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Mini review

A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes

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Introduction

Pore-forming bacterial toxins are being increasingly employed by cell biologists as tools for controlled permeabilization of cell membranes. For many reasons, these agents are usually superior to digitonin or saponin, since the effects of the latter are normally difficult to predict, control or monitor. The toxins are easy to handle; the two most widely used agents, *S. aureus* α-toxin and streptolysin-*O* (SLO), are stable and can be stored over long periods provided that they are present in highly purified form. No special procedures are required to activate the toxins, and binding occurs spontaneously even at low temperature. Transmembrane pores formed by bacterial toxins are often fairly well defined in size. Correct choice of toxin and experimental conditions thus enables one to produce stable membrane lesions ranging from approximately 8 Å to 300 Å in size. It becomes possible to manipulate the intracellular ionic milieu, to introduce small molecules such as nucleotides, or even to apply very large macromolecules, such as antibodies, to the cytoplasm. Pore-forming toxins applied to intact cells seldom, if ever, pass the plasma membrane to attack intracellular membranes. Finally, since pure preparations of pore-forming toxins are devoid of enzymatic (proteolytic) activity, they cause no alteration of cell constituents.

The first publication presenting the principle of using *S. aureus* α-toxin and SLO to permeabilize membranes for studying the minimal requirements for secretion in chromaffin cells appeared in 1985 [1]. Since then, over 50 publications have appeared reporting work in which these two toxins have been used as permeabilizing agents, and the numbers are steadily increasing. In fact, utilization of these toxins is becoming something approaching a routine method. However, many experiments are done in a highly empirical fashion, and in good faith on the assumption that there are no serious hazards or pitfalls. Inasmuch as we are interested in propagating the use of these toxins, it appears timely to point out

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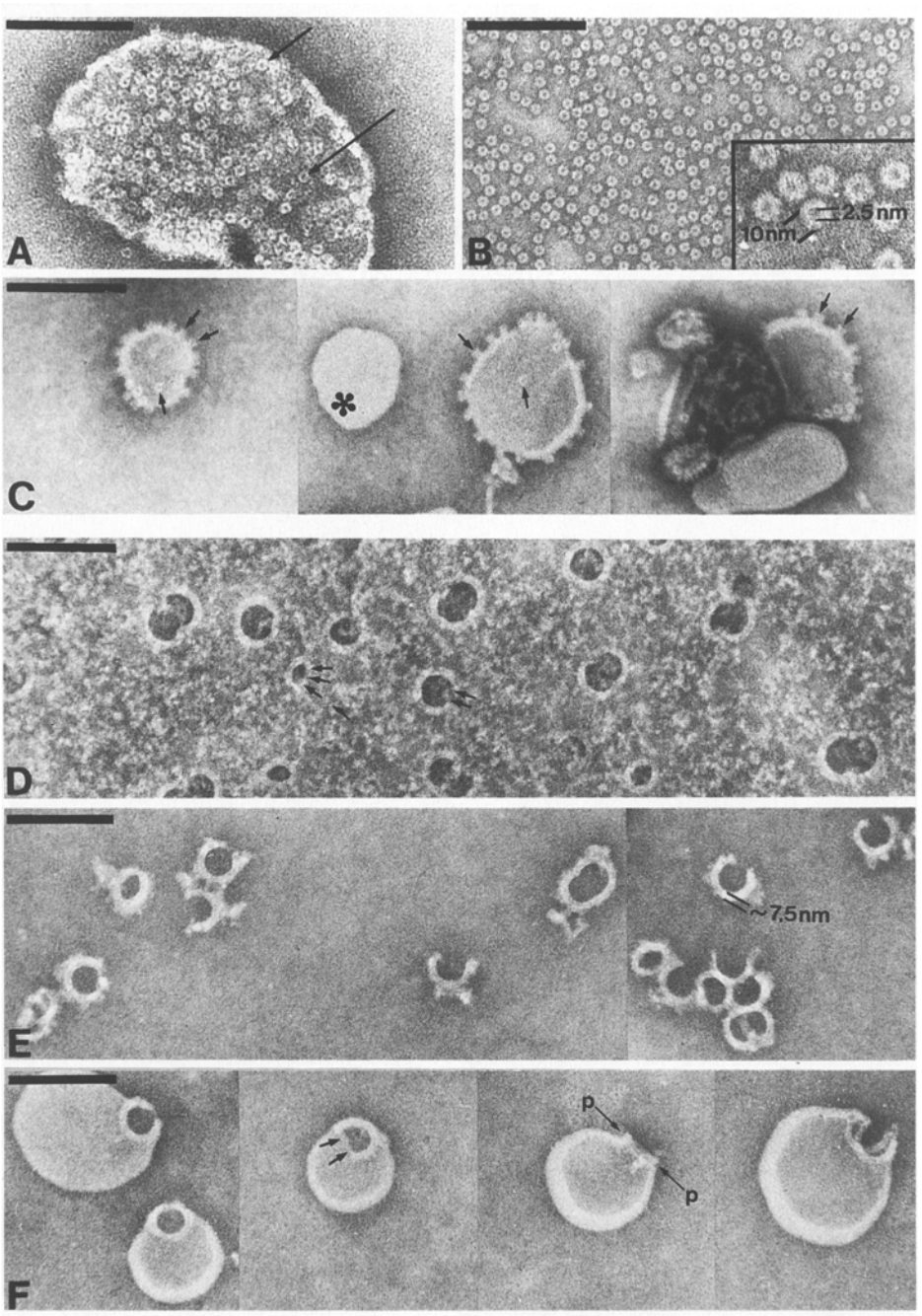

