PORE-FORMING TOXINS (PFT2014)

a meeting in memory of Gianfranco Menestrina

28-30 August 2014, Trento (Italy)

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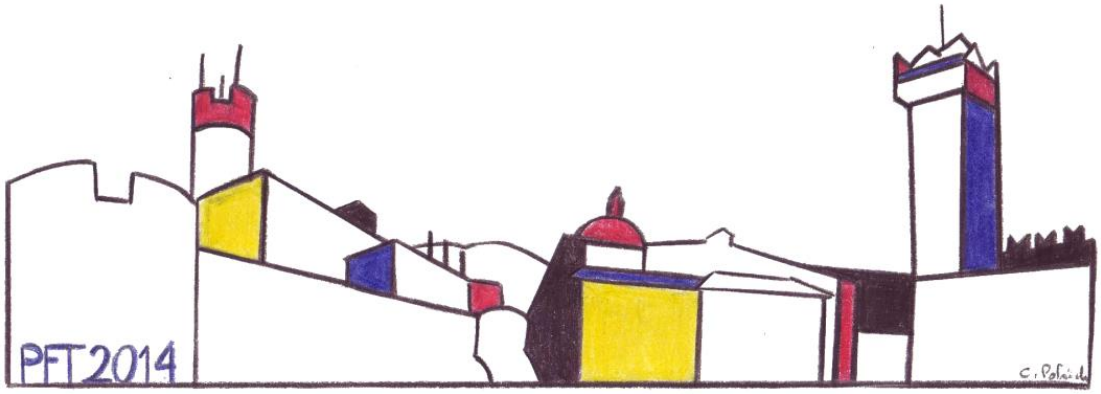

Program – Abstracts – Participant List

PFT2014

Pore-Forming Toxins:

a meeting in memory of Gianfranco Menestrina

28-30 August 2014, Trento, Italy

Science Museum, Corso del Lavoro e della Scienza 3, Trento

Humanities Hub of Bruno Kessler Foundation, via S. Croce 77, Trento

Scientific Committee
Gregor Anderluh (National Institute of Chemistry, SI)
Hagan Bayley (University of Oxford, UK)
Mauro Dalla Serra (CNR-Institute of Biophysics & Bruno Kessler Foundation, IT)
Franco Gambale (CNR-Institute of Biophysics, IT)
Cesare Montecucco (University of Padova, IT)
Cecilia Pederzolli (Bruno Kessler Foundation, IT)
Gilles Prévost (University of Strasbourg, FR)

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L01 In memory of Gianfranco Menestrina

On July 8th 2004, while attending a scientific meeting, I received an unexpected phone call from a desperate friend announcing that Gianfranco Menestrina had died in a motorcycle accident.

I was devastated and totally lost for a long time, unable to figure out whether the news was just a nightmare or a horrible reality I had to face.

Although ten years have passed since that day, the memory of Gianfranco's death is still painful because he was a good friend of mine as, for more than 25 years, we have shared several scientific, institutional and family experiences.

His scientific skills applied to the characterization of membrane transport mediated by peptides as well as bacterial and animal toxins have been widely recognized.

Here I wish to share some aspects of Gianfranco's personality which are less well known.

Gianfranco was very talented both in adopting innovative experimental techniques as well as in the theoretical interpretation of the data. Since we co-authored six papers, I had the opportunity to directly experience not only his rigorous approach but also his capability to extract every tiny piece of information hidden in the details of the raw data. In my career, I have rarely encountered this talent in other people to such a degree.

Gianfranco was a member of the Scientific board of the Institute of Cybernetics and Biophysics of the Italian National Research Council for almost ten years (from 1993 to 2002), in the period when I was the Director of the Institute. During the periodic board meetings, he often demonstrated great awareness of the scientific objectives of the Institute, which he was keen to address on the basis of rigorous evaluations but also using, when needed, intelligent and flexible criteria. He never missed a board meeting , despite the several scientific engagements he used to have both in Trento or abroad. I deeply miss the relaxing dinners we used to have together the evening before each meeting, talking about our families, science, politics and management.

We also collaborated within the Italian Society for Pure and Applied Biophysics. In the summer of 2004, he was the strongest candidate for Presidency of the Society, to be elected during the biennial meeting of the Society in September 2004. Therefore his premature death was a great loss not only for his group but also for the entire Italian biophysics community.

Despite the sadness of this anniversary, today we can see that Gianfranco's legacy was not dispersed as he trained his students and collaborators on a solid scientific basis that allowed the group to survive his passing. Presently the group comprises researchers competing at international level in the field of Pore-forming Toxins as well as in other biophysical topics.

This meeting in itself and the presence of so many renowned scientists and friends of Gianfranco's demonstrate how much he was loved and appreciated.

Goodbye Gianfranco and "… *la terra ti sia lieve*".

Your friends and colleagues

Trento, August 2014

Program

Thursday, 28 August at the Science Museum of Trento (MUSE)

12.00 – 14.00 *Registration and light brunch at the Science Museum of Trento (MUSE)*
14.00 – 14.30 *Welcome address*

Session 1 ***Chairs: Peter Maček, Cesare Montecucco***
14.30 – 15.00 **L01 Franco Gambale**: *Opening lecture*
In memory of a missing friend, Gianfranco Menestrina a brilliant biophysicist
15.00 – 15.30 **L02 Gilles Prévost**: The long way of some staphylococcal leukotoxins to form pores
15.30 – 16.00 **L03 Gregor Anderluh**: Extended superfamily of actinoporins, pore-forming proteins from sea anemones

16.00 – 16.45 *Coffee Break*

16.45 – 17.15 **L04 Teresa Frisan**: Carcinogenic properties of the bacterial genotoxins
17.15 – 17.30 **S01 Matej Butala**: The *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin with a truncated CdtB subunit
17.30 – 17.45 **S02 Michel Popoff**: Pore-forming activity of clostridial binary toxins
17.45 – 18.00 **S03 Kristina Sepčič**: Ostreolysin A-mCherry protein as a tool for tracking cholesterol/sphingomyelin-rich membrane domains

18.30 – 20.00 *Guided visit at the Science Museum of Trento (MUSE)*
20.00 – 23.30 *Aperitif offered on the MUSE terrace and Congress Dinner at MUSE*

Friday, 29 August at the Bruno Kessler Foundation (FBK)

Session 2 ***Chairs: Hagan Bayley, Gilles Prévost***
08.30 – 08.45 **S04 Qiu-Xing Jiang**: CryoEM study of the pore-forming mechanism for human RegIII αlectin
08.45 – 09.00 **S05 Peter Greimel**: Structural insight into mushroom derived homologues of actinoporins
09.00 – 09.30 **L05 Yechiel Shai:** Antimicrobial peptides: pore former or else (**EBSA talk**)
09.30 – 10.00 **L06 Alex Tossi:** Oligomerization and pore formation by the human cathelicidin LL-37
10.00 – 10.15 **S06 Tea Lenarcic**: Biophysical characterization and crystal structure of HaNLP3, a nontoxic NLP protein from *Hyaloperonospora arabidopsidis*
10.15 – 10.30 **S07 Katia Cosentino**: Single molecule approach to study pore formation in membranes by Bcl-2 proteins

