J. Membrane Biol. 127, 195–203 (1992)

The Journal of
Membrane Biology

Differential Sensitivity of Pneumolysin-Induced Channels to Gating by Divalent Cations

Y.E. Korchev*, C.L. Bashford, and C.A Pasternak
Department of Cellular & Molecular Sciences, Division of Biochemistry, St. George's Hospital Medical School, University of London, London SW17 ORE, United Kingdom

Summary. The induction of channels across planar lipid bilayers by purified, recombinant pneumolysin (a hemolytic protein from *Streptococcus pneumoniae*) has been studied by measuring increases in electrical conductivity. Pneumolysin-induced channels exhibit a wide range of single channel conductances (<50 pS to >1 nS at 0.1 M KCl). Channels can be categorized on the basis of their $K^+ : Cl^-$ selectivity: the smallest channels are strongly cation selective, with t_+ (the cation transference number) approaching 1.0; the largest channels are unselective ($t_+ \approx 0.5$). Channels tend to remain open at all voltages (−150 to 150 mV); only the smallest channels exhibit any rectification.

In the presence of divalent cations (1–5 mM Zn^{2+}; 10–20 mM Ca^{2+}), small (<50 pS) and medium-sized (50 pS to 1 nS) channels are closed in a voltage-dependent manner (more closure at higher voltages); at 0 voltage channels reopen. Overall selectivity is reduced by divalent cations, compatible with small, selective channels being closed preferentially to large, nonselective ones.

It is concluded that a single molecular species (pneumolysin) induces multiple-sized channels that can be categorized by cation : anion selectivity and by their sensitivity to closure by divalent cations.

Key Words pneumolysin · ion channels · lipid bilayers · gating · divalent cations · toxin

Introduction

Streptococcus pneumoniae is a common cause of pneumonia, meningitis and otitis media [1]. A putative pathogenic determinant synthesized by this organism is a 53,000 mol wt hemolytic protein called pneumolysin, that shares several properties with hemolytic proteins produced by *Streptococcus pyogenes* (streptolysin O), *Clostridium perfringens* (perfringolysin or theta toxin) and other Gram positive bacteria [20]. These include oxygen lability and a requirement for cholesterol, in addition to their capacity to damage membranes by the creation of relatively large pores [5]. The leakage of metabolites through such pores [21], as well as through smaller pores induced by *Staphylococcus aureus* α toxin [3, 6, 9] and other agents [2], is prevented by divalent cations like Ca^{2+} or Zn^{2+}. Prevention of leakage is not due to the displacement or pore-forming agent from the cell membrane [2, 6], but to the maintenance of potential pores in a nonleaky state [13, 17]. In the case of *S. aureus* α toxin [2, 12, 13] and *C. perfringens* θ toxin [13], conductivity measurements across planar lipid bilayers show that toxin-induced channels are closed by Ca^{2+} or Zn^{2+} and can be re-opened by reducing the applied voltage to zero.

Because the gene for pneumolysin has been cloned, sequenced [23] and expressed in *E. coli* [15], it is possible to generate site-directed mutants [18]. The similarity between pneumolysin and *C. perfringens* toxin (perfringolysin) [22] makes an investigation of the divalent cation sensitivity of pneumolysin and its mutants an obvious approach for trying to elucidate the mechanism(s) by which divalent cations affect toxin-induced channels. Here we show that highly purified recombinant pneumolysin isolated from *E. coli* which is to all intent identical with native pneumolysin isolated from *S. pneumoniae* [15], induces a wide spectrum of different-sized channels across planar lipid bilayers that can be distinguished on the basis of their cation/anion selectivity and that have a differential sensitivity to closure by divalent cations. [The word channel is used in an operational sense only, to indicate particular conductance states that flicker between an open and a closed configuration; the word is not meant to imply the existence necessarily of discrete protein assemblies analogous to those that constitute endogenous ion channels (e.g. [11]).] Some of these results have been presented in brief at a recent meeting [17*a*].

* *Permanent address:* Institute of Cytology, Academy of Sciences, 9064 St. Petersburg, Russia.

Materials and Methods

Toxin

Recombinant pneumolysin (C428), prepared and purified as described [15], was used throughout; SDS-PAGE indicates this preparation to consist of a single major band as assessed by Coomassie blue (Fig. 2*B* of ref. 15) and by N-terminal analysis (15). It was kindly donated by Dr. G.J. Boulnois.

Lipids

Dioleoyl phosphatidylcholine (DOPC), asolectin, cholesterol and ergosterol were obtained from Sigma.

Methodology

Planar bilayers were formed according to the technique described by Montal and Muller [16] or that of Schindler [19]. The apparatus consisted of two Teflon chambers (capacity 0.1 ml each) connected by an aperture (10–20 μm diameter) across a 10 μm thick Teflon film. Ag/AgCl electrodes were used; the electrode connected to virtual ground was in the chamber to which pneumolysin was added (*cis*). Voltage signs refer to the *cis* compartment; at positive potential cations flow from *cis* to *trans*. KCl buffered to pH 7.4 with 5 mM HEPES was used throughout; the concentration of KCl was 0.1 M in both chambers, unless selectivity was being measured, in which case the solution in *trans* chamber contained 0.01 M KCl. All experiments were performed at room temperature. The cation selectivity or transference number (t_+) was calculated from the corrected, applied potential (ψ^0, the null potential) which gave zero transmembrane current

$$\psi^0 = \psi_m^0 - \psi_c^0 \tag{1}$$

where ψ_m^0 is the null potential of the membrane containing pneumolysin and ψ_c^0 is the null potential of the chamber with no membrane separating the aqueous compartments. t_+ and ψ^0 are related according to the expression:

$$\psi^0 = (2t_+ - 1)\frac{RT}{F}\ln\frac{[K^+]\,trans}{[K^+]\,cis} \tag{2}$$

where R, T and F have their usual meanings. This analysis corrects for contributions of half-cell potential at the Ag/AgCl electrodes [*via* Eq. (1)] and assumes that, in the KCl solutions employed here, the diffusion potential across the open aperture is zero.