Fig. 1. **A** Negatively stained fragment of rabbit erythrocyte lysed with staphylococcal α-toxin. Numerous 10-nm ring-shaped structures are seen over the membrane (*arrows*). **B** Isolated toxin hexamers in detergent solution. **C** Lecithin liposomes carrying reincorporated α-toxin hexamers. The hexamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (*arrows*). Characteristically, liposomes that escape incorporation of the toxin are impermeable to the stain. **D** Negatively stained erythrocyte membrane lysed by streptolysin-*O*

some important but generally unknown or unappreciated facts that should be considered.

Some prototypes of pore-forming bacterial toxins

S. aureus α-toxin was the first bacterial toxin recognized to damage mamalian plasma membranes by forming transmembrane pores [14, 15, 21]. In the past decade, over 50 other bacterial toxins have been identified as pore formers [14]. They differ in their mode of primary interaction with membranes, in the mode of pore formation, and also with respect to the size of the pores they create. In the following, we will provide a simplified overview on the action of three pore formers, i.e. *S. aureus* α-toxin, SLO, and *E. coli* haemolysin (HlyA). *S. aureus* α-toxin is an apparently unique toxin not known to have any extensive homology to any other protein. SLO and HlyA represent prototypes of toxin families, each encompassing at least 15 members with similar properties. Each of the three toxins has characteristic pore-forming properties. In most cases, one of the three should fulfil the demands set by a given experiment.

S. aureus α-toxin

This toxin is produced as a single-chain polypeptide of M_r 34 000. At low concentrations (below 5 μg/ml), the toxin monomer binds to as yet unidentified, protease-resistant acceptor sites that are present on most nucleated cells, albeit in widely varying numbers [17, 25]. Cells lacking the acceptor(s) cannot be permeabilized by low doses of this toxin. When applied at high concentrations (50–200 μg/ml), α-toxin non-specifically adsorbs to lipid bilayers and can then produce pores in almost any cell.

Pore formation occurs when six membrane-bound monomers collide and oligomerize to form hexamers (Fig. 1). Hexamerization is thought to trigger conformational changes, leading to insertion of amphiphilic domains into the lipid bilayer and to generation of pores [4, 15, 20, 21, 26, 37, 40]. It is not known whether the specific acceptor molecules are involved in formation of the channels. If this were indeed so, it would imply that the pores formed by toxin binding at low concentrations to the acceptors may differ in composition and function from pores formed at high toxin doses. Toxin hexamers are stable in SDS unless heated, and they present in all cases as a homogeneous protein band of M_r 200 000. The toxin hexamer is insensitive to destruction by proteases at neutral pH.

