J. Membrane Biol. 143, 143–151 (1995)

The Journal of
Membrane
Biology

Staphylococcus aureus Alpha-Toxin-Induced Pores: Channel-like Behavior in Lipid Bilayers and Patch Clamped Cells

Y.E. Korchev*, G.M. Alder, A. Bakhramov, C.L. Bashford, B.S. Joomun, E.V. Sviderskaya***, P.N.R. Usherwood****, C.A. Pasternak**

Division of Biochemistry, Department of Cellular & Molecular Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, United Kingdom

Received: 15 July 1994/Revised: 5 October 1994

Abstract. The conductance of pores induced by *Staphylococcus aureus* α-toxin in Lettre cells has been compared to that in bilayers composed of synthetic lipids or Lettre cell membrane constituents. Previously described characteristics of toxin-induced conductance changes in lipid bilayers, namely rectification, voltage-dependent closure, and closure at low pH or in the presence of divalent cations (Menestrina, 1986) are displayed also in bilayers prepared from Lettre cell membranes and in patch clamped Lettre cells. It is concluded that endogenous proteins do not affect the properties of α-toxin-induced channels significantly and that the relative lack of ion channels in Lettre cells makes them ideal for studies of pore-forming toxins by the patch clamp technique.

Key words: Alpha toxin — Ion channel — Lettre cell — Patch clamp — Planar bilayer — *Staphylococcus aureus*

* *Present address:* Dr. Korchev is on leave of absence from the Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia

** *Present address:* Department of Pharmacology and Clinical Pharmacology, St George's Hospital Medical School, London, United Kingdom

*** *Present address:* Department of Anatomy, St George's Hospital Medical School, London, United Kingdom. Dr. Sviderskaya is on leave of absence from the Physiology Institute, University of St. Petersburg, Russia

**** *Present address:* Department of Life Science, University of Nottingham, University Park, Nottingham, NG7 2RD United Kingdom

Correspondence to: C.A. Pasternak

Introduction

The pathogenic determinants in many microbial diseases are pore-forming toxins secreted by the relevant organism (Jeljaszeiwicz & Wadstrom, 1978; Bhakdi & Tranum-Jensen, 1987). The properties of toxin-induced pores have been studied by investigating both the leakage of low molecular weight metabolites from intact cells (Impraim et al., 1980; Thelestam, 1983) and the movement of ions across defined membranes as vesicles (liposomes) or planar bilayers (e.g., Schein, Kagan & Finkelstein, 1978; Tosteson & Tosteson, 1978; Tokuda & Konisky, 1979; Stein & Lieb, 1986; Menestrina, Bashford & Pasternak, 1990). Measurement of conductivity changes induced by pore-forming toxins across planar lipid bilayers has been particularly instructive in defining some of the properties of the pores (e.g., Menestrina, 1986). However, there are some discrepancies. In the case of *Staphylococcus aureus* α-toxin-induced pores, for example, sensitivity to divalent cations in whole cells is independent of membrane potential (Pasternak, Bashford & Menestrina, 1989), whereas in bilayers it is voltage dependent (Menestrina, 1986). In view of this, a study of toxin-induced changes in the conductivity of intact cells is desirable.

The presence of endogenous ion channels in the plasma membrane of most cells complicates interpretation of toxin-induced conductivity changes. It is not surprising, therefore, that only a few patch clamp studies of toxin-treated cells have been made (e.g., Yoshii et al., 1987; Bussey, 1991). Lettre cells, which are an ascites form of murine tumor cells (Lettre et al., 1972), are exceptional in that they have few endogenous channels, yet maintain a resting transmembrane potential of around −50 mV (Bashford & Pasternak, 1984, 1990). Since these cells respond to a number of bacterial and other

pore-forming proteins (Bashford et al., 1986; Bashford et al., 1988*a*; Menestrina et al., 1990), they should prove a useful experimental system. Here we report on studies with *S. aureus* α-toxin, a well-characterized protein (Thelestam & Blomqvist, 1988; Bhakdi & Tranum-Jensen, 1991) that is a common determinant of infectious disease in man and cattle (Easmon & Adlam, 1983); part of its pathogenicity may be related to the induction of pores across the plasma membrane of susceptible cells that results in an inflow of Ca^{2+} (Pasternak, 1986) with consequential triggering of cellular events (Suttorp et al., 1985; Bhakdi & Tranum-Jensen, 1991). *S. aureus* α-toxin induces pores in bilayers prepared from synthetic phospholipids, which show several characteristic features (Bashford et al., 1986, 1988*b*; Menestrina, 1986; Menestrina et al., 1990). These include: slight anion selectivity; voltage-dependent closure that is influenced by divalent cations or by protons; a single channel conductance at low voltages (in 0.1 M KCl at pH 7.4) of approximately 50 pS; and rectification at higher voltages that is abolished by divalent cations or protons. Our present results show that these features are also displayed by toxin-induced pores in bilayers prepared from Lettre cell membranes and in Lettre cells in the whole-cell-clamp recording mode. They indicate the usefulness of Lettre cells for such studies and show that, at least for *S. aureus* α-toxin, the nature of the membrane lesion is independent of the surrounding molecules.

Materials and Methods

Chemicals

S. aureus α-toxin was a highly purified sample donated by Dr. J.P. Arbuthnott (University of Strathclyde, Glasgow) or a purified sample donated by Dr. K.D. Hungerer (Behringwerke, Marburg, Germany): the two samples gave a similar pattern of metabolite leakage from Lettre cells, measured as described by Bashford et al. (1986). Dioleoyl phosphatidylcholine (DOPC) and glycerol monolein (GMO) were obtained from Sigma; diphytanoyl phosphatidylcholine (DPhPC) was obtained from Avanti Polar Lipids.

Lettre Cells

Lettre cells were grown intraperitoneally in mice as an ascitic suspension (Bashford et al., 1986). Cells were removed after seven days and washed in HEPES-buffered saline (HBS: 150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, pH 7.4). For the preparation of cell membranes, the cells were suspended in 1 mM $NaHCO_3$ containing 0.2 mM $MgCl_2$. For patch clamp studies cells were suspended in HBS (5×10^6 cells/ml) and 0.1 ml of the suspension pipetted into 35 mm plastic petri dishes or on to glass coverslips coated with concanavalin A (Bashford & Pasternak, 1990). The dishes or coverslips were left for at least 10 min at room temperature before removing any cells still left in suspension. HBS (1 ml) was then added to each dish prior to patch clamping.