After several pilot studies, a mixture of DOPC: ergosterol (1 : 2 molar ratio) was found to give stable channels with pneumolysin and was used for most of the experiments reported herein; EDTA (0.1 mM) was generally added as this appeared to facilitate formation of pneumolysin-induced channels.

Results

Characterization of Channels

Pneumolysin added to bilayers induces channels of widely varying size. Typical recordings from three different membrane preparations are illustrated in Fig. 1. In trace *a*, channels with an approximate conductance of 35 pS, as well as smaller fluctuations, are seen. Such channels (<50 pS) will be designated "small." Trace *b* shows channels of approximate conductance of 300 pS, designated as "medium" (>50 pS to <1 nS). Channels of approximate conductance of 12 nS are seen in trace *c*; such channels (>1 nS) are designated "large." A common feature of all three types of channel is that they tend to be in the open state much of the time. There is no obvious relationship between amount of pneumolysin added and the number of channels or their particular conductance. The frequency of finding small, medium or large channels in any given membrane is indicated in Table 1.

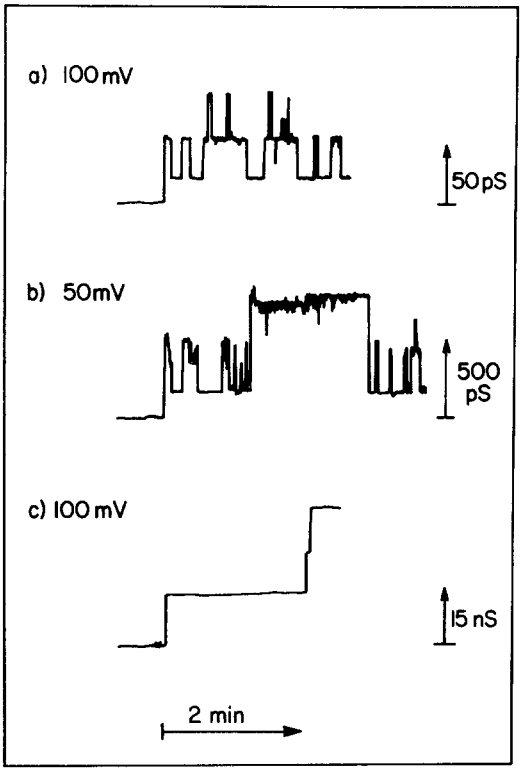


Fig. 1. Pneumolysin-induced channels in DOPC : ergosterol planar lipid bilayers. Pneumolysin (50 μg/ml final concentration) was applied to DOPC : ergosterol (1 : 2 mol/mol) bilayers bathed in 0.1 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) at 25°C. Records from three separate membranes with applied potential held at the value indicated are presented. Small channels (conductance <50 pS), medium channels (conductance 50–1000 pS) and large channels (conductance >1000 pS) are shown in traces *a*, *b* and *c*, respectively.

The current-voltage characteristics of membranes containing the various types of channel were investigated by following current when the voltage is being continuously varied between −150 and +150 mV (with a triangular wave form) (Fig 2); in this

Table 1. Frequency of small, medium or large channels in membranes

Channel size	Frequency (%)
Small	48
Medium (+*S*)	37
Large (+*M*+*S*)	15

The frequency of finding small (*S*), medium (*M*) or large channels [as defined in the text] in >100 membranes analyzed is given. For technical reasons the simultaneous presence of smaller-sized channels in membranes containing medium or large channels is difficult to assess.

instance asymmetric solutions of KCl were used, so that a measure of the cation/anion selectivity can also be deduced (from the voltage at which the current is zero). As is evident from the traces depicted in Fig. 2, membranes containing different categories of small channels can be distinguished by whether there is rectification or not. Small channels having high selectivity ($t_+ \approx 1$) are rectified (less current at negative voltage; more current at positive voltage), whereas small channels having somewhat less selectivity ($t_+ \approx 0.9$) are not. There is also some evidence of more current at voltages $> +100$ mV or < -100 mV in the case of membranes containing several medium-sized channels, compatible with voltage-dependent opening of additional channels. Membranes with large channels show little selectivity, rectification, or voltage-dependent opening.

Effect of Divalent Cations

When Ca^{2+} is present in the same chamber (*cis*) as pneumolysin, the effect is to cause voltage-dependent closure by steps that correspond to small and medium-sized channels; closure occurs at positive voltage only. This is illustrated in Fig. 3*A*, which also shows that closure is reversible, in the sense that switching polarity reopens the channels. If Ca^{2+} is added to both chambers (*cis* and *trans*), closure occurs at negative as well as positive voltages (compatible with Ca^{2+} being required to be drawn into the channel in order to effect closure); this is illustrated in Fig. 3*B*, which again shows reversibility (merely reducing voltage to zero reopens channels). In general, Ca^{2+} concentrations of 10 mM or more are necessary to observe clear-cut effects.