(SLO), showing numerous curved rods 25–100 nm long and approximately 7.5 nm wide with inner radius of curvature of 13–16 nm. Most rods are approximately semicircular, often joined in pairs at their ends. Dense accumulations of stain are seen at the concave side of the rods. When these do not form closed profiles, the stain deposit is partly bordered by a "free" edge of the erythrocyte membrane (*arrows*). **E** Negative staining of isolated SLO oligomers, showing numerous curved rod structures identical to those found in toxin-treated membranes. **F** Purified SLO complexes reincorporated into cholesterol-free lecithin liposomes. The toxin oligomers form holes in the liposomes (*unlabelled arrows*); *p* indicates a lesion seen in profile. *Scale bars*, 100 nm in all frames. Sodium silicotungstate was used as negative stain in **B**–**F**. Uranylacetate was used in **A**. Reproduced from Bhakdi and Tranum-Jensen [9]

Functional consequences of hexamer formation in cell membranes

In erythrocyte membranes, toxin hexamers form pores of apparently uniform size with a functional diameter of approximately 1.5 nm regardless of the dose of toxin applied. Many nucleated cells also become permeabilized, with concomitant appearance of toxin hexamers, and data accrued in the past have indicated that these pores have approximately the same size [15]. However, this simple model now needs to be modified. It has recently become clear that formation of toxin hexamers in nucleated cells may evoke one of three effects, depending on dose and cell type (unpublished data). First, hexamers may be entirely devoid of permeabilizing properties. This is, for example, the case with human granulocytes treated with low to moderate doses of the toxin (up to 20 μg/ml). Second, pores created by hexamers may be very small, allowing flux of only monovalent ions (and not of calcium). This has been observed with lymphocytes and keratinocytes treated with low doses of the toxin (30–1000 ng/ml). Third, α-toxin pores may be large enough to permit flux of calcium and small macromolecules (e.g. nucleotides). This occurs even at low concentrations in the case of platelets and monocytes [10, 11], and it is generally the case when the cells are treated with high toxin doses (over 50–100 μg/ml).

The causes underlying these heterogeneous effects have not been delineated. We are considering the possibility that they are related to the interaction of the toxin with specific binding sites as opposed to non-specific absorption at high concentrations. Possibly, certain cell membranes contain constituents that under certain circumstances can restrict or even prevent pore formation. The heterogeneous effects of α-toxin on nucleated cells harbour obvious consequences for the use of this toxin as a permeabilizing agent.

Comments on the use of alpha-toxin as a permeabilizing agent

1. Commercially available toxin preparations should be checked for purity using SDS-PAGE, and also checked for the possible presence of contaminating DNase and proteases. We have detected severe contaminants in the past.
2. Pilot experiments must first be performed to assess the overall effect of the toxin on the respective cell target. We recommend the following procedure. Treat cells with 0.1, 1, 10 and 100 μg/ml toxin for 60 min at 25–37 °C. Thereafter, measure cellular ATP levels (using for example the simple luciferase assay). ATP depletion is a reliable indication that pore formation has occurred. To obtain an indication on the size of the pores, add trypan blue, eosin or propidium iodide, and determine whether one of these markers enters the cells. We favour propidium iodide, since influx renders cells brightly fluorescent, and quantification can easily be undertaken by flow cytometry (see e.g. [5, 33]). If no influx occurs, the pores are of the small type and they probably do not permit calcium influx. The latter can be confirmed by determining cellular accumulation of ^{45}Ca. Often, it will be found that high concentrations (50–200 μg/ml) of α-toxin must be applied in order to generate the larger pores. High doses will then be necessary for investigating the dependence of a given process on calcium or nucleotide.
3. Whenever α-toxin does produce the required type of pore, its use has many advantages. The pure dried protein is very stable and can be stored for years

without significant loss of activity at −20 °C. It is soluble in water or aqueous buffer and is stable over a wide pH range (5–9). No ionic requirements have been noted for membrane binding or pore formation, and permeabilization can thus be performed in the presence of chelating agents. Permeabilization is most efficient at room temperature, but can also be achieved at 4 °C or 37 °C.