Lettre Cell Membranes

Lettre cell membranes, enriched in plasma membrane constituents, were obtained as described by Graham and Coffey (1979). Cells (approx. 3×10^9) from three mice were allowed to swell in 45 ml of 1 mM $NaHCO_3$ containing 0.2 mM $MgCl_2$ and disrupted by hand using a tight-fitting Dounce homogenizer until >95% were broken (20–30 passages). Nuclei were removed by centrifugation at $1{,}000 \times g$ for 5 min and the supernatant was diluted in 2 M sucrose to give a final concentration of 0.25 M sucrose. This suspension was layered on to a gradient of sucrose and centrifuged in a BXIV zonal rotor exactly as described by Graham and Coffey (1979). Successive fractions were assayed for Na^+/K^+-dependent ATPase, NADPH/cytochrome c reductase and succinate/cytochrome c reductase to identify fractions enriched in plasma membrane, endoplasmic reticulum and mitochondria, respectively (Graham & Coffey, 1979). Fractions enriched in Na^+/K^+-dependent ATPase (two- to ninefold enrichment over activity in whole Lettre cell homogenate) were pooled, spun to remove sucrose and resuspended in buffered saline; aliquots were used directly or after freezing at –70°C for up to one month.

Bilayer Experiments

The apparatus consisted of two Teflon chambers (capacity 0.12 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 α toxin was added (*cis*). Voltage signs refer to the *trans* compartment; at positive potential cations flow from *trans* to *cis*. KCl buffered to pH 7.4 with 5 mM HEPES was used throughout; the concentration of KCl was 100 mM in both chambers, unless selectivity was being measured, in which case the solution in the *trans* chamber contained 10 mM KCl. All the experiments were performed at room temperature (20–25°C).

Planar bilayers were formed from synthetic lipids as described by Montal and Müller (1972). Lipid monolayers were first prepared either by spreading 2–3 μl of lipid solution in hexane (0.5 mg/ml) on the air-water interface or by addition of 5 μl of liposome suspension (5 mg/ml) to each chamber with 100 μl buffer. A bilayer was then formed by lowering and raising the monolayers past the aperture.

Planar bilayers were prepared from Lettre cell membranes using the procedure of Schindler (1980). We added 5–10 μl of Lettre cell membranes (5 mg protein/ml) to each chamber with 100 μl of buffered KCl solution; after waiting 20–40 min for the monolayers to form on the surface, a bilayer was then formed by lowering and raising the monolayers past the aperture.

A solution of α-toxin (1–2 μl containing 1 mg protein/ml in 0.16 M NaCl) was used in both cases.

Preliminary Patch Clamping Experiments (in Nottingham)

Patch pipettes were pulled from hematocrit glass on a horizontal puller to give tip resistances of *ca.* 7 MΩ. The pipettes were not fire-polished before use, but they were coated with Sylgard resin to improve their capacitance properties. Cell-attached patches and inside-out patches were studied. In both cases the patch pipettes contained HBS (mM: 150 NaCl, 5 KCl, 1 $MgSO_4$ and 5 HEPES; pH 7.4) ± α-toxin. For whole-cell recordings the patch pipettes were filled with (mM) 80 KCl, 50 NaCl, 6.2 $MgSO_4$, 0.77 EGTA, 11 glucose, 5 NaOH, 1 KH_2PO_4 and 10 HEPES; pH 7.4 (5 mM ATP was sometimes added to this saline). For such recordings, toxin was applied either in the pipette or in the bath or in both environments. In all experiments the bath saline was HBS.

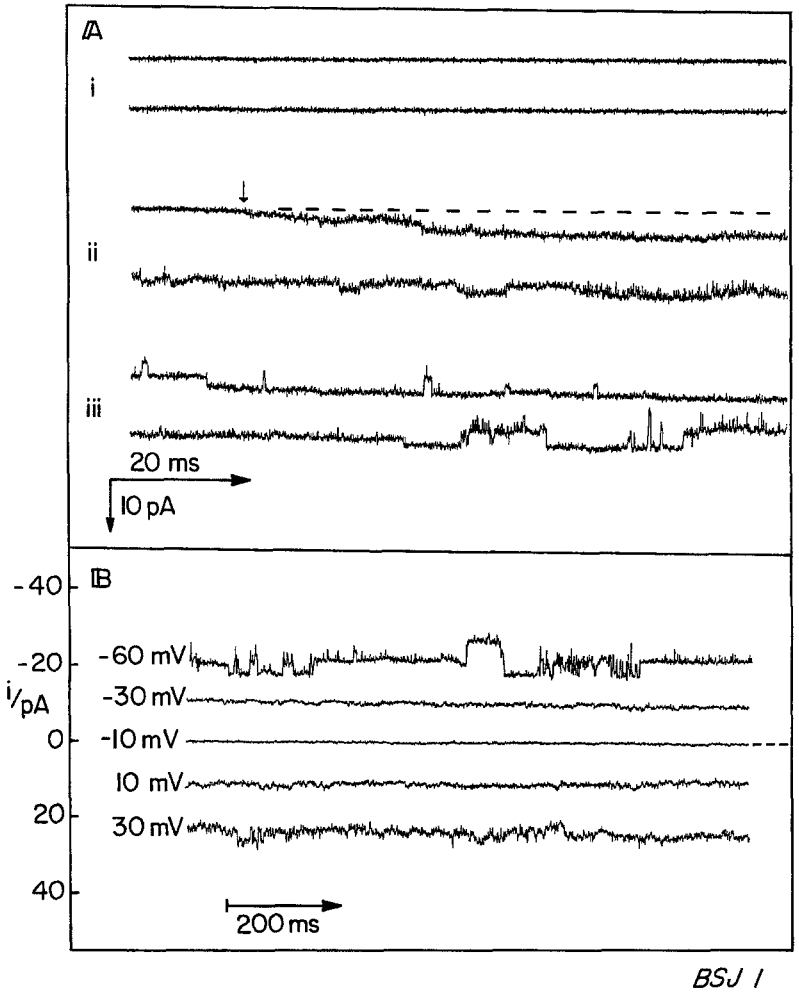

Fig. 1. *S. aureus* α-toxin-induced channels in Lettre cells (whole-cell mode). (*Cell 1: A*) 10 μg/ml α-toxin in the patch pipette; pipette potential −130 mV. No channel openings are seen 12 min (trace *i*) after establishing whole-cell recording mode; at 12.5 min (trace *ii*) openings (downwards) appear (arrow; the dashed line indicates the initial, low conductance condition); by 13 min (trace *iii*) long-duration openings with some brief closures to lower conductance levels are evident. (*Cell 2: B*) 100 μg/ml α-toxin in the patch pipette. Channel activity was measured 30 min after establishment of the recording mode. Channel currents are seen to reverse at *ca.* −10 mV; channel openings are upwards at positive potentials and downwards at negative potentials.