With Zn^{2+} the effects are similar to those with Ca^{2+}, except that concentrations in the range of 1–5 mM are sufficient to elicit a response. In addition, Zn^{2+} typically tends to open (or insert) additional channels before causing closure. Figure 4*A* shows a representative trace, with Zn^{2+} present on the *cis*

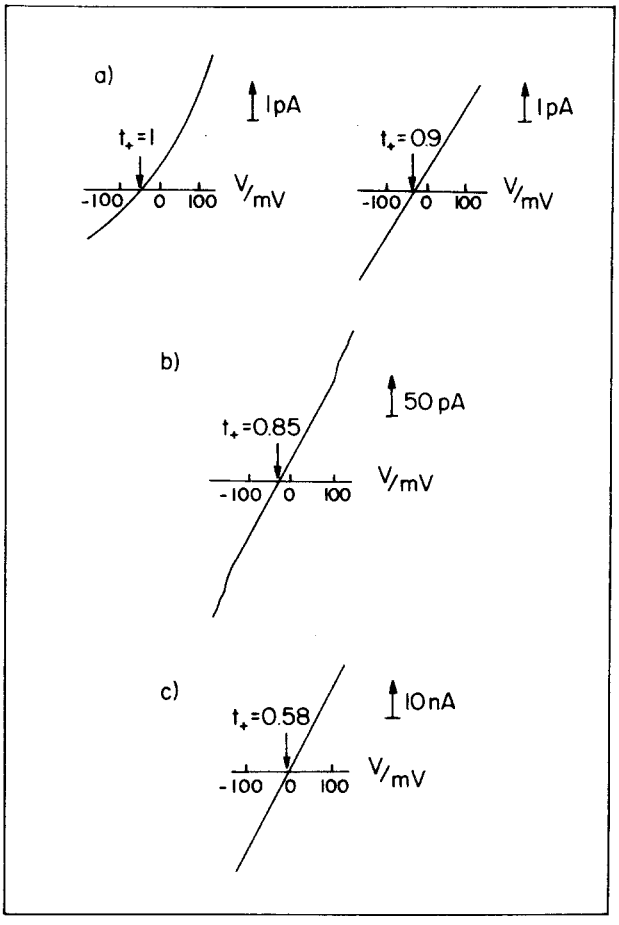


Fig. 2. Current-voltage characteristic of membranes containing pneumolysin-induced channels. Pneumolysin (5–50 μg/ml final concentration) was applied to DOPC : ergosterol bilayers bathed in 0.1 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) on the side to which pneumolysin was added (*cis* side) and 0.01 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) on the opposite (*trans*) side. The records of current with respect to applied voltage were measured using triangular changes in applied potential between ± 150 mV at a speed of 2 mV/sec. Potentials are expressed relative to the potential required for zero current (null potential, ψ_c^0) between the chambers in the absence of the membrane (i.e., through the Teflon orifice). Cation transference numbers (t_+) were calculated from the null potential in the presence of a pneumolysin-containing membrane (ψ^0) according to the relationship

$$t_+ = \frac{59 + \psi^0}{118}$$

(*see* Eq. (2) in Materials and Methods). The two traces labeled *a* were from two membranes, each of which appeared to contain a single small pneumolysin channel (26 and 21 pS, respectively); trace *b* was from a membrane containing 4–6 medium (200–500 pS) channels; trace *c* was from a membrane containing about 10 large (10–20 nS) channels.

side only; if Zn^{2+} is present on both sides, closure occurs at negative voltage also (*not shown*). The rapidity of closure by Zn^{2+} is to be noted (>90% within 2 min); since channels reopen almost com-