4. The toxin attaches exclusively to the plasma membrane and does not reach intracellular organelles. Pores created by the toxin do not permit egress of macromolecules from the cytoplasm. Therefore, cellular mechanisms (e.g. for secretion) and enzyme cascades remain intact over extended time periods. Controlled manipulation of the intracellular ionic composition, as well as introduction of nucleotides, is feasible.
5. At very high concentrations (100–200 μg/ml), membrane vesiculation may occur that is accompanied by release or loss of intracellular proteins. Monitoring the release of LDH is a simple and useful method to detect this process.
6. When applied at concentrations above 10 μg/ml, only a very small fraction of α-toxin will bind, and it is therefore theoretically possible to retrieve supernatants for re-use.

E. coli haemolysin (HlyA)

This toxin is a water-soluble single-chain protein of M_r 107 000 [18, 31, 41] that appears to be acylated with (a) fatty acid(s) [27]. It seems to bind non-specifically to lipid bilayers, and most cells can therefore be permeabilized. Toxin preparations from our laboratory always contain calcium in high concentrations (approximately 100 mM) because supplementation of bacterial culture medium with $CaCl_2$ enhances the yield of active toxin [7]. Therefore, experiments in which Ca^{2+} is a critical factor must be carefully planned and controlled.

The toxin has useful properties. Wide variations in pore size as observed with α-toxin have not been noted. The toxin binds rapidly to generate pores of 1–2 nm diameter [5–7]. These pores permit rapid flux of ions and nucleotides, but not of proteins. The appearance of transmembrane pores can easily be monitored using propidium iodide as a marker [5, 7]. Cells treated with 1–5 HU/ml (100–500 ng/ml) of HlyA are usually permeabilized within minutes and, in the presence of propidium iodide, assume a red fluorescence that can be detected by fluorescence microscopy or flow cytometry. Trypan blue or eosin can also be used.

Comments on the use of HlyA as a permeabilizing agent

HlyA has not been used as a permeabilizing agent in any published study to date. However, it will probably replace α-toxin in the future when the latter fails to create Ca^{2+}-permissive pores. The following comments may be of use to those wishing to use HlyA.

1. For unknown reasons, this toxin is very labile and loses activity even at −20 °C. Toxin preparations must therefore be stored at −80 °C in small aliquots. They can be thawed and re-frozen once or twice. It is advisable to perform haemolytic titrations to check the activity of the toxin before use.
2. Very low doses of the toxin can trigger G-protein-dependent processes [24]. In neutrophils, this is accompanied by generation of IP3 and, hence, probably by

mobilization of intracellular Ca^{2+}. These effects, however, are only observed within a very narrow range of toxin concentrations (below 1 HU/ml). Higher doses (usually 1–10 HU/ml) should always be used in permeabilizing experiments in order to avoid unpredictable cellular events.

3. Depending on the source, HlyA preparations may contain high concentrations of Ca^{2+}. If permeabilization is to be undertaken in the absence of free Ca^{2+}, a sufficient amount of EGTA or EDTA must be added. It is noteworthy that active toxin contains tightly bound Ca^{2+} that is apparently removed only slowly by EDTA. Thus, it may be possible to achieve permeabilization in the presence of EGTA or EDTA, provided the toxin has not been exposed to the chelating agents beforehand. Once Ca^{2+} has been removed, HlyA toxin is inactive [16, 30].
4. The HlyA pore is destroyed by proteases [32]. Proteases may be present in cell preparations, or they may be secreted from the cells in response to rises in intracellular Ca^{2+} during permeabilization.
5. HlyA preparations inevitably contain varying amounts of contaminating LPS.

HlyA could emerge as an excellent agent to create defined pores 1–2 nm in diameter. Its use should be considered when α-toxin fails to work. The toxin is not commercially available, but can be obtained from research groups. Cells that have been shown to have been permeabilized by HlyA under retention of cytoplasmic proteins include human granulocytes [5], monocytes [8], endothelial cells [36], renal tubular epithelial cells [28], keratinocytes and hepatocytes (unpublished data).