"Single-channel" and "whole-cell" recordings were made using a List EPC 7 patch clamp amplifier. The latter was coupled to a Sony VCR using a pulse code modulation unit modified to give a uniform frequency response from dC to 20 kHz. Data analyses were undertaken off-line using a Masscomp MC 550 computer (Kerry et al., 1987), with recordings of channel data being filtered at 10 kHz (8-pole Bessel filter) on playback. All recordings were made at 22°C, unless otherwise stated.

Further Patch Clamping Experiments (in London)

The technique was in general similar to that used in Nottingham. Borosilicate glass pipettes (Clark Electromedical Instruments) with a resistance of 2–4 mΩ were filled with pipette solution. Whole-cell currents were amplified (List, EPC-7) and analyzed by pCLAMP software (Axon Instruments). The pipette potential was stepped from a holding potential of 0 mV to different levels ranging from −140 to +120 mV with 20 mV increments each of 1 sec duration. The interpulse interval was 3 sec.

Fresh cells attached to concanavalin A-coated coverslips were placed into a 100 μl chamber and a continuous flow of solution through the chamber (at about 3 ml min^{-1}) was started 2 min before recordings. Test solutions were applied by switching manually to another reservoir connected to the inflow. Solution exchange was completed 20 sec after switching. The pipette solution contained (mM) 45 CsCl, 80 CsOH, 20 BAPTA, 9.2 $CaCl_2$ (final free Ca^{2+} concentration 0.1 μM), 6 creatine phosphate, 2 $MgCl_2$, 10 HEPES (final pH 7.0). The bathing solution contained (mM) 125 CsCl, 10 HEPES, 1.2 $MgCl_2$, 12 glucose at pH 7.4 and during experiments was replaced by perfusion with the required solution. A solution of α-toxin (1–2 μl containing 10 mg protein/ml) was applied to the 100 μl chamber and removed as soon as additional (i.e., toxin-induced) current appeared. All experiments were performed at room temperature (20–25°C).

Results

Patch Clamp Recordings of Untreated Lettre Cells

Preliminary studies (in Nottingham) were carried out in Ca^{2+}-free medium. These showed that single channel openings were absent in five recordings from cell-attached patches and two recordings from inside-out patches, each of which lasted for more than one hour. Many other recordings made in these modes, but of shorter duration than the above, were also notable for the absence of channel activity even when large potential differences (±199 mV) are imposed across a membrane patch. It is thus confirmed that the surface membrane of

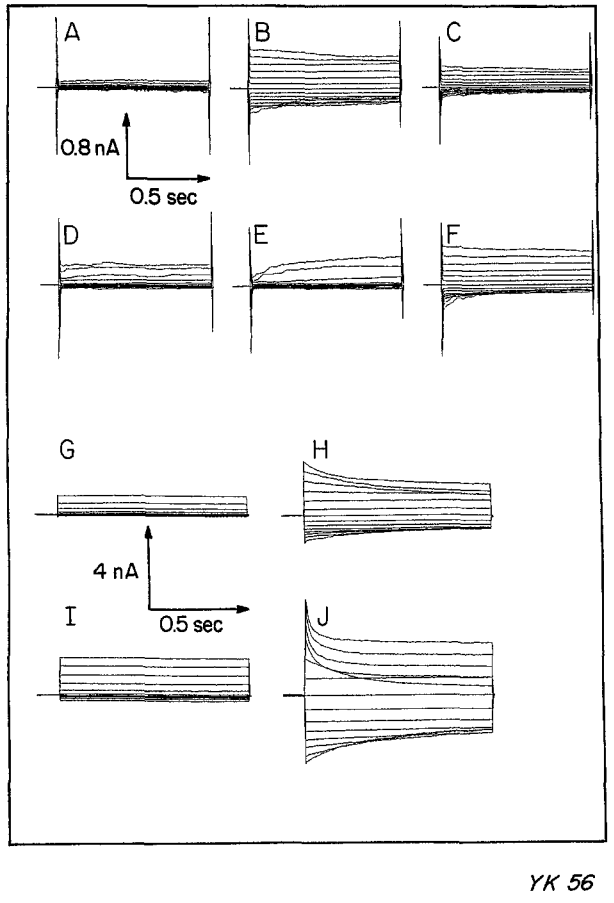

Fig. 2. Whole-cell currents from Lettre cells. α-Toxin was applied at 100–200 μg/ml in the medium and removed when additional current was detected. The applied potential was stepped from a holding potential of 0 mV to different levels ranging from −140 to 120 mV with 20 mV increments for 1 sec. The interpulse interval was 3 sec. (*Cell 1*) Trace *A:* control, before α-toxin, at pH 7.4; Traces *B–F:* after α-toxin without (*B*) or with successive additions of final concentrations of 0.4 mM Zn^{2+} (*C*), 1 mM Zn^{2+} (*D*), 3 mM Zn^{2+} (*E*) followed by Zn^{2+}-free control (*F*). (*Cell 2*) Traces *G* and *H:* before (*G*) or after α-toxin (*H*) at pH 7.4; Traces *I* and *J:* before (*I*) or after α-toxin (*J*) at pH 5.0.

a Lettre cell contains few active endogenous ion channels under these conditions (Bashford & Pasternak, 1984, 1990). This conclusion is supported by the results of studies made in the whole-cell recording mode where the input resistance for Lettre cells is up to 60 GΩ. In most (19 out of 21) whole-cell recordings, single channel currents were not seen over long (>1 hr) recording periods and during imposition of a wide range of membrane potentials, although in two cells a channel of low conductance (*ca.* 0.5 pS) and low open probability was observed. The resting potential was zero in the whole-cell recording mode with patch pipettes without ATP, whereas when 5 mM ATP was present, resting potentials of *ca.* −30 mV were observed.