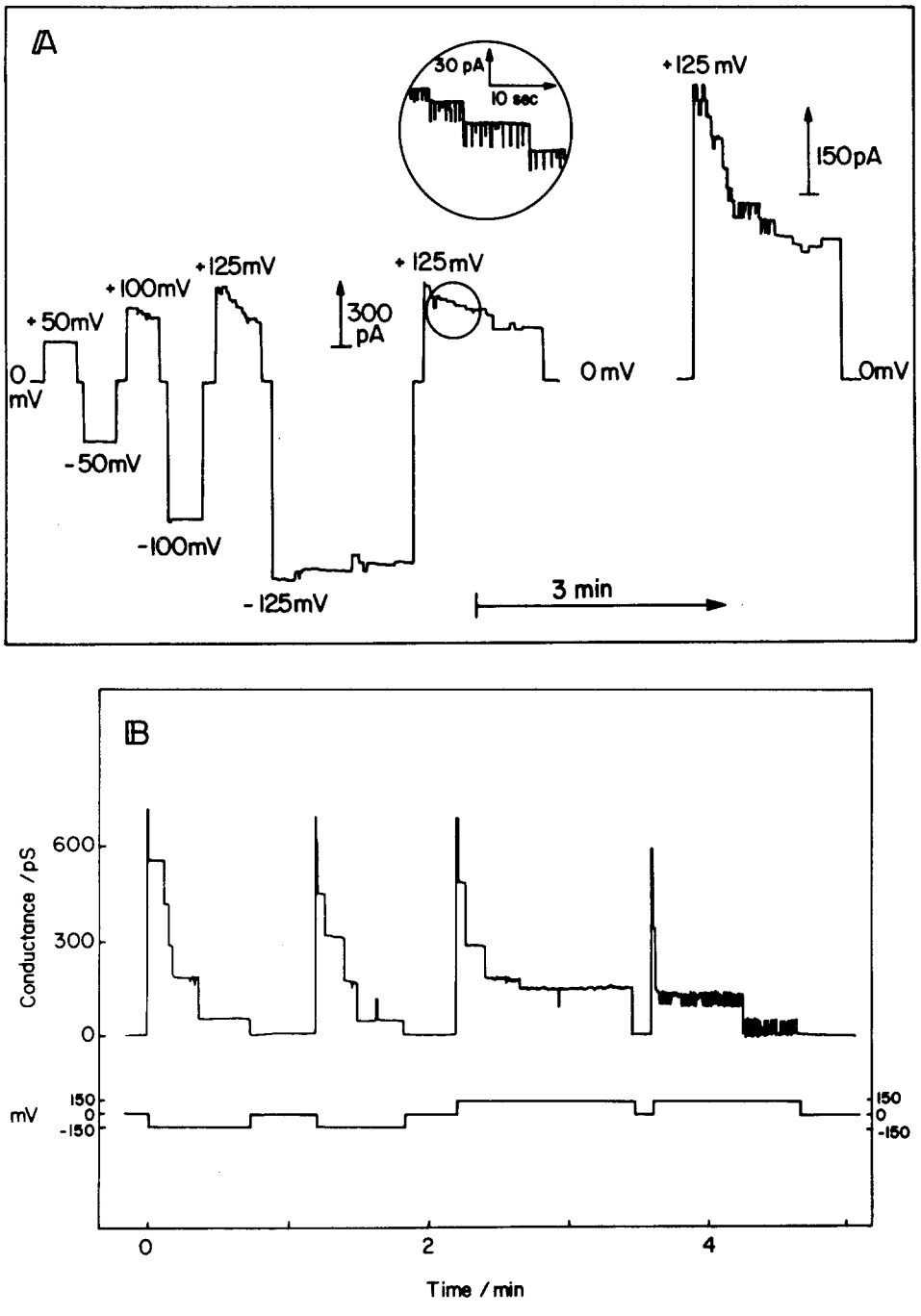


Fig. 3. Closure of pneumolysin-induced channels by Ca^{2+}. Pneumolysin (50 μg/ml (*A*); 0.25 mg/ml, (*B*)) in DOPC : ergosterol (1 : 2) membranes bathed in 0.1 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) at 28°C and 20 mM $CaCl_2$ (*cis* side only, *A*) or 10 mM $CaCl_2$ (*cis* and *trans*, *B*). The inset in *A* is an expanded view of the trace as indicated; traces are records of current obtained at the applied voltages indicated. (*B*) The upper trace shows membrane conductance (indicated as positive irrespective of the sign of the applied potential) obtained at the voltages indicated by the lower trace.

pletely after switching to negative voltage (Fig. 4*B*), it is possible that much of the initial closure at positive voltage is missed by the relatively slow response time of the apparatus, though other explanations for such apparent rectification are possible. Removal of Zn^{2+} by EDTA (right part of trace in Fig. 4*B*) restores the open configuration of channels, with equivalent conductivity at positive and negative voltages. The "ragged" appearance of the membrane conductance shown in Fig. 4*B* is due to open-

