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Transport of Cephalosporin Antibiotics Across the Outer Membrane

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Antibiotic resistance is emerging in Gram-negative bacteria worldwide. The outer membrane of Gram-negative bacteria is a formidable selective barrier, and a major factor in broad-spectrum antibiotic resistance. Influx of antibiotics into the Periplasm of bacteria is facilitated by proteins that form channels in the outer membrane. We studied the influx of several cephalosporin antibiotics through the major Escherichia coli porins OmpF and OmpC.

Conductance measurements through purified single porins reconstituted in artificial lipid bilayers allowed us to count the passage of single antibiotic molecules. Statistical analysis of transport events yields the kinetic parameters at the single molecular level. Fluorescence steady-state measurements were used to quantify the interaction between the antibiotics and the porin channels and verify the calculation of translocation kinetics. For the first time, we have been able to characterize facilitated translocation of several β-lactams through OmpC (most expressed porin in vivo) and quantified the distinguishable permeation properties of ceftriaxone, ceftazidime and cefpirome through both outer membrane porins OmpF and OmpC - concluding a stronger interaction with OmpF than OmpC for all three cephalosporins -especially ceftazidime-.

Our approach may be of great benefit to the understanding of porin-drug interaction at the molecular scale and may contribute to the rational design of more efficient antibiotics.

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Molecular Basis of Enrofloxacin Affinity to a Membrane Channel of E. Coli - When Binding Does Not Imply Translocation

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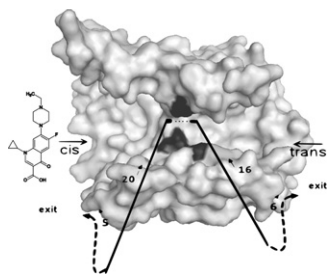
The molecular pathway of enrofloxacin, a fluoroquinolone antibiotic, across the main outer membrane channel OmpF of E. coli is investigated. Through high-resolution ion current fluctuation analysis we count single enrofloxacin channel penetrations, showing a binding to OmpF comparable to the affinity-enhanced translocation through substrate specific channels. A single point mutation D113N increases the dissociation rate 30 times, making the interaction comparable to other antibiotics, corresponding to their weaker binding in a non-specific channel. Molecular dynamics simulations elucidate translocation barriers: WT OmpF has two symmetric binding sites for enrofloxacin located at each channel entry separated by a large barrier in the centre, inhibiting antibiotic translocation. Removal of the negative charge on 113 removes the central barrier shifting both peripheral binding sites to one central site enabling translocation. Fluorescence steady-state measurements confirm simulations. Our results demonstrate that a single mutation of the porin results in a substantial modification of translocation. This example demonstrates how translocation through a channel depends not only on the strength of the substrate-channel interaction, but on a local affinity site counteracting the conformational entropy change at the smallest constriction.



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Permeation of Antibiotics through Bacterial Porins: Screening for Influx on a Single Molecular Level

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Chip based automated patch clamp technique provides an attractive biophysical tool to quantify solute permeation through membrane channels. Proteo-Giant Unilamellar Vesicles (GUVs) were used to form stable lipid bilayer across the micrometer sized hole and single channel recordings were achieved with very low background noise. Influx of antibiotics into the periplasm of gram-negative bacteria is facilitated by porins that form channel in the outer membrane. Influx of two major class of antibiotics- cephalosporin and fluroquinolones through major *E.coli* porins OmpF and OmpC was investigated. Ion current fluctuations through porins in the presence of penetrating antibiotics revealed thermodynamic and kinetic parameters of substrate binding from which we calculated flux. We have been able to show rapid and efficient screening of antibiotics through bacterial porins at a single-molecule scale. In vitro activity of antibiotics was determined by microbiological assays which correlates with the results obtained from lipid bilayer measurements. In addition, molecular modelling provided details on the interaction of the molecules with the channel surface, revealed the preferred orientation of the antibiotic along its pathway and the position of affinity sites. Our approach may contribute to the rational design of new antibiotics against clinical bacterial strains for the most efficient delivery to target sites.

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Probing the Molecular Mechanism of Passive Transport

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The passive transport of small molecules across the plasma membrane is the major pathway by which orally delivered drugs enter circulation and a major route of delivery into target cells. While molecular dynamics simulations suggest that molecules diffusing through lipid bilayers are subject to a complex, dynamic environment, the analytical framework for describing passive transport continues to treat the membrane as a uniform material. The study of passive transport is complicated by the shortcomings of current experimental techniques, in which transport tends to be dominated by diffusion through a stagnant layer adjacent to the membrane.