Further studies of Lettre cells were carried out (in London) with Ca^{2+}-containing media, as well as with bilayers prepared from Lettre cell membranes (*see below*). In this case the input resistance at negative potentials is 0.5 to 50 GΩ; nevertheless some current is observed at positive potentials, which appears to be due to Ca^{2+}-activated K^+ channel activity (Pershadsingh, Johnstone & Laris, 1978); in the presence of Cs^+ in place of K^+ in the patch pipette and bath solutions (Benham et al., 1986) it is largely suppressed (e.g., Fig. 2*A*), and all subsequent recordings were made with 125 mM Cs^+ present in bath and pipette. Under these conditions the resting potential is −68 ± 15 mV (n = 5) and the input resistance exceeds 6 GΩ. In other media, with less Ca^{2+}-buffering and with or without cation gradients (i.e., Cs^+/Cs^+, Na^+/Cs^+, Na^+/K^+), the mean membrane potential is −36 ± 12 mV (n = 15).

Patch Clamp Recordings of α-Toxin-treated Lettre Cells

The preliminary studies in the whole-cell recording mode showed openings that occur within minutes of the addition of toxin (Fig. 1*A*). Therefore, the membrane current rapidly increases to the point where it is not easy to observe single channel openings. From some such records (Fig. 1*B*), open channel conductances of around 80 pS (at +30 or −60 mV) were calculated.

By treating cells with the dye erythrosin prior to patch clamping, it is possible to distinguish two classes of cell: those that exclude the dye (as did 98% of cells not treated with α-toxin) and those that take up dye (30, 52 and 100% after 30 min at 37°C in the presence of 10, 50 and 100 μg α-toxin/ml, respectively). Interestingly, even cells that had not taken up dye show high currents. This confirms results on leakage of phosphoryl [^{3}H]choline from α-toxin-treated Lettre cells, which show that cells require 30–100 times more α-toxin to elicit uptake of dye than to elicit leakage of phosphoryl [^{3}H]choline. In other words, as with pore-forming agents such as complement (Micklem et al., 1988) or hemolytic Sendai virus (Bashford, Micklem & Pasternak, 1985), the apparent size or stability of membrane lesions increases with the amount of pore-forming agent added.

Further experiments confirmed the induction of ion current by α-toxin, as illustrated in Fig. 2*B,H,J;* in these instances single channel events could not be resolved. At voltages in excess of ±100 mV the instantaneous current decreases towards a steady-state value, particularly at low pH (Fig. 2*J*); instantaneous current is restored by switching to a new potential via zero, as it is in lipid bilayers (Menestrina, 1986). The α-toxin-induced current is higher at positive potentials (rectification), confirming the preliminary results (Fig. 1*B*) and shown in greater detail in Fig. 7 (*below*).

Zn^{2+} reversibly decreases the α-toxin-induced current (Fig. 2*C–E*). Higher resolution records of the

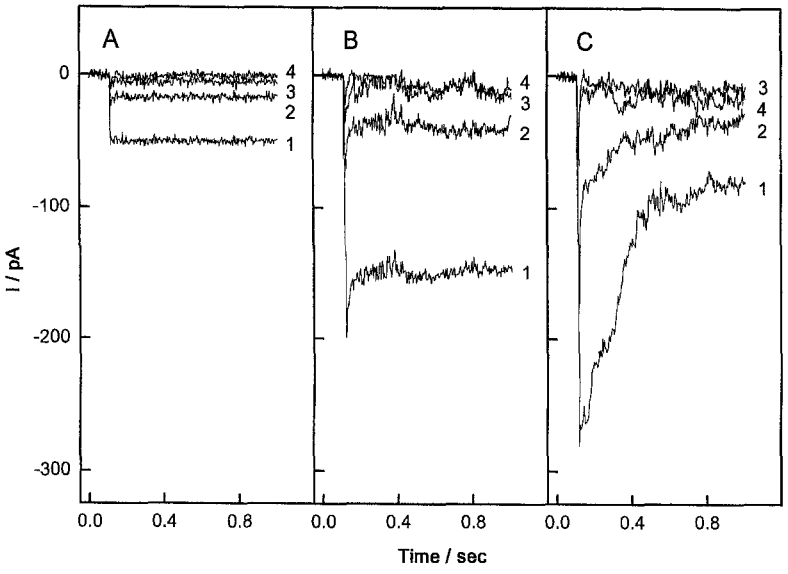

Fig. 3. Effect of Zn^{2+} on α-toxin-induced whole-cell currents in Lettre cells. The applied potential was stepped from a holding potential of 0 to −20 mV (*A*), −80 mV (*B*) or −140 mV (*C*) for 1 sec in the absence or presence of the following final concentrations of Zn^{2+}: 1, 0 mM; 2, 0.4 mM; 3, 1 mM; 4, 3 mM. α-Toxin was applied at 150 μg/ml in the medium and removed when additional current was detected. Currents observed in control cells without α-toxin have been subtracted in each case.

α-toxin-induced current (i.e., with toxin-free current subtracted) at negative potentials are shown in Fig. 3. These traces also show voltage-dependent closure of α-toxin-induced channels (Fig. 3*C vs. B vs. A*). The effects of Zn^{2+} at positive potentials (e.g., Fig. 2*E*) are complex because such potentials drive the Zn^{2+}, supplied only in the bath, out of the channels. The reduction of the α-toxin-induced current by Zn^{2+} is accompanied by a change of resting membrane potential (Fig. 4). In the absence of Zn^{2+}, the membrane potential falls from about −55 to about −8 mV on the addition of α-toxin. As increasing levels of Zn^{2+} are applied, the resting potential recovers; removal of Zn^{2+} returns the potential to its low level. However, restoration of the initial membrane potential is not complete, as Zn^{2+} alone induces some leakiness in Lettre cells (current traces not shown; *see also* Bashford et al., 1988*b*).