10.30 – 11.30 *Coffee Break & Posters*

11.30 – 12.00 **L07 John Kasianowicz**: *Oral presentation in memory of Oleg Krasilnikov:* Nanopore-based single molecule detection and characterization
12.00 – 12.30 **L08 Roland Benz**: AB type of toxins of *Bacillus anthracis and Clostridium botulinum*: differences and similarities
12.30 – 13.00 **L09 Rodney Welch:** *Escherichia coli* hemolysin LPS–dependent and –independent cytotoxic activities

13.00 – 14.20 *Lunch at Orso Grigio*

Session 3 ***Chairs: Gregor Anderluh, Roland Benz***

14.30 – 15.00 **L10 Hagan Bayley**: Polymers through protein pores: single-molecule experiments with nucleic acids, polypeptides and polysaccharides

15.00 – 15.30 **L11 Stefan Howorka:** Membrane-spanning DNA nanopores

15.30 – 16.00 **L12 Mark Wallace:** Parallel optical sequencing of DNA using protein nanopores

16.00 – 17.30 *Coffee Break & Posters*

17.30 – 18.00 **L13 Toshihide Kobayashi:** Pore-forming toxins as tools to image lipids

18.00 – 18.15 **S08 Jan Behrends**: Exploring the biological variability of pore-forming beta-barrel membrane protein assembly with parallel nanopore microarray recordings

18.15 – 18.30 **S09 Tereza Dolejsova**: Characterization of membrane pores formed by colicin U from *Shigella boydii*

18.30 – 18.45 **S10 Nadja Hellmann**: Pore formation by α-toxin from *S. aureus*: addressing cell-specific and general aspects

18.45 – 19.00 **S11 Armando Carpaneto**: The plant vacuole as a biological model system to study the functional properties of exogenous channels and transporters

Free Evening

Saturday, 30 August at the Bruno Kessler Foundation (FBK)

Session 4 ***Chairs: Franco Gambale, Rodney Welch***

08.45 – 09.00 **S12 Daniel Ladant**: Translocation of *Bordetella pertussis* adenylate cyclase (CyaA) toxin across a tethered lipid bilayer

09.00 – 09.30 **L14 Robert Gilbert:** MACPF/CDC proteolipid pores

09.30 – 10.00 **L15 Michelle Dunstone:** A new model for pore formation by cholesterol-dependent cytolysins

10.00 – 10.15 **S13 Peter Maček**: Pore complex of ostreolysin A and a MACPF/CDC-like protein, pleurotolysin B, on cholesterol/sphingomyelin membranes

10.15 – 10.30 **S14 Neval Yilmaz**: Assembling of lysenin on sphingomyelin-containing membranes

10.30 – 10.45 **S15 Gintaras Valincius**: Reconstitution of cholesterol dependent cytolysins into tethered bilayer membranes

10.45 – 11.45 *Coffee Break & Posters*

11.45 – 12.00 **S16 Oneda Leka**: Diphteria toxin conformational switching at acidic pH

12.00 – 12.15 **S17 Marco Pirazzini**: Thioredoxin - Thioredoxin Reductase system is present on synaptic vesicles and its inhibition prevents the neuroparalysis induced by botulinum neurotoxins

12.15 – 12.45 **L16 Cesare Montecucco:** How clostridial neurotoxins translocate into the cytosol of nerve terminals?

12.45 – 14.00 *Closing remarks and light brunch together*

Abstracts

Lectures (L)

L02 The long way of some staphylococcal leukotoxins to form pores

G. Prévost[1], M.Y. Tawk[1], G. Zimmermann-Meisse[1], J.-L. Bossu[2], C. Potrich[3], T. Bourcier[1], M. Dalla Serra[3], B. Poulain[2], E. Jover[1]

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A growing number of cell receptors, are identified for staphylococcal bicomponent leukotoxins. These findings raise the new question of whether the receptors are normally activated or barely used as docking points favouring the formation of a pore. To elucidate if the *Staphylococcus aureus* Leukocidin of Panton-Valentine and the g-Haemolysin HlgC/HlgB act through the C5aR as agonists, antagonists or differ from the C5a complement derived peptide, we explored activities on C5aR expressing cells. Both the two staphylococcal leukotoxins equally bind the C5aR in neutrophils and in stable transfected cells and initiate an intracellular Ca^{2+} mobilization. However, this Ca^{2+} recruitment is different in nature to the C5a triggered intracellular Ca^{2+} transient. The g-haemolysin HlgC/HlgB needs the presence of robust intracellular acidic Ca^{2+} stores to evoke a rise of free intracellular Ca^{2+}, while the Panton-Valentine Leukocidin LukS-PV/LukF-PV directly alters reticular Ca^{2+} stores. The specificity of the signalling is brought by the presence of HlgB. As observed through immune-fluorescent labelling, the internalization of the C5aR with the two leukotoxin components (S- and F-subunits) is required for the initiation of intracellular Ca^{2+} mobilization. Electrophysiological techniques on living cells, in the presence of 1mM Ca^{2+}ext, showed that the LukS-PV/LukF-PV, contrary to HlgC/HlgB did not change the membrane resistance of C5aR expressing cells, which was confirmed in planar lipid bi-layers and in liposomes. Our observations suggest that the pore formation should take place in internal compartments, at least for some of these leukotoxins.

L03 Extended superfamily of actinoporins, pore-forming proteins from sea anemones

G. Anderluh

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Actinoporins are pore-forming toxins from sea anemones characterised by specificity for membranes containing sphingomyelin. They were purified from different sea anemones and one of the most studied examples is equinatoxin II from the sea anemone *Actinia equina*. Biochemical and biophysical characterisation of equinatoxin II and other actinoporins properties and membrane interactions has allowed insight into their molecular mechanism of action. It is known that membrane attachment and sphingomyeling recognition is achieved by an aromatic rich region and that the most important part for the formation of the pore is the N-terminal amphipatic region of the molecule. Currently much attention is focused on characterisation of pore structure and interactions of actinoporins with different lipid domains and these will be discussed in the talk. It is also clear that at least five different protein families exhibit significant structural similarity to actinoporins. I will present these examples and discuss what is known about the ligand binding specificity and role of these proteins for the toxicity of producing organisms.

L04 Carcinogenic properties of the bacterial genotoxins

R. Guidi[1], L. Del Bel Belluz[1], L. Levi[1], M. Rhen[2], S. Fazle[2], S. Puiac[2], M.G. Masucci[1], M. Rhen[2], T. Frisan[1]

Departments of [1]Cell and Molecular Biology, [2]Microbiology Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden; [3]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA; [4]Institute for Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover, Germany.

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Chronic inflammation and infection are associated with an increased risk of cancer development, however the mechanisms by which bacteria contribute to carcinogenesis are still poorly characterized. Several Gram-negative human pathogens produce the cytolethal distending toxin (CDT), which induces DNA damage in the target cells. The effects of intoxication are similar to those evoked by ionizing radiation, a well-characterized genotoxic stress, known to be carcinogenic.