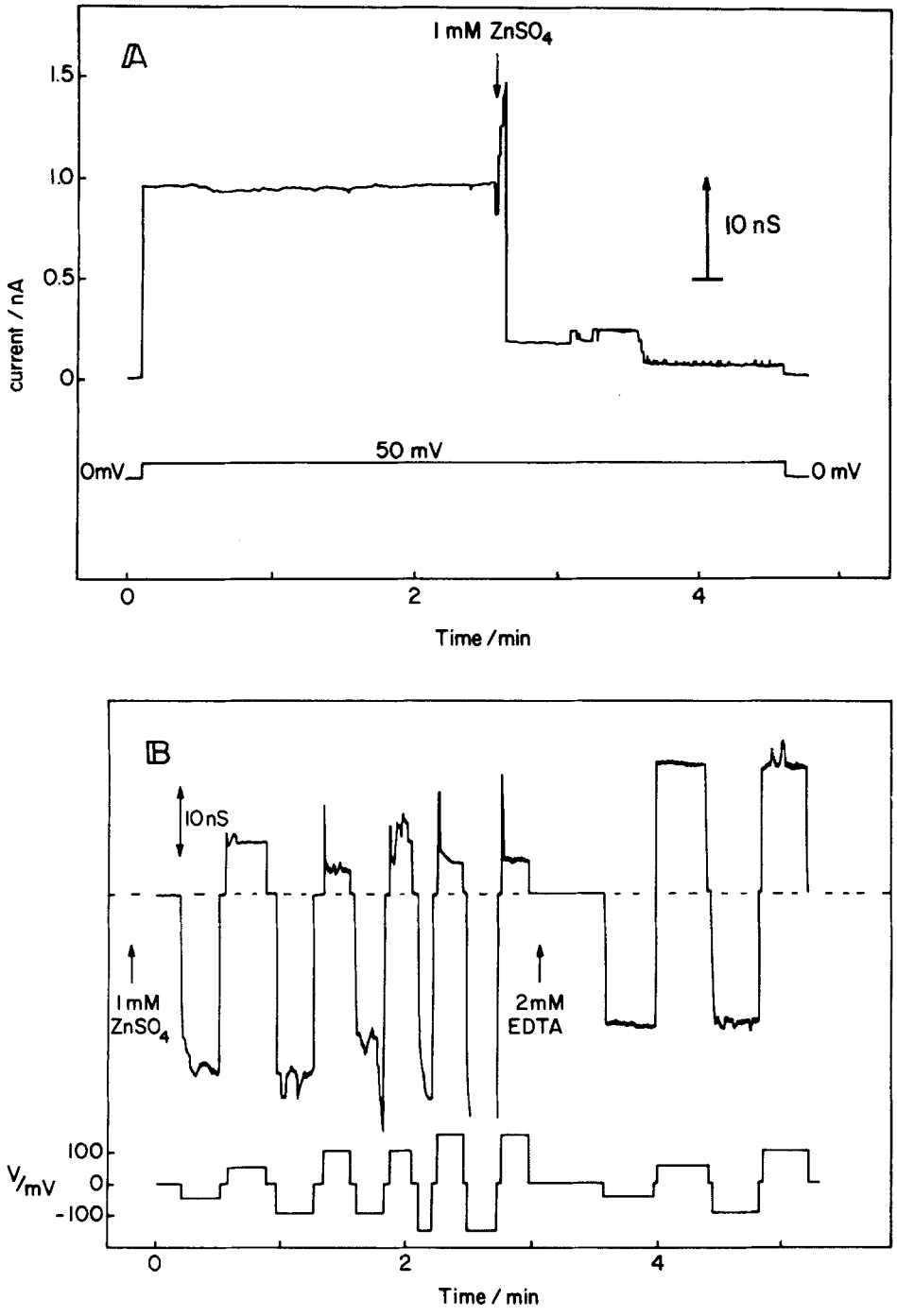


Fig. 4. Closure of pneumolysin-induced channels by Zn^{2+}. Pneumolysin (25 μg/ml) was applied to DOPC : ergosterol (1 : 2) membranes bathed in 0.1 M KCl, 0.005 M HEPES, 0.1 mM EDTA, pH 7.4 (with KOH) at 28°C: 1 mM $ZnSO_4$ (*cis* side), and 2 mM EDTA were subsequently added as indicated. (*A*) Membrane current (upper trace) is shown at +50 mV, applied as indicated (lower trace). (*B*) The conductance of the membrane (upper trace) is shown at a series of applied potentials (indicated in the lower trace); the dotted line indicates zero conductance (obtained at zero applied potential).

ing and closing of pneumolysin channels rather than generalized membrane "leakiness"; when the Zn^{2+} was removed by chelation stable conductance was restored.

The effect of divalent cations on rectification and closing of pneumolysin-induced channels is further illustrated in Figs. 5 and 6. Figure 5 shows the effect of Ca^{2+} (*cis*) on the various-sized channels, identified on the basis of their selectivity. Comparison with Fig. 2 shows that highly selective ($t_+ \approx$

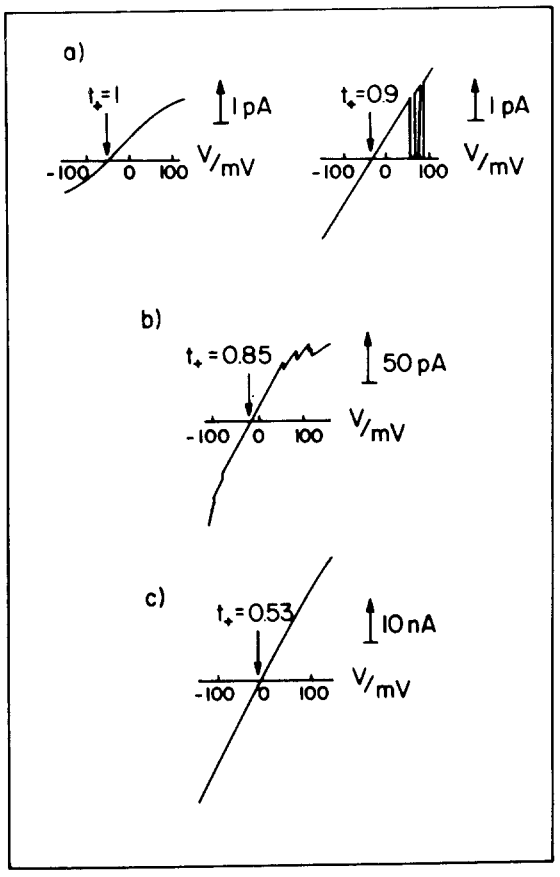


Fig. 5. Current-voltage characteristic of pneumolysin-induced channels in the presence of Ca^{2-}. Pneumolysin (5–50 μg/ml) was applied to DOPC : ergosterol (1 : 2) bilayers as described in the legend to Fig. 2, except that in this case the solution bathing the *cis* side of the membrane also contained 10 mM $CaCl_2$. Potentials between ± 150 mV were applied as a triangular wave (2 mV/sec) and t_- calculated as described in the legend to Fig. 2. The two traces labeled *a* were from two membranes, each of which appeared to contain a single, small channel (13 pS and 20 pS, respectively); trace *b* was from a membrane containing 4–6 medium (200–300 pS) channels; trace *c* was from a membrane containing about 10 large (10–20 nS) channels.

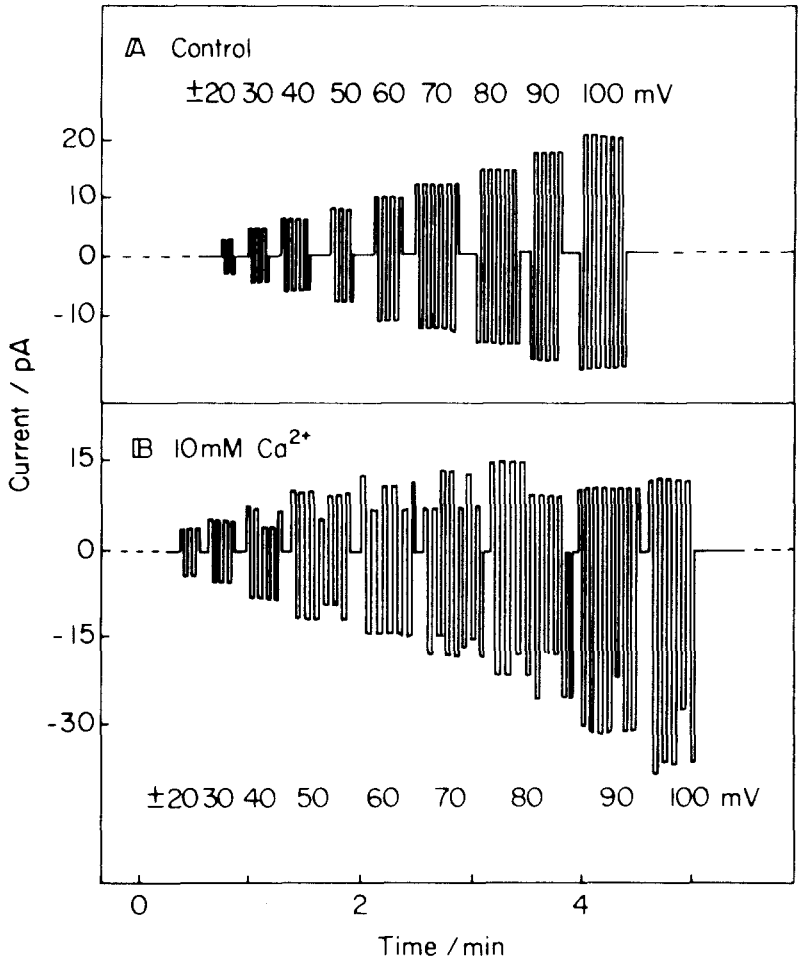
1) small channels have a different current-voltage characteristic in the presence of Ca^{2+} due to closure at positive voltage. The same is true of the less selective small channels ($t_+ \approx 0.9$) and medium-sized channels ($t_+ \approx 0.85$), but not of the large channels ($t_+ \approx 0.53$) which are unaffected.

