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Effects of Erythrocytes Treated with Alpha Hemolysin of *E.Coli* on Endothelial Cells

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Uropathogenic strains of *E. coli* deliver the toxin alpha-hemolysin (HlyA) to optimize the host environment for the spread of infection. It was reported that at high concentrations, the toxin forms pores in eukaryotic membranes, leading to cell lysis, while lower concentrations might interfere with host-cell-signaling pathways, causing apoptosis. In the present investigation we demonstrate that a relatively low concentration of HlyA induces morphological changes and phosphatidylserine (PS) externalization of human erythrocytes. On the other hand, the unacylated nonhemolytic form of HlyA, ProHlyA induces similar morphological changes but no PS externalization. We performed osmoscan experiments to test the effect of both proteins on erythrocytes structure. HlyA treated erythrocytes show increased membrane fragility and cell volume as well as diminished cytoplasmic viscosity and S/V ration. ProHlyA-treated erythrocyte are not different from control ones. Since PS exposure of erythrocytes is known to induce cell adhesion, we used a dynamic cell adhesion platform to study the consequences of HlyA vs ProHlya exposure of erythrocytes on their adhesion to human endothelial cells (HMEC). Results indicate that HlyA-treated erythrocytes adhere more to endothelial cells than Pro-treated erythrocytes at low flux (0.5 din). At higher fluxes (1 and 2 din), however, HlyA-treated erythrocytes detached easily than control ones, indicating that the adherence is weak. We also study the efflux of ATP from erythrocytes treated with both toxins by luciferin-luciferase luminescence. Results demonstrate that HlyA induces the efflux of ATP while ProHlyA does not. Since PS exposure was suggested to simultaneously increase extracellular ATP and adhesion to the vascular endothelium, and erythrocyte derived ATPe can alter the caliber of the vascular lumen, future experiments will be designed to relate HlyA induced efflux of ATP of erythrocytes with their adhesion and interaction with endothelial cells.

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Reversible Permeabilization of Cell Membranes via Lysenin Channels

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The selective nature of the cell-membrane hinders the permeability of drugs, fluorescent probes, and other macromolecules into living cells. To introduce foreign molecules as well as preserve cell viability, reversible permeabilization of the cell membrane is required. To achieve this aim, several techniques have been proposed including microinjection, electroporation, optoporation, and lipophilic and peptide carriers. These methods are either highly invasive, inefficient, limited to single-cell, require sophisticated instruments, or need tedious conjugation process. Therefore, we proposed a simple yet efficient method of controlled transport of exogenous molecules into viable cells using the pore-forming toxin lysenin. Lysenin inserts a large conducting pathway into the lipid bilayer membrane containing sphingomyelin, hence allowing cell-impermeable molecules to cross the membrane barrier. In addition, the lysenin pore is irreversibly blocked by biologically inert chitosan molecules. In this way, the lysenin-induced pore formation and the activity act as a nano-valve. In our work, we temporarily permeabilized mammalian cells ATDC5 with lysenin channels and loaded the membrane-impermeable fluorescent dye propidium iodide. The process was blocked by the addition of chitosan and the viability of the cells was assessed by using viable-cell indicators. Similarly, we employed lysenin channels to introduce the cell-impermeant actin marker phalloidin into the cells. These results indicate that lysenin channel can be used as a simple and efficient tool to deliver bioactive molecules into living cells.