We are focusing on how CDT intoxication alters processes involved in the regulation of genomic integrity, cell cycle progression, cell survival as well as cytoskeleton dynamics and tissue remodelling *in vitro* and *in vivo*.

We demonstrated that chronic exposure to sub-lethal doses of CDT in mammalian cells promoted the acquisition of malignant properties, such as genomic instability in the absence of significant alterations of cell cycle distribution, apoptosis or senescence. This phenotype was associated with impaired activation of the DNA damage response. Cell survival in response to DNA damaging agents is required for tumor initiation /progression, and we showed that cells exposed to the active CDT depend on sustained activity of the p38 MAP kinase pathway as well as on DNA damage-dependent integrin-mediated survival signals.

To dissect the role of the CDT in *in vivo* tumor development, we produced a *Salmonella typhimurium* strain expressing S. typhi CDT-like toxin, known as typhoid toxin (TT), and as control, we used an isogenic strain carrying a mutant inactive. Both strains successfully infected the immunocompetent sv129 mouse strain for more than 2 months. We are currently analyzing the infection burden, the histopathology, the survival and the profile of the immune response in chronically infected animals. We are also characterizing the capacity of TT to modulate the host gut microbiota, since intestinal bacteria are known to play a crucial role in body homeostasis, inflammation and cancer onset in humans.

Our results indicate that chronic exposure to CDT promotes the characteristic traits of tumor initiation/progression, alters the normal DNA damage responses and promotes cell survival, contributing to unravel the molecular mechanism(s) of bacterial-induced carcinogenesis.

L05 Antimicrobial peptides: pore formers or else (*EBSA talk*)

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All types of living organisms including humans and plants produce a large repertoire of gene-encoded cell-lytic peptides that serve as part of their innate immunity to pathogen invasion. They are known also as antimicrobial peptides (AMPs). Most AMPs are characterized by their relatively high hydrophobicity and a net positive charge. However, they have different lengths and a variety of secondary structures including linear and cyclic forms. Despite these diversities most of them are active at μM concentrations, suggesting that they have a common non-receptor mediated target. Indeed, studies have revealed that a major target of most AMPs is the negatively-charged bacterial membrane. These studies support a 'carpet' mechanism for membrane lysis by many native and de-novo designed AMPs. The 'carpet' mechanism describes a situation in which the positively charged peptides first associate with the negatively charged cell membrane through electrostatic interactions and cover it in a carpet-like manner followed by changes in their secondary structures and orientation toward the membrane. In the second step, after a threshold concentration has been reached, the peptides insert into the membrane and permeate it. Continuous membrane permeation can lead to membrane disruption and micellization. An early step before the collapse of the membrane packing may include the formation of transient holes in the membrane. Such holes were described as toroidal pores when the peptide is long enough to span the membrane. Before reaching the inner target membrane they need to traverse the lipopolysaccharide (LPS) or lipotechoic actid (LTA), the protecting cell walls of Gram-negative and Gram-positive bacteria, respectively. We showed that the ability to traverse this barrier depends upon the composition of LPS and LTA, as well as, the biophysical properties of the peptides. Based on the "carpet" mechanism, we designed novel families of diastereomeric (containing D- and L-amino acids) peptides and nano-structured ultra-short lipopeptides. These compounds show cell specific killing activity against a variety of target cells. They have promising properties, which make them attractive templates for the development of future antibiotics with new modes of action, to which it will be difficult for pathogens to develop resistance.

L06 Oligomerization and pore formation by the human cathelicidin LL-37

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LL-37 is a multifunctional innate immune effector, with a membrane-directed antimicrobial activity and receptor-mediated pleiotropic effects on host cells. Sequence variations in its primate orthologues suggest two types of functional features have evolved; those like LL-37 form an amphipathic helical structure and self-assembles under physiological conditions, while those like rhesus RL-37 only adopt this structure in the presence of bacterial membranes. The first type of peptide has a lower and more medium-sensitive antimicrobial activity than the second, but an increased capacity to stimulate host cells. Oligomerization strongly affects the mode of interaction with biological membranes and consequently both cytotoxicity and receptor-mediated activities. We have explored the effects of LL-37 self-association by using obligate, disulfide-linked dimers with either parallel or anti-parallel orientations. These had an increased propensity to form stacked helices in bulk-solution and when in contact with either anionic or neutral model membranes. The antimicrobial activity against Gram-positive or Gram-negative bacteria, as well as the cytotoxic effects on host cells, strongly depended on the type of dimerization. To investigate the extent of native oligomerization we replaced Phe5 with the photoactive residue p-Benzoyl-L-Phenylalanine (Bpa), which on UV irradiation enabled covalent cross-linking and allowed us to assess the extent of oligomerization in both physiological solution and in model membranes. Furthermore, Phe residues in different positions throughout the native sequences of LL-37 and RL-37 were systematically replacing with the non-invasive fluorescent and IR probe p-cyanophenylalanine. Steady-state and time-resolved fluorescence studies confirmed that LL-37, in contrast to RL-37, forms oligomers with a loose hydrophobic core in physiological solutions, which persist in the presence of biological membranes. This correlated with a distinctly different mode of bacterial membrane permeabilization, as determined using a flow cytometric method involving impermeant fluorescent dyes linked to polymers of defined sizes.

L07 Nanopore-based single molecule detection and characterization

J.J. Kasianowicz[1], A. Balijepalli[1,2], J.W.F. Robertson[1], J. Ettedgui[1], D.L. Burden[3], S. Kumar[4], J.J. Russo[4], C. Fuller[4], J. Ju[4], & J.E. Reiner[5]

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Our interest in using nanometer-scale pores for the electronic detection, characterization, and quantification of individual molecules was borne from understanding the mechanism of receptor channels, and keen observations on the alpha-hemolysin ion channel's gating activity (Gianfranco Menestrina) and the interaction of polymers with that channel (Oleg Krasilnikov). Since that time, we demonstrated how nanopores can discriminate between subtlety different ions, separate synthetic polymers (Fig. 1), detect proteins, screen for therapeutic agents against bacterial toxins, and potentially sequence DNA (Fig. 2). In addition, we recently showed that single nanopores can also be used to study the thermodynamic and kinetic properties of molecules, one at a time.

Supported by the NIH NHGRI, NSF, & NIST Office of Law Enforcement Standards.