Figure 6 illustrates the result of an experiment in which the current response to rapid switching between positive and negative voltages of increasing magnitude is followed. Figure 6*A* shows that in the absence of divalent cations, there is no rectification; in this instance small, highly selective channels (*see* Fig. 2, left-hand trace) are either absent or overshadowed by nonrectifying channels. On addition of Ca^{2+} (Fig. 6*B*), a clear-cut rectification at voltages in excess of 40 mV is apparent. This is due to an increase at negative voltages as well as a decrease at positive voltages. In Fig. 6*C* the current records of Fig. 6*B* are shown as the conductances at the relevant voltages. The distribution of conductances is compatible with the presence of three medium sized (≈500 pS) channels at ±100 mV in this experiment.

An experiment that confirms that it is small and medium-sized, — i.e., selective — channels, and not large — i.e., non-selective — channels that are predominantly closed by divalent cations is shown in Fig. 7. In this instance, the mean selectivity of channels was t_+ 0.73 at the start of the experiment. On the addition of 1 mM Zn^{2+}, selectivity falls to $t_+ \approx 0.58$, with a concomitant decrease in current. Further addition of Zn^{2+} (5 mM) causes a further fall in selectivity, with little decrease in current; this effect is compatible with closure of a significant number of small or medium-sized, selective channels, that contribute relatively little current in comparison with the residual current carried by predominantly large, nonselective channels.

Discussion

The main conclusions to be drawn from the present experiments are two-fold. First, pneumolysin induces a spectrum of different-sized channels across planar lipid bilayers, as summarized in Table 2. This finding is in good agreement with that reported previously for tetanolysin, another hemolytic oxygen-labile, cholesterol-requiring toxin that forms large pores in cell membranes [7]. Thus tetanolysin forms channels with conductance ranging from ≈10–400 pS (at 0.145 M NaCl), with a conductance of 28 pS showing the highest frequency. Channels are cation selective with transference number (t_+) of 0.83; in the light of the present results, this is likely to be a mean value. The formation of channels is not voltage or concentration dependent but does require the presence of sterol, as found for pneumolysin also. A similar dependence of pore formation on sterol presence is also shown by nystatin [24]. The conclusion [7] that "tetanolysin acts by causing lipid perturbations . . . rather than formation of structural channels" is not inconsistent with our own views on pore formation by agents as diverse as triton or polylysine [2]. Nevertheless, the fact that pore-forming proteins such as *S. aureus* α toxin [2, 12], perfringolysin [13], lymphocyte cytolysin [4] and pneumolysin (this paper) all form channels that are closed in a voltage-dependent and voltage-reversible manner by divalent cations in a way similar to that by which divalent cations affect voltage-gated channels such as the Na^+ channel [8,



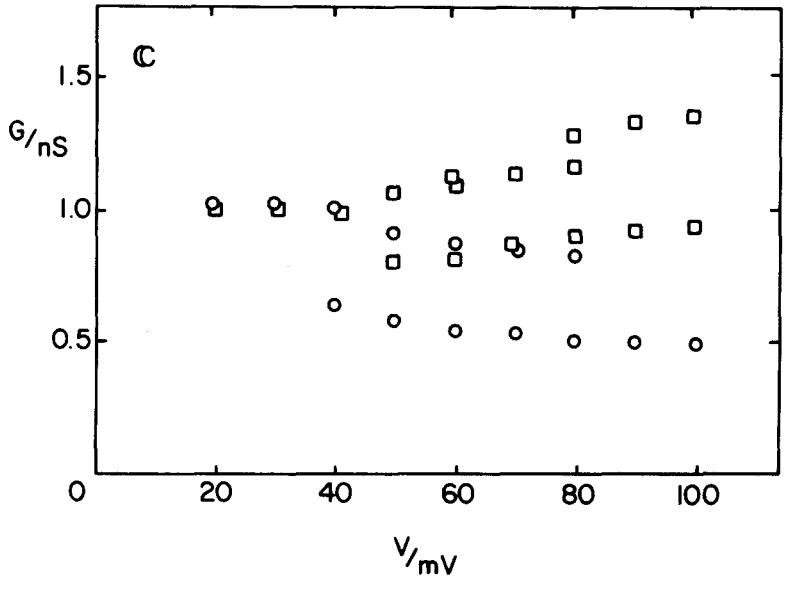


Fig. 6. Rectification/closure of pneumolysin-induced channels in the presence of Ca^{2+}. Pneumolysin (50 μg/ml) was applied to a DOPC : ergosterol (1 : 2) membrane bathed in 0.1 M KCl, 0.005 M HEPES, 0.1 mol EDTA pH at 7.4 (with KOH) without (*A*) or with (*B*) 10 mM $CaCl_2$. Voltage was switched between positive and negative poles from 20 to 100 mV. (*C*) Conductance values are plotted of the experiment illustrated in *B* (○, conductance at positive potentials; □, conductance at negative potentials). The conductance levels indicate that the membrane contained three medium channels (≈500 pS) and some smaller channels. The preparation of pneumolysin used for this experiment was not the native form, but a mutant in which a W at position 433 is replaced by F (F433); the properties of this mutant are the same as those of the native toxin, in-so-far as the behavior of individual channels is concerned.