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Studying Binding, Conformational Transition and Assembly of E. Coli Cytolysin a Pore Forming Toxin by Single Molecule Fluorescence

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Pore forming toxins (PFT) belong to a class of bacterial toxin proteins that form nano-scaled pores on target cell's membrane and cause unregulated efflux of ions and biomolecules leading to cell death. They are released in a water-soluble conformation which upon membrane exposure undergoes large structural rearrangement. This membrane bound monomer further oligomerizes and forms of a complete pore. While high resolution structural information of complete pores are available, our understanding of early events of PFT binding and assembly is incomplete owing to the highly dynamic nature of the aforementioned processes. In this study, we use single molecule tracking and spectroscopy to understand the dynamics of Cytolysin A (ClyA), a prototypical α-PFT from E. coli, on lipid bilayer membranes. Binding of ClyA to PEG-cushioned supported bilayer was rapid and reached saturation within a few seconds. Diffusional analysis of particle trajectories showed existence of two discrete mobility states exhibiting 'fast' and 'slow' motions. Binding of PFT proteins was invariably in the 'fast' mobility state that was followed by transitions of the single monomer between the two states. The slow moving population was significantly enhanced in cholesterol containing bilayers. We argue that the change in mobility is a consequence of structural transition to an assembly competent intermediate, the protomer state. Preliminary analysis indicates that cholesterol enhances conformational transition by direct binding to the N-terminus of ClyA leading to stabilization of the protomer-like state. This stabilization directly translates to increased formation of higher order structures at high concentrations of protein as measured by analysis of single molecule photobleaching trajectories of pre-formed ClyA pores. Therefore, we propose a molecular mechanism for selective pore formation in eukaryotic membranes driven by conformational selectivity in the presence of cholesterol.

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Role of the Tryptophan-Rich Motif of Listeriolysin O in Membrane Binding

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Listeriolysin O (LLO) is a major virulence factor of the Gram-positive pathogenic bacteria, *Listeria monocytogenes*. It is secreted as a soluble monomer that binds to cholesterol rich membranes, oligomerizes and forms pores. LLO belongs to a group of cholesterol-dependent citolysins (CDCs) that are composed of four structural domains. Mutations in tryptophan-rich domain 4 identified several amino acids as neccessary for binding to cholesterol-rich lipid membranes. However, the exact mechanism at the molecular level is still unclear. LLO was expressed with fluorine labelled tryptophan to obtain greater insight into the role of the tryptophans in LLO binding to cholesterol. The presence of cholesterol-rich membrane bilayers induced significant ^{19}F chemical shift change in LLO, indicating that the toxin bound to the membranes. Moreover, the relatively narrow resonance is likely from a single population, which indicates that LLO is fully partitioned into the lipid bilayer. ^{31}P NMR spectra of d_{31}-POPC/cholesterol vesicles with and without LLO were similar, indicating no loss of lipid bilayer integrity but the ^{2}H spectra showed that LLO had an impact on the order of the lipid acyl chains. Finally, ^{13}C CPMAS revealed a greater broadening of the 3,4-$^{13}C_2$ cholesterol than the lipid carbons in the presence of LLO, suggesting that LLO was preferentially bound to cholesterol.

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Dimerization, a Key Step for Pore Formation of Fragaceatoxin C, an Actinoporin from the Sea Anemone Actinia Fragacea

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Actinoporins are produced by sea anemones, and are excellent models of eukaryotic α-pore forming toxins. Recent research on actinoporins has mainly focused on the oligomerization step on the membrane and the detachment of the N-terminal region. Crystallographic data points suggested the key role of a small hydrophobic protein-protein interaction surface for actinoporins oligomerization and pore formation in membranes. Here, we demonstrated that disrupting the key hydrophobic interaction between V60 and F163 (fragaceatoxin C (FraC) numbering scheme) in the oligomerization interface of FraC, equinatoxin II (EqtII) and sticholysin II (StII) impairs the pore formation activity of these proteins. We combined molecular dynamics simulations with biochemical and biophysical tools to design, obtain and characterize the double mutants FraCV60D/F163D, EqtIIV60D/F163D and StIII58D/I161D which contain mutations in the oligomerization interface. We predicted that these mutations in the oligomerization interface would disrupt dimerization and therefore pore formation. Consequently, the double mutants completely lacked the activity of the wild type proteins, although they maintained the main structural properties of actinoporins and the