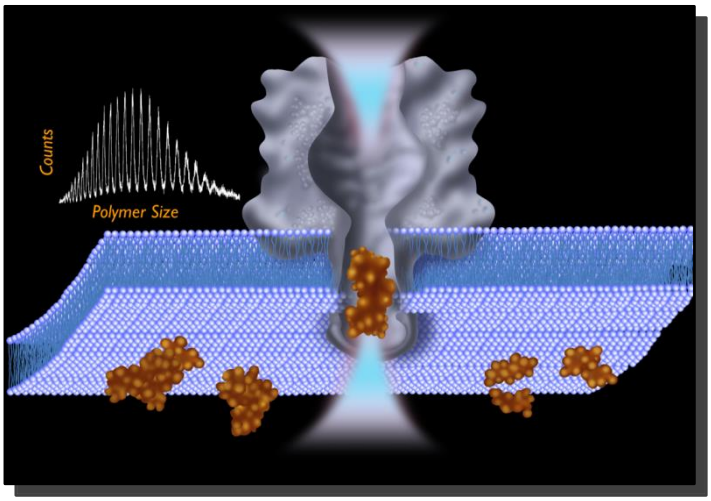

Fig. 1. Single molecule "mass spectrometry".

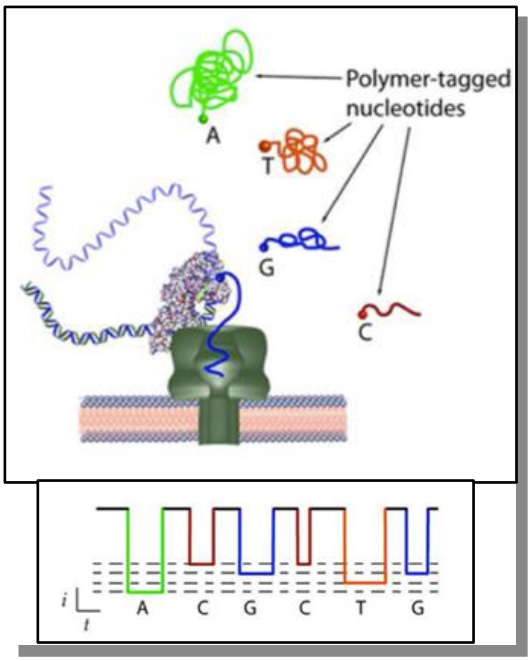

Fig. 2. Nanopore-based DNA sequencing by synthesis

L08 AB type of toxins of *Bacillus anthracis* and *Clostridium botulinum*: differences and similarities

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Binary toxins of the $AB_{7/8}$-type are among the most potent and specialized bacterial protein toxins. The B subunits multimerize to form a pore that binds with high affinity host cell receptors and the enzymatic A subunit. This allows the endocytosis of the complex and subsequent injection of the A subunit into the cytosol of the host cells following acidification of the endosomes. Prominent examples for AB-types of toxins are the tripartite anthrax toxin from *Bacillus anthracis* comprising one binding component (PA) and two toxins; lethal factor (LF) and edema factor (EF) and C2-toxin from *Clostridium botulinum* with one binding component (C2II) and an enzymatic component (C2I) acting as an NAD-dependent ADP-ribosyltransferase on arginine177 of monomeric G-actin. The binding components of AB-types of toxins share a high homology in particular in a domain of the proteins that is responsible for channel formation in biological and artificial membranes. Channel formation has been observed for all binding components of AB-types of toxins (Bachmeyer et al., 2001. Orlik et al., 2004) It is definitely a prerequisite for intoxication of target cells (Bachmeyer et al., 2001). PA and C2II form both highly cation-selective channels in lipid bilayer membranes that are blocked by the corresponding enzymatic components but show also some affinity for not related enzymatic components, i.e. some crossing over between anthrax and C2-toxin. Here we report that the addition of an N-terminal His_6-tag to various proteins increased their binding affinity to the anthrax protective antigen (PA) PA_{63}-channels, irrespective if they are related (EF, LF, C2I) or unrelated (gpJ, EDIN) to the $AB_{7/8}$-family of toxins (Beitzinger et al., 2012). Interestingly, this increase of affinity was shown to be highly voltage-dependent when the voltage at the trans-side, the side opposite to the addition of the proteins had a negative sign. His_6-EDIN exhibited voltage-dependent increase of the stability constant for binding by a factor of about 25 when the *trans*-side corresponding to the cell interior was at -70 mV. Surprisingly, the *Clostridum botulinum* toxin C2II-channel did not share this feature of PA. Cell-based experiments demonstrated that addition of an N-terminal His_6-tag promoted also intoxication of endothelial cells by C2I or EDIN via PA but not via C2II, which represents an important difference between anthrax PA and *Clostridum botulinum* C2II. The results presented demonstrate that addition of His_6-tags to several factors increase their binding properties to PA and property to intoxicate cells. This may define binding component of anthrax toxin as molecular-syringe devices in order to deliver enzymatic activities into host cells.

Bachmeyer, C., Benz, R., Barth, H., Aktories, K., Gibert, M. and Popoff, M.R. (2001). Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes and Vero cells: inhibition of channel function in chloroquine and related compounds *in vitro* and toxin action *in vivo*. *FASEB J.* **15:** 1658-1660.

Orlik, F, Schiffler, B. Benz R. (2005) Anthrax toxin protective antigen: inhibition of channel function by chloroquine and related compounds and study of binding kinetics using the current noise analysis. *Biophys J.* 88(3):1715-24.

Beitzinger C, Stefani C, Kronhardt A, Rolando M, Flatau G, Lemichez E, Benz R. (2012) Role of N-terminal His_6-Tags in binding and efficient translocation of polypeptides into cells using anthrax protective antigen (PA). *PLoS One.* ;7(10):e46964.

L09 *Escherichia coli* hemolysin LPS-dependent and –independent cytotoxic activities

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The role of lipopolysaccharide (LPS) in the structure and activity of the *Escherichia coli* hemolysin (HlyA) is the subject of controversy. There has not been a rigorous demonstration that the HlyA polypeptide can be purified free of LPS while maintaining full HlyA-dependent cytotoxic activities. We cannot separate active HlyA protein from LPS by traditional liquid chromatographic methods or by a variety of affinity capture methods. However, we have developed a purification scheme where the protein in concentrated culture supernatants is denatured by boiling in the presence of sodium dodecyl sulphate (SDS). The material is subjected to SDS gel electrophoresis, the HlyA protein is eluted from the gels and HlyA renatured after removal of the SDS. HlyA prepared in this manner is cytolytic and free of detectable LPS by chemical and physical assays. We previously demonstrated that HlyA secreted in an *E. coli rfaC* deep rough mutant background is nearly inactive against erythrocytes. It is proposed that the adverse effect of rough LPS molecules on HlyA activity occurs when negatively charged, rough LPS aggregates with the HlyA polypeptide after secretion into the medium. We purified HlyA protein from the *E. coli rfaC* mutant. Removal of the LPS resulted in erythrolytic activity identical to that of HlyA from a wild type *E.coli*. We then compared cytotoxic activities of various HlyA preparations against two cultured cell lines. We used Jurkat cells (immortalized human T-cells) and A498 cells (human renal carcinoma cells). LPS-free HlyA from either HlyA (wt) or *rfaC* backgrounds showed identical specific cytotoxic activities against Jurkat cells. LPS-containing HlyA(*rfaC*) was 50-fold less active than HlyA (wt). In the case of A498 cells, LPS-containing HlyA(wt) and HlyA (*rfaC*) preparations were 100-fold more active than the LPS-free versions of HlyA. We conclude that HlyA association and cytotoxicity against Jurkat cells occurs regardless of associated LPS whereas A498 cytotoxicity is strongly dependent on the LPS-associated HlyA protein. Several hypotheses to explain our results will be presented.