Reducing the pH of the medium in which the Lettre cells are bathed has three effects. First, the membrane current in the absence of α-toxin increases (Fig. 2*I vs. G*); we do not know whether this is due to activation of endogenous K^+ channels. Second, the instantaneous current induced by α-toxin increases (Fig. 2*J vs. H*), especially at negative voltages, which results in a loss of rectification (an effect previously observed with lipid bilayers (Kasianowicz et al., 1991; G.M. Alder, Y.E. Korchev and G. Menestrina, *unpublished observations*). Third, the voltage-dependent decay of toxin-induced current is more marked at pH 5.0 than at pH 7.4 (Fig. 2*J vs. H*).

Reconstituted Lettre Cell Membrane Bilayers

A preparation of Lettre cell membranes, enriched in plasma membrane constituents, was used. Apart from

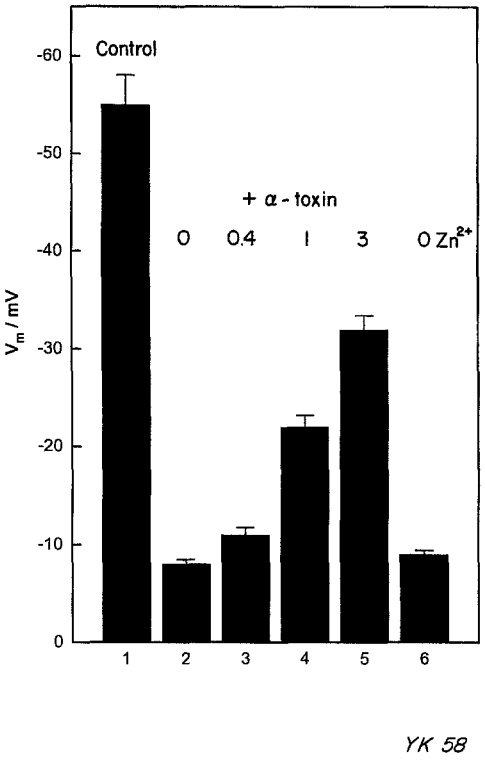

Fig. 4. Effect of Zn^{2+} on Lettre cell potentials in the presence of α-toxin. Membrane potential (resting potential, V_M) was measured every 2 min; error bars represent the SEM of at least three determinations. (*1*) Potential before α-toxin (100–200 μg/ml) was applied and removed as soon as additional current appeared. After 10 min, current reached a plateau and potential was measured (*2*). Then 0.4 mM Zn^{2+} was applied and after 4 min the membrane potential was measured (*3*); removal of Zn^{2+} restored the toxin-induced potential (*2*). Subsequent additions of 1 mM Zn^{2+} (*4*) and 3 mM Zn^{2+} (*5*) are seen to reversibly restore potential. Removal of Zn^{2+} (*6*) returns potential to that seen immediately after treatment with α-toxin (*2*).

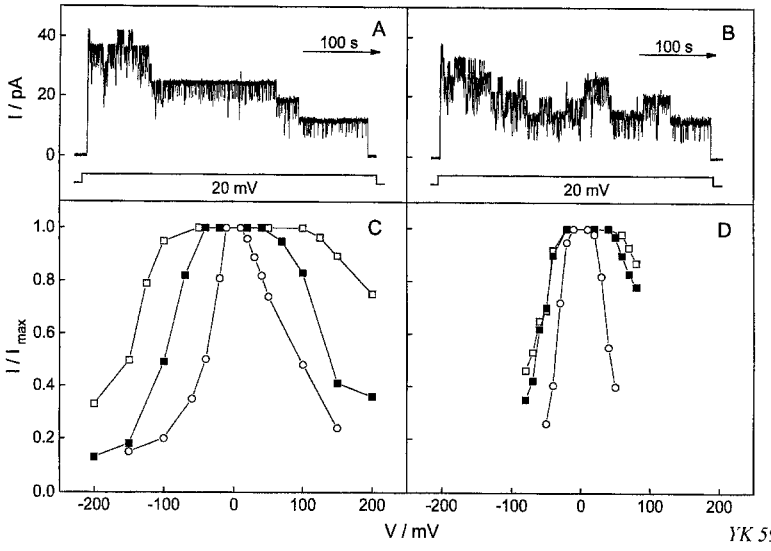

Fig. 5. Voltage dependence of α-toxin-induced currents in bilayers. Bilayers were formed from DPhPC (*A,C*) or from Lettre cell membranes (*B,D*). Current (*I*) 1 min after applying the voltage indicated is expressed relative to the instantaneous current (I_{max}) observed after switching from zero potential. Current record of an α-toxin-treated DPhPC (*A*) or Lettre cell membrane (*B*) bilayer at pH 4.5. Voltage dependence of I/I_{max} for α-toxin-treated DPhPC (*C:* (□) pH 7.4; (■) pH 7.4 + 5 mM Zn^{2+}; (○) pH 4.5) or Lettre cell membrane (*D:* (□) pH 7.4; (■) pH 7.4 + 5 mM Zn^{2+}; (○) pH 4.5) bilayers. α-Toxin present at 15 μg/ml throughout each experiment.

occasional channel activity that may represent the endogenous K^+ channels referred to earlier, very little current in the absence of α-toxin was recorded. In the presence of α-toxin, channels appear with properties similar to those documented for α-toxin channels induced in pure lipid bilayers. These include single channel-like events that are rectified at pH 7.4. At pH 4.5 rectification is abolished and time-dependent closure is clearly evident (Fig. 5, upper traces). Single channel conductances under various conditions, as well as selectivity for anions over cations, are presented in the Table. Current measured 1 min after changing the voltage (*I*) is less than the instantaneous current (I_{max}), an effect which is increasingly evident at progressively higher voltages; this effect is more marked at negative voltages (Fig. 5*C*). Bilayers prepared from Lettre cell membranes (Fig. 5*D*) are more voltage sensitive in this regard than DPhPC bilayers, although their general behavior is similar. Low pH potentiates the effect further, as with patch clamped cells (Fig. 2*J,H*). Zn^{2+} has little effect on Lettre cell membranes (Fig. 5*D*)—compatible with its predominantly voltage-independent action on Lettre cells (Figs. 2*C–E*, 3)—in contrast to its effect in pure lipid bilayers (Figs. 5*A,C*).

