PORE-FORMING TOXINS: ATTACK AND DEFENCE AT THE CELL SURFACE

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The plasma membrane of eukaryotic cells fulfils many functions. Perhaps the most fundamental of these is its role as a barrier. The membrane is the structure that is primarily responsible for what enters the cell (such as ions, and nutrients) and what leaves (metabolic waste products, for example). It is the site at which many chemical signals are recognised (by receptors) and at which downstream signalling pathways originate. The movement of ions across the membrane generates and sustains the membrane potential either by diffusion through ion-specific channels or by the action of pumps [1]. Changes in membrane potential, induced by alterations in ion permeability, play a critical role in excitable tissues. The integrity of the membrane is a prerequisite for the functional, nutritional and metabolic competence of cells and breaches of the membrane can quickly lead to cell death by either necrosis or apoptosis [2]. Our group have been interested in agents, of all types, that compromise membrane integrity and the mechanisms available to cells to protect them against such attacks.

The number of agents that generate membrane damage (Table 1) is large and growing. They range from some enveloped viruses, through bacterial, animal and plant toxins to man-made molecules and environmental pollutants such as heavy metals [3]. The hallmark of the membrane damaging toxins is a sequential series of events that mark increasing damage to the permeability barrier presented by the membrane [4,5]. The first sign of damage is membrane depolarisation - as soon as pores large enough to allow monovalent ions to pass are created, inflow of Na^+ and efflux of Cl^- cause depolarisation. If the pores remain open, the ion gradients dissipate and the internal and external ionic solutions equilibrate. If the pores continue to increase in number and/or size larger molecules will cross the membrane. Thus intermediates of metabolism (eg. nucleotides and sugar phosphates) leak out with consequential loss of metabolic integrity. At a still later stage macromolecules, cell proteins, will leak and the cell will die (by necrosis).

Tab. 1 Agents that form divalent cation-sensitive pores [21]

Viruses	Sendai, Newcastle Disease, Influenza
Bacterial toxins	S. aureus α- and δ-toxin, Streptolysin O, C. perfringens θ-toxin, S. pneumoniae pneumolysin, E. coli haemolysin, *A.* hydropohila haemolysin, C. lacteus cytolysin, B. thuringiensis δ-endotoxin
Animal toxins	Melittin (honey bee), Cytolysin (sea anemone), Latrotoxin (spider venom)
Immune proteins	Activated complement, Cytolysin (perforin)
Synthetic compounds	Polycations, Triton X-100

The common pattern of damage by a wide variety of agents suggested a common mechanism [5]. This notion was reinforced by our repeated observation that leakage induced by membrane-damaging agents could be ameliorated by extracellular divalent cations. Zn^{2+} was especially effective followed by Ca^{2+} and then Mg^{2+}. Protons had a similar action and were active in inhibiting leakage at very low concentrations [5, 6]. The sharing of a common mechanism was further reinforced by the finding that different agents could act synergistically to create divalent cation-sensitive leakage at concentrations at which either partner (on its own) was ineffective.

We reasoned that a common mechanism of action might be revealed by studies of membrane damaging agents in a simple model system. One such is the black lipid membrane - a system in which individual pores in a lipid membrane can readily be detected by electrophysiological techniques [7]. Our results with the bacterial toxins [8] were particularly encouraging. In each case, under the appropriate conditions, addition of toxin led to a time-dependent appearance of gradual increases of membrane current as if ion-conducting channels were being inserted into the membrane. Figure 2 shows data for a wide variety of agents incorporated into lipid bilayers ranging from endogenous ion-channels, through bacterial and animal toxins to other mammalian proteins and artificial substances such as detergents. In the case of the α-toxin from ***Staphylococcus aureus*** the pores induced are slightly voltage-sensitive at physiological pH values. Intriguingly voltage-dependence (closure) was markedly increased by Zn^{2+} and especially by H^{+} (low pH). The leakage behaviour in intact cells

seemed to be reflected by channel-or-pore-closures in a model membrane. A similar story was found with many of the other membrane-damaging toxins and it seems appropriate to classify them as 'pore-forming' agents. We use the word pore in a functional sense - it represents a state of the membrane in which molecules normally unable to permeate now have a route to cross the membrane.