L10 Polymers through protein pores: single-molecule experiments with nucleic acids, polypeptides and polysaccharides

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When polymers move from one cellular compartment to another, they pass through protein pores. Nucleic acids, polypeptides and polysaccharides are all transported in this way stimulating questions about the nature of the transported polymer (diameter, stiffness, branching, charge, charge distribution), the driving force (ΔV, ΔpH, refolding, binding) and how that driving force is coupled (direct coupling v diffusion/ ratchet). We have been investigating all three classes of biopolymer by current recording through individual transmembrane pores. We have not only made interesting fundamental discoveries about the translocation processes, but also found useful applications of our work, for example in nucleic acid sequencing and the discovery of antibacterial agents.

L11 Membrane-spanning DNA nanopores

S. Howorka

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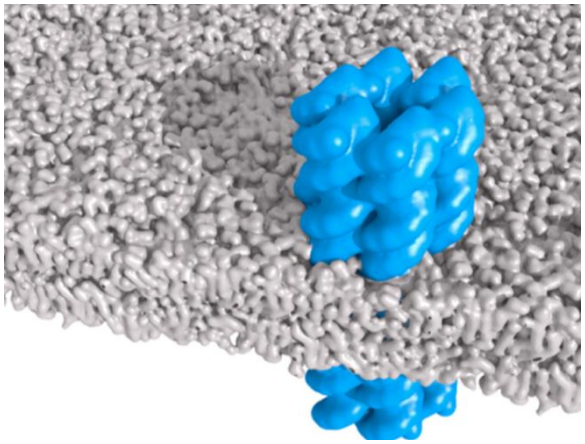
DNA nanotechnology excels at rationally designing functional bottom-up structures. I describe the design and generation of self-assembled DNA-based nanopores that insert into lipid bilayers to support transmembrane water flow. The DNA nanopores consist of a bundle of six hexagonally arranged duplexes which are interconnected by cross-overs. The negatively charged nanobarrels carry lipid anchors to facilitate the pores' insertion into the hydrophobic bilayers. The lipid anchors either neutralize localized negative charges on the DNA backbone[1] or consist of few, large hydrophobic groups [2]. The small membrane-spanning DNA pores merge the fields of nanopores and DNA-nanotechnology and will help open up the design of new molecular devices for applications within single-molecule research and sensing, cell biology, electric circuits, catalysis, and nanofluidics.



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2. Burns, J.R. et al. Lipid Bilayer-Spanning DNA Nanopores with a Bifunctional Porphyrin Anchor. *Angew. Chem. Int. Ed.* 52, 12069–12072 (2013).

L12 Parallel optical sequencing of DNA using protein nanopores

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Pore-forming proteins such as S.aureus alpha-hemolysin can be used to sense the nuclotide-specific blockade in ionic current from single-stranded DNA present within the pore. Commercialisation of these methods have significant potential to increase the throughput of genome sequencing to the point where it can become a routine medical procedure. However, there are practical limits to the required scaling for these methods to sequence an entire genome in a few minutes.

To overcome these limits we use droplet-interface bilayers to enable optical detection of the ionic flux through nanopores present in the bilayer. By optically encoding the ionic flux we are able to parallelise the detection of nucleic-acid binding events within individual nanopores. We report parallel measurement from multiple pores with a maximum density of ~10^5 measurements per mm^2, 3 ms temporal resolution, and sub-pA equivalent amplitude resolution.

L13 Pore-forming toxins as tools to image lipids

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Sphingomyelin (SM) is a major sphingolipid in mammalian cells and is reported to form specific lipid domains together with cholesterol. However, methods to examine the membrane distribution of SM are limited. We demonstrated in model membranes that fluorescent protein conjugates of two specific SM-binding pore-forming toxins, lysenin (Lys) and equinatoxin II (EqtII) recognize different membrane distributions of SM: Lys exclusively binds “clustered” SM composed of 5-6 molecules of the lipid, whereas EqtII preferentially binds “dispersed” SM. Freeze-fracture immunoelectron microscopy showed that clustered and dispersed SM pools formed lipid domains on the cell surface with different sizes. Cholesterol, glycolipids or the membrane concentration of SM affect the SM distribution pattern on the plasma membrane. Using Lys and EqtII as SM distribution-sensitive probes, we revealed the exclusive accumulation of SM clusters in the midbody at the time of cytokinesis. Interestingly, apical membranes of differentiated epithelial cells exhibited dispersed SM distribution whereas SM was clustered in basolateral membranes. Clustered but not dispersed SM was absent from the cell surface of acid sphingomyelinase-deficient Niemann-Pick type A cells. All these data suggest that both the SM content and membrane distribution are crucial for pathophysiological events bringing therapeutic perspective in the role of SM membrane distribution.

L14 MACPF/CDC proteolipid pores

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It was first suggested thirty years ago by Sucharit Bhakdi and colleagues that cholesterol-dependent cytolysins (CDCs) might form membrane pores using incomplete (arc-shaped) oligomers as well as full rings of subunits. A variety of evidence from imaging and functional studies since then has continued to support this idea, among which were important contributions by Gianfranco Menestrina. However, this model for CDC activity has not gained wide acceptance. I will emphasise recent functional and structural data from studies of perforin and pneumolysin which show that MACPF/CDC proteins indeed form pores using arcs of subunits as well as rings, via a prepore-to-pore transition. These proteolipid pores possess valuable characteristics for the carrying out of MACPF/CDC protein biological functions and their biological relevance is supported by studies in living cells. Proteolipid pores in which a toroidal arrangement of lipids plays a key role are also increasingly acknowledged for the separate colicin/Bcl-2 superfamily, underscoring the general importance of toroidal lipid structures in membrane permeabilisation.

L15 A new model for pore formation by cholesterol-dependent cytolysins

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Cholesterol Dependent Cytolysins (CDCs) are important bacterial virulence factors that form large (200-300 Å) membrane-embedded pores in target cells. Currently, insights from X-ray crystallography, biophysical and single particle cryo-Electron Microscopy (cryo-EM) experiments suggest that soluble monomers first interact with the membrane surface via a C-terminal Immunoglobulin-like domain (Ig; Domain 4). Membrane bound oligomers then assemble into a prepore oligomeric form, following which the prepore assembly collapses towards the membrane surface, with concomitant release and insertion of the membrane spanning subunits. During this rearrangement it is proposed that Domain 2, a region comprising three β-strands that links the pore forming region (Domains 1 and 3) and the Ig domain, must undergo a significant yet currently undetermined, conformational change. Here we address this problem through a systematic molecular modeling and structural bioinformatics approach. Our work shows that simple rigid body rotations may account for the observed collapse of the prepore towards the membrane surface. Moreover, our research shows that the CDC toxins use a unique β-barrel architecture to make extremely wide and extremely tall pores. Together, our data provide new and testable model for CDC pore structure.

