gating was now reversed. Pronase present in the opposite compartment was now unable to destroy the pores. (b) Effects of pronase on open channels. 12.5 HU/ml of *E. coli* hemolysin was added to a bilayer clamped at −10 mV in buffer A (arrow). After formation, channels wer locked in the open state at −10 mV. Addition of 17.5 U/ml of pronase to the cis side (arrow in trace) produced total resealing of the membrane within 5 min.

Thus, pronase, trypsin and α-chymotrypsin were all able to completely destroy the pores formed by the toxin when added to the same compartment (cis), but were completely ineffective when added to the trans solution. As shown in Fig. 7b, the inactivating affects were also noted when proteases were added to bilayers containing channels clamped in the conducting configuration, i.e., when at least part of the toxin was in membrane-inserted form. A similar situation has been reported for colicin E_1 [31].

Conclusions

We have found that *E. coli* hemolysin acts on lipid membranes by forming ionic channels and this property accounts for the lytic effects of the toxin on erythrocytes. It is clear that specific membrane 'receptors' are not required for the toxin to generate the transmembrane pores. The large single-channel conductance is entirely compatible with the noted osmotic lysis of erythrocytes that follows rapid efflux of cellular K^+. It accounts for the finding that the toxin is 10^3–10^4-times more effective than the ionophore A23187 in translocating Ca^{2+}. The channel is

clearly cation-selective and is probably filled with water. The voltage-dependence of pore-opening accounts for its effects on viable cells which bear the correct inner potential to turn on the channels. At variance with earlier reports [13], but in agreement with more recent results [14], we found that channel activity does not require the presence of Ca^{2+} or other divalent cations in the solution. The *E. coli* hemolysin pore resembles colicin channels in that it acts probably as a monomer to form open channels that are ohmic, are cation-selective, display a conductance proportional to the conductivity of the solution, and open at a rate which is exponentially determined by the applied voltage. That pores are created by protein monomers is further indirectly supported by the absence of ultrastructural 'lesions' on toxin-lysed erythrocyte membranes which is in fact an indication that pores have an effective mass lower than 200 kDa, the lower limit of electron microscopic detection.

The pores are sensitive to the action of proteolytic enzymes on the cis, but not on the trans side of planar lipid membranes. In this respect, *E. coli* hemolysin pores, like those formed by colicins, again differ from the voltage-independent and proteinase-resistant oligomeric channels formed by *S. aureus* α-toxin or complement. However, in contrast to pore-forming colicins, *E. coli* hemolysin channels have a larger conductance and do not require either the presence of negatively charged lipids in the membrane, or the existence of a large transmembane potential to open. It is noteworthy that we have not found any sequence homologies between *E. coli* hemolysin and any pore-forming colicin. The mode of action of *E. coli* hemolysin may emerge as a prototype for other pore-forming exotoxins of Gram-negative organisms that act on mammalian target membranes.

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