Comparison between Lettre Cells, Reconstituted Lettre Cell Membrane Bilayers and DPhPC Bilayers

As indicated in the preceding sections, the effects of adding α-toxin are broadly similar in all three systems. For example, in each system negative voltages are more effective than positive voltages at decreasing I/I_{max} (Fig. 6). The greater sensitivity of Lettre cells compared with DPhPC bilayers is not due to the difference in medium used for whole-cell experiments, since that medium gave the same result as 0.1 M KCl when applied to DPhPC bilayers (open *vs.* closed squares in Fig. 6).

Table. Conductances and selectivity of single α-toxin channels

	Lettre cell membranes	DPhPC bilayers
Conductance at −50 mV and pH 7.4	45.3 ± 4.2 pS	57.7 ± 4.0 pS
at +50 mV and pH 7.4	83.3 ± 7.0 pS	81.7 ± 7.6 pS
at ±50 mV and pH 4.5	233.0 ± 10.2 pS	235.7 ± 16.2 pS
Selectivity (t-)	0.64 ± 0.02	0.62 ± 0.03

Single channel conductance (in 0.1 M KCl) was calculated from traces such as those shown in the upper panels of Figs. 5 and 7. Selectivity for anions over cations, expressed as the transference number (t-), was calculated from the reversal potential (Ψ), where t- is defined as: ½[1 + Ψ/((*RT* ln)/(*F*)) ([Cl^-] *trans*)])/([Cl^-] *cis*)
Number of observations is >20 in every case. α-Toxin (10–20 μg/ml) was applied to the *cis* compartment and removed by perfusion as soon as channel activity was detected.

At pH 7.4 single α-toxin-induced channel-like activity is rectified in bilayers prepared from Lettre cell membranes (Fig. 7*A*) or DPhPC (Fig. 7*B*); note that for Lettre cell membrane bilayers closure at voltages more negative than −40 mV is evident. Figure 7*C* shows the relationship between rectification and applied voltage for Lettre cells, Lettre cell membrane bilayers and DPhPC bilayers, each containing many toxin-induced channels. Such channels in Lettre cells and reconstituted Lettre cell membrane bilayers show greater rectification than in DPhPC bilayers; likewise they show greater voltage sensitivity (Figs. 5 and 6).

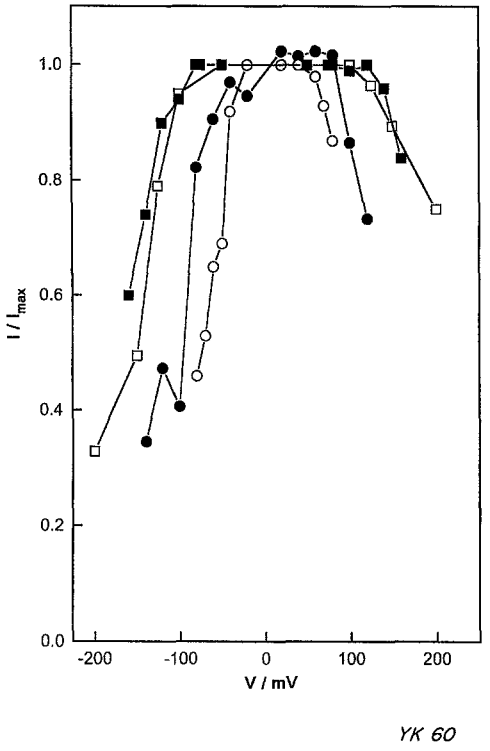

Fig. 6. Voltage dependence of α-toxin-induced currents. Current (*I*) after 1 sec (Lettre cells, ●) or after 1 min (bilayers prepared from DPhPC (□, ■) or from Lettre cell membranes (○)) of applying the voltage indicated is expressed relative to the instantaneous current (I_{max}) observed after switching from zero potential. The medium comprised (mM) 100 KCl, 5 HEPES, pH 7.4 (○, □) or 125 Cs^+, 45 Cl^-, 20 BAPTA, 10 HEPES, 6 creatine phosphate, 2 Mg^{2+}, and 0.1 μM Ca^{2+} (*trans*) and 125 mM Cs^+, 12 mM glucose, 10 mM HEPES, 1.2 mM $MgCl_2$ (*cis*) at pH 7.4 (●, ■). α-Toxin present at 15 μg/ml throughout each experiment.

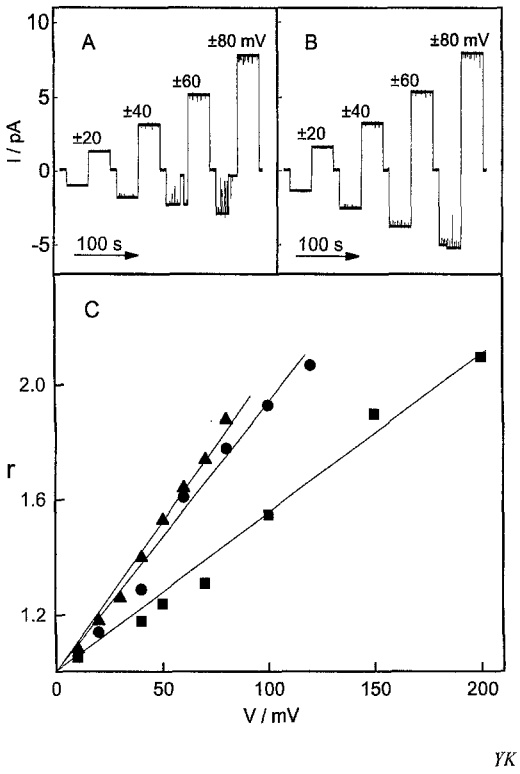

Fig. 7. Rectification of α-toxin-induced currents in bilayers. (*A*) α-Toxin-treated bilayers prepared from Lettre cell membranes. (*B*) α-Toxin-treated bilayers prepared from DPhPC. (*C*) Rectification expressed as the ratio, *r*, of instantaneous currents at positive (I_{+v}) to negative (I_{-v}) voltages observed after switching from zero to positive and negative potential (i.e., I_{+v}/I_{-v}) in α-toxin-treated DPhPC bilayers (■), Lettre cell plasma membrane bilayers (▲) and patch clamped Lettre cells (●).