L16 How clostridial neurotoxins translocate into the cytosol of nerve terminals?

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Tetanus neurotoxin (TeNT) and botulinum neurotoxins (seven different serotypes: BoNT/A, /B, /C, /D, /E, /F and /G) cause a prolonged blockade of neuroexocytosis by entering nerve terminals and performing a specific proteolysis of the SNARE proteins which form the core of the neuroexocytosis nanomachine thus causing neuroparalysis. Their cellular mechanism of intoxication consists of four steps: a) binding, b) endocytosis inside acidic vesicles, c) membrane translocation of the metalloprotease L chain, d) cleavage of either VAMP, or SNAP-25 or syntaxin (1). Steps b) and c) are ill-known.

We recently found that: 1) BoNT/A is endocytosed inside synaptic vesicles; 2) toxin positive vesicles, on average, contain < 1.5 toxin molecules/vesicle; 3) the translocation across the membrane into the cytosol of TeNT and BoNT/C and /D is strongly temperature dependent and it is very low (BoNT/D) or null (BoNT/C) at 20 C; 4) at 37^{o} C the membrane translocation of TeNT, BoNT/A, /C and /D is accomplished within minutes; 5) the NADPH-thioredoxin reductase-thioredoxin system is the main responsible for the reduction of the interchain disulfide bridge of TeNT and the BoNTs which is absolutely required for intoxication; 6) the NADPH-thioredoxin reductase-thioredoxin system is located on the cytosolic face of synaptic vesicles; 7) a novel class of inhibitors of Clostridial neurotoxins that act on this redox system in vitro and in vivo have been identified.

A model for the process of membrane translocation will be presented and discussed.

Selected Orals (S)

S01 The *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin with a truncated CdtB subunit

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Aggregatibacter actinomycetemcomitans (*Aa*) is an oral commensal bacterium often found in association with aggressive forms of periodontitis (DiRienzo, 2014). Fifteen *Aa* strains from patients with generalized advanced chronic periodontitis have been surveyed for virulence factor genes: *ltxA* (leukotoxin), *cdtABC* operon (cytolethal distending toxin, CdtABC), *apaH,* and *flp1* (adherence factors, biofilm formation). We found two *cdtABC*-positive strains harbouring an aberant *cdtB* gene, coding for a truncated form (deletion of 70-amino acids) of the subunit B of the heterotrimeric CdtABC. This deleted region includes H160, one of the two histidines that are essential for DNase activity of various Cdts. The wild-type and truncated CdtB have been cloned and expressed in yeast cells to assess toxicity. Suprisingly, despite missing the catalytic residue H160, the truncated CdtB significantly decreased yeast growth. Moreover, this truncated protein, overexpressed in *E. coli*, exhibited *in vitro* DNase activity, comparable to the wild-type toxin subunit B. Further experiments are in progress to characterize enzymatic activity and cytotoxicity of this novel form of cytolethal distending toxins.

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S02 Pore-forming activity of clostridial binary toxins

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Clostridial binary toxins are organized into enzyme and binding components (BCs) and include *Clostridium perfringens* Iota toxin, *Clostridium botulinum* C2 toxin, *Clostridium spiroforme* toxin (CST) and *Clostridium difficile* transferase (CDT). BCs of clostridial binary toxins retain the same organization in four domains to that of the protective antigen (PA) from *Bacillus anthracis* toxin and are related to the cholesterol-dependent cytolysins (CDCs) the prototype of which is the Perfringolysin (PFO). BCs recognize specific receptors on the cell surface and both BC and enzymatic components are trapped into endocytic vesicles. Then BCs mediate the translocation of the enzymatic components through a pore forming activity across the endosomal membrane. We have determined the pore-forming domain in Ib, the iota toxin BC. Interestingly, albeit Ib and the other clostridial BCs are structurally related to PFO, Ib and clostridial BC use only one amphipathic β-hairpin from each monomer to built the β-barrel instead of two in PFO. Moreover, clostridial BCs form heptamers instead of large PFO oligomers (40 to 50). Indeed, clostrdiail BCs share functional similarities with β-pore-forming toxins (PFTs) of the aerolysin family, which are heptameric PFTs. BCs are proteolytically activated by removing an N-terminal propeptide. The BC cleaved propeptide is much longer (20 kDa) than those of aerolysin family β-PFTs. Furthermore, BC amphipathic β-hairpin share significant amino acid sequence with the corresponding sequences of aerolysin family β-PFTs. The Ib β-hairpin shows 45% identity with that of *Clostridium perfringens* epsilon toxin from the aerolysin family. Like in PA, Ib and C2-II (C2 toxin BC) contain a Phe residue in the vestibule region of the channel forming domain, which influences channel diameter and conductance (Φ-clamp). In addition, we show that Ib uses a second clamp mechanism located at Thr360. Albeit clostridial BCs share global structural organization with PFTs from the CDC and aerolysin families, they retain specific functional aspects. (Bachmeyer *et al.*, 2001, Knapp *et al.*, 2002, Knapp *et al.*, 2009, Knapp *et al.*, 2010, Kronhardt *et al.*, 2011)

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S03 Ostreolysin A-mCherry protein as a tool for tracking cholesterol/sphingomyelin-rich membrane domains

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Ostreolysin A (OlyA), a ~15-kDa protein from the mushroom *Pleurotus ostreatus* has been shown to bind selectively to membranes rich in cholesterol and sphingomyelin [1]. We prepared OlyA fused at the C-terminal with fluorescent mCherry (OlyA-mCherry) and monitored its binding to cholesterol/ sphingomyelin domains in artificial membrane systems and in membranes of fixed and living Madin-Darby canine kidney (MDCK) epithelial cells [2]. While not cytotoxic to the cells, OlyA-mCherry showed similar lipid binding characteristics to non-tagged OlyA. The staining of the MDCK cells by OlyA-mCherry was abolished by pretreatment of the cells by either methyl-β-cyclodextrin or sphingomyelinase. Double labelling of the MDCK cells with OlyA-mCherry and the sphingomyelin-specific markers equinatoxin II–Alexa488 and GST-lysenin, the cholera toxin B subunit as a probe that binds to the ganglioside G_{M1}, or the cholesterol-specific D4 domain of perfringolysin O fused with EGFP, showed different patterns of binding and distribution of OlyA-mCherry in comparison with these other proteins. Furthermore, OlyA-mCherry was internalised in living MDCK cells, and within 90 min it reached the juxtanuclear region *via* caveolin-1–positive structures. No binding to membranes could be seen when OlyA-mCherry was expressed in MDCK cells. Our results clearly indicate that OlyA-mCherry is a promising tool for labelling a distinct pool of cholesterol/ sphingomyelin membrane domains in living and fixed cells, and for following these domains when they are apparently internalised.