We have developed an approach to probing passive transport that eliminates these artifacts and allows for a study of relationships between molecular structure and transport properties. This approach consists of confocal microscopy of the diffusion of small molecules into giant unilamellar lipid vesicles (GUVs). Experiments and finite element models show that due to small size of GUVs relative to the characteristic diffusive length scale of transported molecules, no significant stagnant layer is established. In addition, confocal imaging allows for observation of the steady-state association of diffusing molecules with the membrane itself, while other technologies only allow for detection of transported molecules.

We have concurrently developed a fabrication technology that yields GUVs in which each leaflet of the bilayer has a different lipid composition. This allows a novel investigation of the relevance of the asymmetry of the plasma membrane to passive transport.

A series of fluorescent molecules of varying hydrophobicity was synthesized. Time-series images of these molecules crossing GUV membranes were captured, and these images were fit to both an analytical model of membrane permeation and a finite element model of permeation with diffusion. Lipid composition was varied to reproduce the range of compositions observed in human plasma membranes.

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Supported Bilayers with Excess Membrane Reservoir (SUPER): Novel Templates for Vesicular and Non-Vesicular Transport Studies

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Our understanding of membrane-localized processes has largely been gained from the use of liposome-based systems. However reactions involving budding and fission of membranes are difficult to analyze using liposome-based systems since their buoyancy imposes a fundamental limitation on separating end-products of such reactions. Supported bilayers formed by liposome fusion on glass represent an attractive solution. Conventional supported bilayers are however deficient in membrane reservoir necessary for membrane budding and fission reactions. We report a novel system of supported bilayers with excess membrane reservoir (SUPER) and have analyzed factors that contribute to their formation. The excess reservoir in this system originates from higher binding affinity of liposomes to glass and depends on the presence of anionic lipids in the membrane and high salt content in the buffer. This template formed on silica beads allows the seamless application of microscopy-based assays to analyze membrane-localized processes as well as sedimentation-based assays to isolate vesicular and non-vesicular products released from the membrane. We demonstrate the utility of SUPER templates by the direct visualization of amphiphile-induced membrane tubulation prior to solubilization and

dynamin-catalyzed membrane fission prior to vesiculation. Our results highlight the general applicability of SUPER templates in analyzing several forms of vesicular and non-vesicular transport processes.

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Nanopore Formation in Cells Exposed to Nanosecond Electric Pulses

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Chemical and physical insults such as electroporation may compromise the membrane integrity and allow leak ion currents through *de-novo* formed lipid pores. These pores are thought to short-circuit the membrane and hinder ion channels' physiological function rather than to complement it. However, here we report that brief electric stimuli can trigger formation of membrane pores with specific behaviors that are traditionally considered to be unique for protein ion channels; still other behaviors of these pores distinguish them from both ''conventional'' electropores and any known ion channels. We found that a single electric shock (600-ns duration, 1 to 5 kV/cm) causes minutes-long increase of membrane electrical conductance due to formation of long-lived, voltage- and current-sensitive, rectifying, cation-selective, asymmetrical pores of nanometer diameter (''nanopores''). Once induced, nanopores oscillate between open and quasi-open (electrically silent) states, followed by either gradual resealing or abrupt breakdown into larger pores, with immediate loss of nanopore-specific behaviors. The formation and extended lifetime of nanopores were verified by non-electrophysiological methods, namely by fluorescent detection of Tl^+ uptake and of phosphatidylserine externalization. Apparently, nanopores are not unique to cell stimulation with nanosecond electric pulses, but may form under various physiological and pathological conditions. Nanopores appear adequately equipped for certain functions that are traditionally ascribed to ion channels. Clear distinction between nanopores- and ion channels-mediated currents may be critical for understanding how these currents are controlled.

Supported by NIH (NCI) R01CA125482.

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Inhibition of Membrane Stretching Prevents Lipid Pore Enlargement in Cells Porated by Nanosecond Electric Pulses (NSEP)

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Recent studies established that nsEP cause formation of stable pores in plasma membrane of mammalian cells. Pores formed by a single nsEP of moderate intensity (e.g., one 600-ns pulse at 3-5 kV/cm) were permeable to alkali cations, but not to a larger propidium cation (van der Waals dimensions 1.6 x 1.4 x 0.8 nm), suggesting that the pore diameter does not exceed 1.4 nm. However, higher nsEP amplitudes and/or exposures to multiple pulses could cause minor propidium uptake. Meanwhile, the mechanism of transformation of initial ''nanopores'' into larger, propidium-permeable pores remains unclear.