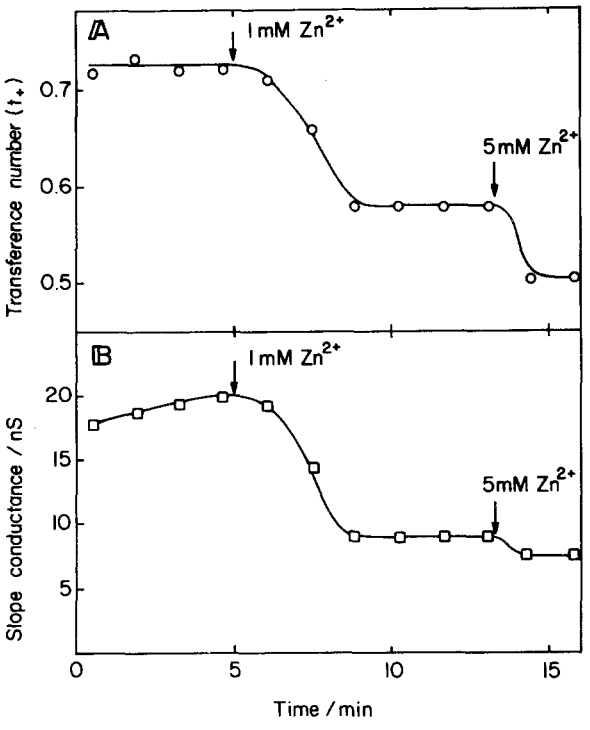


Fig. 7. Effect of Zn^{2+} on the conductance and cation selectivity of a pneumolysin-treated bilayer. Pneumolysin (mutant F433 [*see* legend to Fig 6], 5 μg/ml) was applied to a DOPC : ergosterol (1 : 2) membrane bathed in 0.1 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) on the *cis* side and 0.01 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) on the *trans* side. Voltage between ±150 mV was applied as a triangular wave (2 mV/sec) and transference number [t_+, ○ (*A*)] and slope conductance (□, (*B*)] were calculated at successive null potentials. 1 and 5 mM $ZnSO_4$ (final concentration) were added to the *cis* compartment as shown.

Table 2. Properties of pneumolysin-induced channels in DOPC: ergosterol planar lipid bilayers

Channel size	Conductance (pS)	Cation selectivity (t_+)	Rectification	Closure by divalent cations
Small	<50	1.00	+ +	+ +
	<50	0.93 ± 0.06	−	+ +
Medium	50–1000	0.80 ± 0.06	−	+ +
Large	>1000	0.56 ± 0.06	−	±

Channels induced by pneumolysin (5–50 μg/ml) in DOPC: ergosterol (1 : 2) bilayers bathed with 0.1 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) at room temperature (23–28°C). Single channel conductances were estimated from records similar to those shown in Fig. 1. Cation selectivity (t_+) was assessed with 0.01 M KCl, 0.005 M HEPES, pH 7.4 (with KOH) replacing the 0.1 M KCl medium on the *trans* side: selectivity data from 100 membranes were pooled to give the mean values ± SEM with *n* of 70, 55 and 25 for small, medium and large channels, respectively. Rectification (less current at positive voltages) and closure by divalent cations were assessed from records similar to those shown in Figs. 2–5: − indicates no effect; ± indicates some, variable effect; + + indicates strong, consistent effect.

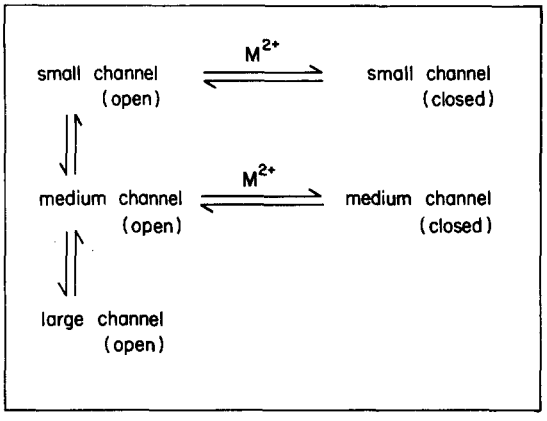


Fig. 8. Model for divalent cation closing of pneumolysin channels.

10], argues for the participation of some defined polypeptide sequences in *trans*-membrane events.

The second conclusion that emerges from the present results is that different-sized channels are differentially sensitive to closure by divalent cations (Table 2). The fact that small channels are closed preferentially to larger ones is not surprising and might suggest some kind of blocking mechanism similar to that by which Ba^{2+}, for example, blocks the Ca^{2+}-activated K^+ channel [14]. Although the voltage characteristics of closure by divalent cations are compatible with the cation being drawn into a pneumolysin-induced channel to block it, there is no evidence to support this. In the case of *S. aureus* α toxin there is evidence to show that closure is not by such blockage [12], and it is therefore more likely that closure in the case of pneumolysin also involves some sort of gating process.

The relationship between the different-sized channels induced by pneumolysin is not clear. It is possible that an equilibrium exists between different-sized channels and their sensitivity to closure by divalent cations, as shown schematically in Fig. 8. Such a scheme would certainly account for closure by apparently large conductance jumps in certain instances (e.g., Fig. 4) if the transition from large to medium channels were fast.

The participation of sterol is worth mentioning in this regard. We used ergosterol, in a 2 : 1 mixture with DOPC, since it gave more reproducible results than cholesterol; the studies with tetanolysin used oxidized cholesterol without any phospholipid [7].