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[2] Skočaj et al. (2014) *PLOS ONE* 9: e92783

S04 CryoEM study of the pore-forming mechanism for human RegIIIα lectin

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Human body-surface epithelia coexist in close association with complex bacterial communities and are protected by a variety of antibacterial proteins. C-type lectins of the RegIII family are bactericidal proteins that limit direct contact between bacteria and intestinal epithelium and thus promote tolerance to intestinal microbiota. RegIII lectins recognize peptidoglycan carbohydrates on their bacterial targets, but the mechanism by which they kill bacteria is unknown. In planar lipid bilayers, we found that the RegIIIa inserted into the membrane and formed conductance pores that are permeable to large ionic moieties. In the presence of negatively charged phospholipid membranes, the RegIIIa formed long filaments, whose cryoEM images allowed us to build a 3D structure for the membrane-bound hexameric pores. With the X-ray structure of the mature RegIIIa lectin, we deduced a pseudoatomic model for the transmembrane pore, and provided a mechanistic understanding of why RegIIIα is bactericidal for Gram-positive but not Gram-negative bacteria. Our structural study and the continuum electrostatic calculations suggested a new charge-segregation mechanism for the stabilization of the hexameric pore in negatively charged membranes. Our findings identify C-type lectins as mediators of membrane attack in the mucosal immune system, and provide detailed insights into an antibacterial mechanism that promotes mutualism with resident microbiota.

S05 Structural insight into mushroom derived homologues of actinoporins

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Lipid rafts, regions of the plasma membrane (PM) rich in sphingomyelin (SM) and cholesterol (Chol), provide a dynamic platform for a multitude of cellular functions. Rafts support the compartmentalization of cellular processes at the PM and are involved for example in signal transduction and membrane trafficking. [1,2] Additionally, it has been proposed that rafts play an important role during cellular entry of a wide range of viruses, bacteria and toxins, as well as to serve as a platform for viral assembly and prion and Alzheimer amyloid formation [3-6].

A range of proteins capable to bind to SM or Chol have been reported. [7] Among them, actinoporins represent a well studied group of widely distributed pore forming toxins (PFT) produced by a large number of sea anemone species. The crystal structure revealed the presence of a β-sandwich core flanked by two helices. Recently, we isolated a structurally homologous protein from mushroom. While it only exhibits a 20% sequence similarity, the crystal structure strongly resembles the typical actinoporin fold. Moreover, it also shares the highly conserved Pro-105 residue of actinoproins close to the choline binding site. The results of our point mutation study, focusing on the choline binding site of this novel mushroom derived protein and its implication on actinoporins, will be discussed.

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S06 Biophysical characterization and crystal structure of HaNLP3, a nontoxic NLP protein from Hyaloperonospora arabidopsidis

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Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) are a group of proteins that are secreted by several phytopathogenic micro-organisms. They trigger leaf necrosis and immunity-associated responses in various dicotyledonous plants. The crystal structure of toxic NLP_{Pya} from *Pythium aphanidermatum* uncovered a central β-sandwich surrounded by α-helices. Tertiary structure-based comparison of the NLP fold revealed structural similarity to actinoporins, a family of cytolytic proteins that act by disrupting the integrity of target cell membrane through pore formation. Fold conservation suggests that the two protein families share a common cytolytic mode of action. Interestingly, HaNLP3 protein, produced by the oomycete *Hyaloperonospora arabidopsidis*, while sharing a high sequence similarity to cytotoxic NLPs, does not induce necrosis. Crystal structure of nontoxic HaNLP3 might thus unravel important features for NLP toxicity.

HaNLP3 was expressed and purified from *Pichia pastoris* as a monomeric protein. Circular dichroism (CD) showed a significant proportion of β-structure (> 40 %). Thermodynamic stability of HaNLP3 was studied by CD spectroscopy and differential scanning fluorimetry. The melting temperature of the protein ranged from 42 ºC to 52 ºC, depending on experimental conditions (pH and concentration of NaCl). In general, the stability of the protein was the highest at pH 6. The presence of salt increased the melting temperature of protein at every pH value for 2 ºC on average. Interestingly, thermal denaturation of the HaNLP3 is irreversible at pH 5.0, but reversible at pH 7.0. When kept in pure water, however, the protein only partially renaturated. The crystal structure of HaNLP3 was determined at 2.8 Å resolution. Although HaNLP3 strongly resembles the overall structure of NLP_{Pya}, it reveals some unique features. It has been shown previously that coordination of a metal ion is crucial for cytolytic activity of NLPs. In HaNLP3, however, the coordination site significantly differs from that of NLP_{Pya}, as well as the conformation of the neighboring three loops. Moreover, an additional nonconserved disulfide bond present in HaNLP3 might contribute to limited conformational flexibility of HaNLP3 and thus potentially the absence of toxicity. Altogether, these data allow a step further towards the understanding of cytolytic activity of NLPs.

S07 Single molecule approach to study pore formation in membranes by Bcl-2 proteins

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The Bcl-2 proteins are essential regulators of the mitochondrial outer membrane permeabilization (MOMP) process by forming pores [1, 2]. These pores allow the release of apoptotic factors into the cytosol, such as cytochrome c, inducing caspase activation and mediating cell death [3]. The dysregulation of this function has an important role in the onset of tumors and neurodegenerative diseases [4].
Despite extensive studies, the molecular mechanism of membrane permeabilization by pore formation remains to be clarified.
Here, we studied the assembling mechanism of Bax, a proapoptotic member of the Bcl-2 protein family, which executes pore formation. By using membrane models and a single molecule approach, we found that Bax molecules assembled mainly in dimers, tetramers and hexamers, supporting the hypothesis of Bax oligomerization by dimer condensation. In addition, in the presence of the antiaptototic Bcl-xL protein, a decrease number of oligomeric Bax species was observed, confirming the inhibitory effect of this protein in inducing mitochondrial apoptosis [5].
Furthermore, in order to elucidate the role of lipids in MOMP, we studied the molecular effects of cardiolipin (CL), a lipid mainly found in mitochondrial membranes, on model systems. We found that CL induced membrane reorganization by formation of non-lamellar structures, similar to those found in mitochondrial contact sites, supporting an involvement of this lipid in membrane permeabilization by pore formation [6].

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S08 Exploring the biological variability of pore-forming beta-barrel membrane protein assembly with parallel nanopore microarray recordings

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Recent studies using pore forming membrane proteins in single-molecule detection have concentrated exclusively on analyte interaction with the so-called "canonic pore", displaying a certain conductance under given ionic conditions and presumably corresponding to a pore structure assembled from a particular number of monomers. For instance, for the pore-forming toxin alpha-hemolysin, a conductance of approximately 1 nS in 1 M KCl at pH 7.5 is thought to correspond to a heptameric assembly, while a smaller conductance level is likely to be due to pores formed by a smaller number of monomers[1-4] Using a recently developed microarray device for parallel and high-resolution recording from suspended microbilayers (Microelectrode cavity array, MECA[5-7] we have begun to study the properties of the lower conductance form of aHL-mediated pores. The adavntage of the parallel recording system for these studies is that single canonical pores can be recorded simultaneously with low conductance forms under identical conditions. We found that low-conductance variants tend to appear after prolonged incubation of monomers in recording solution (3 M KCl). Interestingly, these smaller pores are blocked by poly-ethyleneglycol (PEG) oligomers in a fashion very similar to the larger canonical pores[8], showing a similar resolution of PEG mass[6,9-11]. However, surprisingly, the relative position of the maxima in the histogram of relative residual conductances are shifted to larger values for the smaller pores. This finding has potential implications for the mechanism of the block by PEG, in that it suggests that PEG entry into the pore adds a resistance in series with the resistance of the internal constriction site.