Streptolysin O

SLO is the prototype of a family of toxins that have been termed oxygen-labile or sulphydryl-activatable. This is because the toxin spontaneously loses activity in the presence of atmospheric oxygen, and regains activity upon reduction (e.g. with DDT). Originally, it was thought that this was due to formation of intramolecular disulphide bonds. However, after several toxins were cloned and sequenced [23, 29, 38, 39], it became apparent that each contained a single cysteine residue. Hence, current belief is that this cysteine residue is particularly reactive and forms disulphide bonds with low-molecular-weight moieties that chance to be present. This leads to loss of membrane binding and, hence, of pore-forming activity of the molecule.

The single cysteine residue in SLO (and pneumolysin) can be replaced by alanine without loss of activity [34, 35]. The mutagenized SLO can be isolated from *E. coli* and it is no longer prone to oxygen-dependent inactivation.

SLO is a water-soluble molecule of M_r 69 000 with a pI of 6.4 [12]. It may be isolated from streptococcal culture supernatants, or the recombinant form from *E. coli*. Once purified, the toxin is stable. It can be stored, lyophilized or in solution, at −80 °C for long periods without loss of activity.

Binding of the toxin apparently occurs via interaction with cholesterol. SLO pores are generated when membrane-bound toxin monomers collide via lateral aggregation to form high-molecular-weight oligomers [13]. These can be seen in the electron microscope as partially or fully circularized ring structures with heterogeneous internal diameters [13]. The pores formed by these polymers can

have diameters exceeding 30 nm, which means they allow free passage of proteins across the membranes (Fig. 1).

Comments on the use of SLO as a permeabilizing agent

1. SLO preparations may contain contaminating proteases. These proteases originate either from *Streptococci* or from *E. coli*. Since SLO monomers are prone to proteolytic cleavage, action of such proteases may be reflected by instability of the toxin preparations. For researchers working with adherent cells, the proteases may create complications by causing detachment of the cells. Further, since toxin pores are so large, the proteases could enter the cytoplasm and cause artefacts. Overall, it is therefore worthwhile to check whether a given SLO preparation is protease-contaminated.
2. The action of SLO is extremely rapid, and permeabilization will occur within seconds or minutes. Temporal dissociation of the process of binding from pore formation is often useful; this can be achieved by incubating the cells with the toxin at low temperature (0 °C) and then effecting pore formation by transferring the cells to 37 °C [2]. In this way, the cells can be transferred to a medium of one's choice and there is enough time to handle a series of samples before the permeabilization step takes place. Excessive toxin doses should be avoided (generally >100 HU/ml, corresponding to ~ 1 μg/ml) because pores will then form while the temperature is still 0 °C. Measurement of LDH release is a convenient means of determining when large pores have formed.
3. Even though the pores in the plasma membranes are theoretically large enough to permit SLO to enter the cytoplasm, this is probably rare, because toxin molecules rapidly bind to and are trapped in the plasma membrane. Since membranes devoid of cholesterol cannot bind SLO, many intracellular membranes including large parts of the endoplasmatic reticulum cannot be permeabilized.

We have recently isolated and characterized the action of tetanolysin, which is closely related to SLO. Tetanolysin is now available in highly purified form and free of proteases. This toxin differs from SLO in one important respect, namely that the pore size varies quite widely depending on the toxin dose applied. Thus, it may be possible to produce pores of different sizes by judicious adjustment of toxin concentration. This has recently been exploited in experiments where chromaffin cells were used as targets (unpublished).

Summary and conclusions

Depending on the size of the pores one wishes to produce in plasma membranes, the choice will probably fall on one of the three toxins discussed above. *S. aureus* α-toxin should be tried first when pores of 1–1.5 nm diameter are required. This is generally the case when Ca^{2+} and nucleotide dependence of a given process is being studied. If α-toxin does not work, this is probably due to the fact that the toxin either does not produce pores, or that the pores are too small. In this case, high concentrations of α-toxin should be tried. If this still does not work, we recommend the use of HlyA. When very large pores are to be created, e.g. for

introduction of antibodies into the cells, SLO or another member of this toxin family are the agents of choice. SLO preparations need to be checked for presence of protease contaminants. Tetanolysin currently offers advantages since it is protease-free, and the size of the pores can probably be controlled by varying the toxin dose. Methods for assessing the size of pores created by such agents have been published in the recent literature, and the appropriate papers can be consulted whenever the need arises.

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