Discussion

Three conclusions may be drawn from these results in which the induction of channel activity by a pore-forming toxin has been compared in three different systems, namely, DPhPC bilayers, reconstituted Lettre cell membrane bilayers and patch clamped Lettre cells. First, the low level of ion channel activity in Lettre cells when Ca^{2+}-activated K^+ channels are suppressed makes them ideal for studying pores induced by toxins or other pore-forming agents. The observation that a resting potential of −68 mV in untreated Lettre cells is maintained in the absence of any cation gradient is consistent with a "pumped" rather than a diffusional origin—at least under the in vitro conditions of our experiments—as suggested by Bashford and Pasternak (1984, 1990).

Second, the properties of *S. aureus* α-toxin channels are broadly similar in all three systems. Single channels, which are slightly anion selective, have conductances of *ca.* 50–80 pS (in 0.1 M salt), with rectification (Table, Fig. 7). The ratio of steady-state to instantaneous conductance (Figs. 5, 6) is voltage dependent and approaches a maximum at zero voltage; it is decreased by lowering pH (decreased current due to channel closure, even though the single channel current is increased by low pH). The greater sensitivity of Lettre cells and Lettre cell membranes in this regard may reflect changes in the conformation of the toxin-induced pore. For example, highly voltage-sensitive α-toxin channels can be induced in pure lipid bilayers at neutral pH in the presence of polyethyleneglycol (Y.E. Korchev, C.L. Bashford and C.A. Pasternak, *unpublished results*). In short, many of the properties of α-toxin-induced channels previously documented with other lipid bilayers (Bashford et al., 1986; Menestrina, 1986; Bashford et al., 1988*b*; Krasilnikov et al., 1988; Krasilnikov & Sabirov, 1989; Menestrina et al., 1990; Krasilnikov et al., 1992; Pasternak et al., 1992) are shown also by reconstituted cell membranes and intact cells. Properties such as inhibition by divalent cations or protons are also seen when leakage of ions and metabolites out of cells (Thelestam & Mollby, 1980; Bashford et al., 1984, 1986, 1988*b*) or leakage of dyes into cells (Pasternak et al., 1992) is assessed. This

indicates that for *S. aureus* α-toxin—in contrast to a pore-forming toxin such as that from *Entamoeba histolytica* (Gitler, Calef & Rosenberg, 1984)—metabolic events do not play a role in the induction of pores in cells.

Third, our results confirm the apparent discrepancy between the membrane potential-independent effect of divalent cations on α-toxin-induced pores in cells and the voltage-dependent effect in purely lipid bilayers by extending observations of metabolite leakage (Pasternak et al., 1989) to ion currents and membrane potential. Thus in cells, inhibition of toxin-induced ion currents by Zn^{2+} (Figs. 2, 3) is *not* reversed by switching voltage to zero as it is in bilayers (Menestrina, 1986) (or as is inhibition by protons (Fig. 2)). In addition, Zn^{2+} restores the membrane potential of α-toxin-treated Lettre cells (Fig. 4); such divalent cation-dependent recovery of membrane integrity has been demonstrated for cells treated with hemolytic Sendai virus (Micklem, Nyaruwe & Pasternak, 1985) and lymphocyte perforin (Bashford et al., 1988*a*). The reversibility of Zn^{2+} in abrogating toxin-induced depolarization (Fig. 4) suggests that Zn^{2+} acts directly on the toxin pores rather than by promoting shedding or internalization of damaged pieces of plasma membrane (Ramm et al., 1983; Morgan, Dankert & Esser, 1987). Some of the effect may arise from the ability of Zn^{2+} to perturb the oligomerization of membrane-associated α-toxin in favor of the monomer (C.L. Bashford and A. MacKinnon, *unpublished observations*), which is presumed to be inactive as a pore (e.g., Reichwein et al., 1987; Forti & Menestrina, 1989).

An important aspect of *S. aureus* toxin-induced channels—as of other membrane pores—that is now being addressed is the mechanism by which single channel conductance fluctuates between a high conductance and a low conductance state.

We are grateful to Dr. J.P. Arbuthnott and Dr. K. Hungerer for gifts of *S. aureus* α-toxin, to Dr. T.B. Bolton for collaboration with patch clamped cells and to Dr. J.M. Graham for help with the preparation of Lettre cell plasma membranes. This study was supported by the Cell Surface Research Fund, Medical Research Council, Science and Engineering Research Council, UNESCO (Molecular and Cell Biology Network) and The Wellcome Trust.

References

Bashford, C.L., Alder, G.M., Graham, J.M., Menestrina, G., Pasternak, C.A. 1988*a*. 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

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

Bashford, C.L., Alder, G.M., Menestrina, G., Micklem, K.J., Murphy, J.J., Pasternak, C.A. 1986. Membrane damage by hemolytic viruses, toxins, complement, and other cytotoxic agents. A common mechanism blocked by divalent cations. *J. Biol. Chem.* **261**:9300–9308

Bashford, C.L., Menestrina, G., Henkart, P.A., Pasternak, C.A. 1988*b*. Cell damage by cytolysin. Spontaneous recovery and reversible inhibition by divalent cations. *J. Immunol.* **141**:3965–3974

Bashford, C.L., Micklem, K.J., Pasternak, C.A. 1985. Sequential onset of permeability changes in mouse ascites cells induced by Sendai virus. *Biochim. Biophys. Acta* **814**:247–255

Bashford, C.L., Pasternak, C.A. 1984. Plasma membrane potential of Lettre cells does not depend on cation gradients but on pumps. *J. Membrane Biol.* **79**:275–284

Bashford, C.L., Pasternak, C.A. 1990. Setting of membrane potential. *In:* Monovalent Cations in Biological Systems. C.A. Pasternak, ed. pp. 171–186. CRC, Boca Raton, Florida

Benham, C.D., Bolton, T.B., Lang, R.J., Takewaki, T. 1986. Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *J. Physiol.* **371**:45–67