It is, however, possible to obtain pneumolysin-induced channels — albeit unstable — without any sterol present. Our impression to date, for the role that sterols may play, is that they favor the stability of the large type of channel. This is compatible with the accumulated evidence (e.g. 5, 13, 20, 21) that in cells, pneumolysin and related toxins form large pores that are dependent on the presence of cholesterol in the membrane.

We are grateful to Dr. G. J. Boulnois and T. J. Mitchell for fruitful discussion and supplies of pneumolysin, and to G. M. Alder for technical assistance. YEK is grateful to Dr. A. A. Lev for leave of absence and to the USSR Academy of Sciences and the Global Network for Molecular and Cell Biology (UNESCO) for support of travel and accommodation, respectively. The work was supported by the Cell Surface Research Fund.

References

1. Austrian R. 1984. Pneumococcal infections. *In:* Bacterial Vaccines. pp. 257–288. R. Germanier, editor Academic, London
2. Bashford, C.L., Alder, G.M., Graham, J.M., Menestrina, G., Pasternak, C.A. 1988. Ion modulation of membrane permeability: Effect of cations on intact cells and on cells and phospholipid bilayers treated with pore-forming agents. *J. Membrane Biol.* **103:**79–94
3. Bashford, C.L., Alder, G.M., Patel, K., Pasternak, C.A. 1984. Common action of certain viruses, toxins, and activated complement: Pore formation and its prevention by extracellular Ca^{2+}. *Biosci. Rep.* **4:**797–805
4. Bashford, C.L., Menestrina, G., Henkart, P.A., Pasternak, C.A. 1988. Cell damage by cytolysin. *J. Immunol.* **141:**3965–3974
5. Bhakdi, S., Tranum-Jensen, J. 1988. Damage to cell membrane by pore forming bacterial cytolysins. *Prog. Allergy* **40:**1–43
6. Blomqvist, L., Thelestam, M. 1986. Early events in the action of staphylococcal alpha-toxin on the plasma membrane of adrenocortical Y1 tumor cells. *Infect. Immun.* **53:**636–640
7. Blumenthal, R., Habig, W.H. 1984. Mechanism of tetanolysin-induced membrane damage: Studies with black lipid membranes. *J. Bacteriol* **157:**321–323
8. Cukierman, S., Zinkand, W.C., French, R.J., Krueger, B.K. 1988. Effects of membrane surface charge and calcium on the gating of rat brain sodium channels in planar bilayers *J. Gen. Physiol.* **92:**431–447
9. Harshman, S. Sugg, N. 1985. Effect of calcium ions on staphylococcal alpha-toxin-induced hemolysis of rabbit erythrocytes. *Infect. Immun.* **47:**37–40
10. Hille, B. 1984. Ionic channels of excitable membranes. Sinauer, Sunderland (MA)
11. Krueger, B.K. 1989. Toward an understanding of structure and function of ion channels. *FASEB J.* **3:**1906–1914
12. Menestrina, G. 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: Voltage-dependent inhibition by divalent and trivalent cations. *J. Membrane Biol.* **90:**177–190
13. Menestrina, G., Bashford, C.L., Pasternak, C.A. 1990. Pore-forming toxins: Experiments with *S. aureus* α-toxin, *C perfringens* θ-toxin and *E. coli* haemolysin in lipid bilayers, liposomes and intact cells. *Toxicon* **28:**477–491
14. Miller, C. 1987. Trapping single ions inside single ion channels. *Biophys. J.* **52:**123–126
15. Mitchell, T.J., Walker, J.A., Saunders, F.K., Andrew, P.W., Boulnois, G.J. 1989. Expression of the pneumolysin gene in *Escherichia coli:* Rapid purification and biological properties. *Biochim. Biophys. Acta* **1007:**67–72
16. Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci USA* **69:**3561–3566
17. Pasternak, C.A. 1989. The protective role of extracellular calcium ions. *In:* Biochemical Approaches to Cellular Calcium. E. Reid, G.M.W. Cook, and J.P. Luzio, Editors. pp. 379–386. Royal Society of Chemistry, Cambridge (UK)

17*a*. Pasternak, C.A., Alder, G.M., Bashford, C.L., Korchev, Y.E., Pederzolli, C., Rostovtseva, T.K. 1992. Membrane damage: Common mechanisms of induction and prevention. *FEMS Microbiol. Immunol. (in press)*

18. Saunders, F.K., Mitchell, T.J., Walker, J.A., Andrew, P.W., Boulnois, G.J. 1989. Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for in vitro activity. *Infect. Immun.* **57:**2547–2552
19. Schindler, H. 1980. Formation of planar bilayers from artificial or native membrane vesicles. *FEBS Lett.* **122:**77–79
20. Smyth, C.J., Duncan, J.L. 1978. Thiol-activated (oxygen-labile) cytolysins. *In:* Bacterial Toxins and Cell Membranes. J. Jeljaszewicz and T. Wadstrom, editors. pp. 129–183. Academic, London
21. Thelestam, M., Mollby, R. 1980. Interaction of streptolysin O from *Streptococcus pyogenes* and theta-toxin from *Clostridium perfringens* with human fibroblasts. *Infect. Immun.* **29:**863–872
22. Tweten, R.K. 1988. Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens:* Significant homology with the genes for streptolysin O and pneumolysin. *Infect. Immun.* **56:**3235–3240
23. Walker, J.A., Allen, R.L., Falmagni, P., Johnson, M.K. and Boulnois G.J. 1987. Molecular cloning, characterization and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. *Infect. Immun.* **55:**1184–1189.
24. Woodbury, D.J., Miller, C. 1990. Nystatia-induced liposome fusion. A versatile approach to ion channel reconstitution into planar bilayers. *Biophys. J.* **58:**833–839

Received 11 September 1991; revised 14 January 1992