How might such a diverse selection of materials produce membrane pores? In the case of bacterial toxins negative stain electron microscopy and other biochemical techniques suggested that the water-soluble, monomeric form of the toxin bound to the target membrane and then oligomerised to produce a structure with a central pore [9]. An analogous process had been suggested for the mechanism by which the C9 component of complement, at the heart of humoural defence in man [10,11] and the cytolytic protein of cytotoxic T-cells (known as perforin or cytolysin) damage cells [12]. The crystal structure of the heptameric (pore-form) of *S. aureus* α-toxin is available [13] and shows the presence of a central pore with a minimum diameter, ~2nm, that approximates that predicted by leakage experiments and the conductance of α-toxin induced ion channels [7,14]. The α-toxin pore depends on β-structure rather than α-helixes, and in this regard, resembles the porin molecules embedded in the outer membrane of certain bacteria [15]. Each monomer provides a loop of β structure sufficiently long to cross the lipid bilayer. Interactions between 7 monomers generate a very stable 14 stave barrel which represents the pore structure. Pore-forming β-domains are not nearly so easy to identify in primary sequences as α-motifs.

Our recent work has addressed the question of how such a robust structure as the pore of α-toxin may exhibit the 'open' to 'closed' transitions so characteristic of the much narrower endogenous ion channels. The key to our understanding came from the observation that the 'closed' state of the *S. aureus* α-toxin channel still conducted ions [16], albeit to a much lesser extent than the 'open' state. Furthermore the exclusion of ions from the 'closed' state was greater than that for uncharged molecules. It seems more appropriate in the case of induced pores to refer to 'low conducting' and 'high conducting' states rather than 'closed' and 'open' pores [16].

One reason for the anomalous behaviour of ions in narrow pores could be the effects of surface charge in a confined geometry [17, 18]. The idea is that fixed surface charges within the pore attract mobile counter ions. The effective concentration of mobile counter ions in the pore can greatly exceed their concentration in the bathing solution, particularly at low ionic strength. Pore conductance reflects, predominantly, the number of mobile ions in the pore and is not limited by ion-diffusion in the bathing solutions. Consistent with this explanation is the non-linear dependence of single pore conductance on ionic strength [18, 19]. An additional consequence of this model is that the ionic

selectivity of the pore should reflect the surface charge - negative fixed charges would confer cation selectivity and positive fixed charges anion selectivity. Removal of fixed charge, by titration with acid or base should abolish ion selectivity. We have found [18,19] that all these features are found in a number of artificial pores [19].

The explanation for switching of pores between 'high' and 'low' conducting states might also lie in the properties of the fixed surface changes. Were the responsible groups able to change their state of ionisation in concert, then the channel would change its macroscopic behaviour. We considered the possibility that, because of their proximity in the confined geometry of a narrow pore, surface groups may ionise (or associate) in a quasi-cooperative manner. We have developed a simple model [19] to show that for a hexagonal array of ionisable groups such co-operative behaviour will occur when ionisation of one group changes the proton concentration at neighbouring groups by half a pH unit. In support of this model we note that switches of ion current from 'low' to 'high' conductivity coincide with switches of ion selectivity from 'low' to 'high'. This primitive mechanism may have been the original 'switch' subsequently honed and refined by evolutionary processes to generate the array of ion channels now found in biological membranes.

Our surface charge model of ion channel switching does not preclude the existence of other mechanisms - see for example Hille [20]. The increasing number of high resolution structures of membrane proteins informs the debate about switching mechanism. In the case of *S. aureus* α-toxin it is quite difficult to imagine how the heptameric structure (assumed to be the 'high-conducting' state) can switch to a low conducting configuration. Modelling indicates that a hexamer (the structure originally proposed based on the analysis of electron micrographs) could have a smaller pore compatible with the observed ionic conductance and permeability to non-electrolytes [14]. However, it remains difficult to imagine how such an apparently robust structure as the α-toxin heptamer (or hexamer) could easily change its state of oligomerisation.

This review has indicated that many agents can increase the permeability of cell membranes. Pore-forming agents may be used in a hostile manner by organisms seeking to sustain their biological niche or in a defensive manner, as a means of repelling invading organisms. In all cases environmental conditions, for example the presence of certain cations ($H^+ > Zn^{2+} > Ca^{2+} > Mg^{2+}$) can ameliorate the propensity to form pores. The cell surface remains at the sharp end of the conflict between host and parasite or host and environmental pollutant.

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