Bhakdi, S., Tranum-Jensen, J. 1987. Damage to mammalian cells by proteins that form transmembrane pores. *Rev. Physiol. Biochem. Pharmacol.* **107**:147–223

Bhakdi, S., Tranum-Jensen, J. 1991. Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev.* **55**:733–751

Bussey, H. 1991. K1 killer toxin, a pore-forming protein from yeast. *Mol. Microbiol.* **5**:2339–2343

Easmon, C.S.F., Adlam, C. 1983. Staphylococci and Staphylococcal Infections, vols. 1 and 2. Academic, London

Forti, S., Menestrina, G. 1989. Staphylococcal alpha-toxin increases the permeability of lipid vesicles by cholesterol- and pH-dependent assembly of oligomeric channels. *Eur. J. Biochem.* **181**:767–773

Gitler, C., Calef, E., Rosenberg, I. 1984. Cytopathogenicity of *Entamoeba histolytica*. *Philos. Trans. R. Soc. Lond. B* **307**:73–85

Graham, J.M., Coffey, K.H.M. 1979. Tissue-culture cell fractionation. Fractionation of cellular membranes from ^{125}I/lactoperoxidase-labelled Lettre cells homogenized by bicarbonate-induced lysis: Resolution of membranes by zonal centrifugation and in sucrose and metrizamide gradients. *Biochem. J.* **182**:173–180

Impraim, C.C., Foster, K.A., Micklem, K.J., Pasternak, C.A. 1980. Nature of virally mediated changes in membrane permeability to small molecules. *Biochem. J.* **186**:847–860

Jeljaszewicz, C., Wadstrom, T. 1978. Bacterial Toxins and Cell Membranes. Academic, New York

Kasianowicz, J.J., Moore, C.R., Zimmerberg, J., Pasternak, C., Bashford, C.L., Parsegian, V.A. 1991. Two-sided protonation of the alpha toxin channel inferred from the effects of transmembrane pH gradients. *Biophys. J.* **59**:458*a* (*Abstr.*)

Kerry, C.J., Kits, K.S., Ramsey, R.L., Sansom, M.S., Usherwood, P.N. 1987. Single channel kinetics of a glutamate receptor. *Biophys. J.* **51**:137–144

Krasilnikov, O.V., Sabirov, R.Z. 1989. Ion transport through channels formed in lipid bilayers by *Staphylococcus aureus* alpha-toxin. *Gen. Physiol. Biophys.* **8**:213–222

Krasilnikov, O.V., Sabirov, R.Z., Ternovsky, V.I., Merzliak, P.G., Tashmukhamedov, B.A. 1988. The structure of *Staphylococcus aureus* alpha-toxin-induced ionic channels. *Gen. Physiol. Biophys.* **7**:467–473

Krasilnikov, O.V., Sabirov, R.Z., Ternovsky, V.I., Merzliak, P.G., Muratkhodjaev, J.N. 1992. A simple method for the determination of the pore radius of ion channels in planar lipid bilayer membranes. *FEMS Microbiol. Immunol.* **5**:93–100

Lettre, R., Paweletz, N., Werner, D., Granzow, C. 1972. Sublines of the Ehrlich-Lettre mouse ascites tumour. A new tool for experimental cell research. *Naturwissenschaften* **59**:59–63

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

Menestrina, G., Bashford, C.L., Pasternak, C.A. 1990. Pore-forming toxins: experiments with *S. aureus* alpha-toxin, *C. perfringens* theta-toxin and *E. coli* haemolysin in lipid bilayers, liposomes and intact cells. *Toxicon* **28:**477–491

Micklem, K.J., Alder, G.M., Buckley, C.D., Murphy, J., Pasternak, C.A. 1988. Protection against complement-mediated cell damage by Ca^{2+} and Zn^{2+}. *Complement* **5:**141–152

Micklem, K.J., Nyaruwe, A., Pasternak, C.A. 1985. Permeability changes resulting from virus-cell fusion: temperature-dependence of the contributing processes. *Mol. Cell. Biochem.* **66:**163–173

Montal, M., Müller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Morgan, B.P., Dankert, J.R., Esser, A.F. 1987. Recovery of human neutrophils from complement attack: Removal of the membrane attack complex by endocytosis and exocytosis. *J. Immunol.* **138:**246–253

Pasternak, C.A. 1986. Effect of pore formers on intracellular calcium. *Cell Calcium* **7:**387–397

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.* **105:**83–92

Pasternak, C.A., Bashford, C.L., Menestrina, G. 1989. Mechanisms of attack and defence at the cell surface: the use of phospholipid bilayers as models for cell membrane. *Biosci. Rep.* **9:**503–507

Pershadsingh, H.A., Johnstone, R.M., Laris, P.C. 1978. Influence of (DL)-propranolol and Ca^{2+} on membrane potential and amino acid transport in Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* **509:**360–373

Ramm, L.E., Whitlow, M.B., Koski, C.L., Shin, M.L., Mayer, M.M. 1983. Elimination of complement channels from the plasma membranes of U937, a nucleated mammalian cell line: temperature dependence of the elimination rate. *J. Immunol.* **131:**1411–1415

Reichwein, J., Hugo, F., Roth, M., Sinner, A., Bhakdi, S. 1987. Quantitative analysis of the binding and oligomerization of staphylococcal alpha-toxin in target erythrocyte membranes. *Infect. Immun.* **55:**2940–2944

Schein, S.J., Kagan, B.L., Finkelstein, A. 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. *Nature* **276:**159–163

Schindler, H. 1980. Formation of planar bilayer from artificial or native membrane vesicles. *FEBS Lett.* **122:**77–79

Stein, W.D., Lieb, W.R. 1986. Transport and Diffusion Across Cell Membranes. Academic, London

Suttorp, N., Seeger, W., Dewein, E., Bhakdi, S., Roka, L. 1985. Staphylococcal alpha-toxin-induced PGI2 production in endothelial cells: role of calcium. *Am. J. Physiol.* **248:**C127–C134

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

Thelestam, M. 1983. Membrane damage by staphylococcal alpha-toxin to different types of cultured mammalian cell. *Biochim. Biohys. Acta* **762:**481–488

Thelestam, M., Blomqvist, L. 1988. Staphylococcal alpha toxin—recent advances. *Toxicon* **26:**55–65