One mechanism potentially responsible for nanopore enlargement could be membrane stretching caused by osmotic cell swelling following nsEP exposure. In isoosmotic and even hyperosmotic bath media, membrane permeabilization to small inorganic ions leads to equalization of their concentrations inside and outside of cells. However, larger molecules cannot escape the porated cell interior, creating additional osmotic pressure. To prevent nsEP-induced swelling, the bath buffer can be supplemented with a membrane-impermeable compound, which would prevent the increase of intracellular osmolarity.

We demonstrated that addition of sucrose (8-40 mM) to the bath buffer decreases or eliminates propidium uptake by nsEP-exposed cells (60-ns, 30 pulses at 30 kV/cm). However, addition of isosmotic amount of NaCl caused little or no protective effect. These data are indicative of the fact that membrane stretching by osmotic cell swelling could indeed be the cause of nanopores enlargement. This hypothesis is being additionally tested to exclude a potentially biasing effect of sucrose and NaCl addition on the efficiency of initial nanopore opening.

The work was supported by R01CA125482 from the National Cancer Institute.

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Lipid Raft and Arf6-GTPase Dependent Endocytosis of the hERG Potassium Channel

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The voltage-gated hERG potassium channel is critical for the repolarisation phase of the cardiac action potential. Genetic mutations leading to a decrease in the number of hERG channels at the cell surface lead to potentially fatal Long QT syndrome which is reflected by an elongated QT interval in the ECG. It is therefore important to understand the trafficking mechanisms that regulate the surface density of hERG. Here we have investigated the endocytic pathway of an epitope (hemagglutinin A) tagged hERG, expressed in HeLa cells, using techniques in molecular cell biology, electrophysiology and biochemistry. Our results demonstrate that the majority of the hERG channel is rapidly internalized from the plasma membrane in a dynamin and clathrin independent manner. Endocytosis of hERG is dependent on cholesterol rich lipid rafts and ADP-ribosylation factor 6 (Arf6). Depletion of cholesterol from cell membrane by treatment with methyl-β-cyclodextrin and disruption of Arf6 activity by over-expression of inactive Arf6 mutants or aluminum fluoride resulted in an inhibition of hERG endocytosis, leading to an increase in hERG currents. Majority of the internalized hERG channel was found in lipid rafts isolated by density gradient centrifugation, identified by the presence of raft marker proteins including flotillin and the co-expressed GFP-tagged GPI anchor protein. Raft associated caveolin, Rac-1 and RhoA-GTPase do not appear to be required for hERG endocytosis. A small fraction of hERG, however, appears to undergo endocytosis via clathrin mediated endocytosis. Following internalization the channel enters Rme1 positive recycling endosomes and also Lamp1-positive late endosome/lysosomal compartments. The significance of lipid-raft and Arf6 dependent endocytosis of hERG in cardiac physiology has yet to be understood. This work was funded by the British Heart Foundation.

1945-Pos

High-Resolution Frap of the Cilium-Localized Somatostatin Receptor 3 Reveals the Presence of a Lateral Diffusion Barrier at the Cilium Base

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It is well known that certain proteins localize to the cilia of cells where they perform various specialized functions, such as sensing the physical environment of the cell. Protein localization to subcellular compartments is important for normal cell function and mutations preventing protein localization to cilia are implicated in devastating human pathologies including blindness, deafness, infertility, obesity and many others.

While the mechanisms for transport of proteins to cilia are well-studied, the mechanisms for maintaining their localization to cilia are not understood. One proposed mechanism for the latter predicts the existence of a selective barrier at the base of the cilium that regulates the free movement of both water-soluble and membrane-bound proteins into and out of the ciliary compartment. We directly tested this hypothesis by examining the mobility of the murine somatostatin receptor 3 protein (SSTR3), a G-protein-coupled receptor that naturally localizes to the ciliary membrane of inner-medullary collecting duct (IMCD3) cells.

Using multiphoton fluorescence recovery after photobleaching (MPFRAP) we estimated the diffusion coefficient and measured the equilibration time of SSTR3-EGFP or SSTR3-PAGFP fusion proteins in IMCD3 cell cilia. We found that SSTR3 fusion proteins rapidly, equilibrate along the length of cilia without change in the total mass, indicating that the membrane-bound protein is highly mobile but remains confined inside the cilium. This finding is consistent with the hypothesis that lateral membrane diffusion at the base of the cilium is constrained.