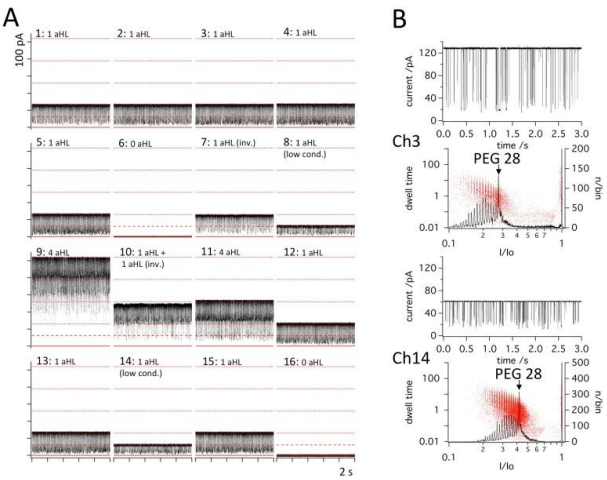

Figure 1: A: Alpha-Hemolysin (aHL) protein nanopores reconstituted on an automatically formed 4 x 4 bilayer array recorded (Tecella Triton 16-channel amplifier) at +40 mV microelectrode potential in the presence of polydisperse poly(ethyleneglycol) (PEG) of mean mass 1500 g/mol) and of an excess of purified PEG28 as a marker) in 3 M KCl (pH 7.5). 11 out of 16 array elements contain a single pore, of which 2 (8,14) are low conductance variants and one (7) is inversely oriented which can be inferred from the smaller conductance. Downward deflections of the current signify entry of a single PEG oligomer into the pore. **B:** Traces recorded from the channels indicated using an Axopatch 200B amplifier that could be switched between channels operated at 10 kHz bandwidth with the corresponding histograms of relative residual conductances (black lines). Red dots show the dwell time distribution (left axis). Note the shift of the maxima to higher relative conductance values for the smaller pore in channel 14 as compared to the canonical pore from channel 3.

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S09 Characterization of membrane pores formed by colicin U from *Shigella boydii*

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Colicin U is a protein toxin produced by *Shigella boydii*, serovars 1 and 8. This protein is active against strains of Gram-negative genera *Shigella* and *Escherichia.* The three-steps mechanism of colicin U interaction with the target cells includes A) binding to the surface receptors OmpA, OmpF and core lipopolysacharide, B) translocation through outer membrane via TolA, -B, -Q or –R proteins and C) final antibacterial lytic action. A high degree of homology can be observed between the C-terminal domain of colicin U and pore-forming colicins A and B. Therefore, colicin U probably also undergoes the same dramatic conformation change from soluble protein to pore-forming protein during its penetration into cytoplasmic membrane.

Using single channel recording on the planar lipid membranes we previously confirmed that colicin U is able to create very stable pores with defined conductance (7,5 pS, 0,1M KCl, pH 6), when negative potential >40mV was applied, and that calcium ions did not affect activity of the colicin. In our present research we have focused on the influence of solutions with various pH values on the properties of the colicin pores and their membrane-incorporation activity. We have observed increased membrane activity of colicin U at lower pH accompanied by changes in pore dynamics. Colicin pore properties are also considerably influenced by membrane composition. Furthermore, we have closely studied the permeability of pores for different electrolytes. We have found that the conductance of pores is strongly affected by both cations and anions, which is in accordance with the observed low ion selectivity of pores.

S10 Pore formation by α-toxin from *S. aureus*: addressing cell-specific and general aspects

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The α-toxin from *S. aureus* is one of the most-studied toxins. Still, the basic mechanism of membrane-protein interactions is poorly understood. The toxin is able to lyse pure lipid membranes if they contain either sphingomyelin or phosphatidylcholine, since these two lipids seem to constitute the primary binding partner. Concentrations necessary to lyse liposomes of about 100 nm diameter are 200 nM and above, depending on the composition. In particular, sphingomyelin in the liquid-disordered phase increase oligomerisation efficiency (Schwiering 2013). In contrast, certain cells are lysed at low or sub-nanomolar concentrations. A protein-based receptor has been suggested to explain this finding, with ADAM10 being the present candidate (Wilke 2010). However, direct physical interaction between ADAM10 and a-toxin was not shown so far. Furthermore, in the last years experimental evidence is accumulating which points towards a role of cellular responses for lysis, involving for example the ATP-gated P2X-channels (Skals 2011). Rabbit erythrocytes are lysed at sub-nanomolar concentrations of a-toxin. We investigate at present, to what extent a cellular response as suggested for other erythrocytes is responsible for this high susceptibility. The experiments indicate so far a possible role of P2X, but not for ADAM10 as receptor and PS-exposure seems to be a consequence of hemolysis rather than a trigger. If cellular amplification takes place then even one or two pores could be enough to lead to lysis. In order to address whether this could occur for a-toxin from *S. aureus*, we developed a model to scale the information obtained from lysis of liposomes to the situation found for cells with their much larger surface. First results indicate that with the same binding and oligomerisation constants which lead to a few pores per liposome also a few pores per erythrocyte are obtained at a 1000x lower overall toxin concentration. Further screening of parameters and concentrations is under way. Modeling the pore formation process will help to combine information from different type of experiments to yield a more quantitative understanding of the underlying mechanism.

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S11 The plant vacuole as a biological model system to study the functional properties of exogenous channels and transporters.

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Plant cells have something that animal cells have not: a large intracellular compartment, the vacuole, which has been investigated for long time. The central vacuole can occupy up to 90% of the cell volume and, differently from intracellular organelles from animal cells as lysosomes or endosomes, is easy to isolate. Because of its large dimension (up to 40 µm diameter) it can be successfully studied using classical electrophysiological techniques such as the patch-clamp. We had the idea that the vacuolar membrane could be used as a convenient model to characterise the functional properties of channel forming peptides. Therefore we verified that the phytotoxic lipodepsipeptide Syringopeptin 25A from *Pseudomonas syringae* pv *syringae* was able to form ionic pores in sugar beet vacuoles and we performed a detailed biophysical analysis. Recently we extended the use of plant vacuoles in the expression and functional characterisation of animal intracellular transporters, named rat CLC-7, and channels, i.e. human TPC2. Since endo-lysosomal transporters and channels are still largely unexplored because their intracellular localization makes them difficult to study, we believe that this novel approach can be seen as a powerful system for the investigation of the molecular mechanisms of exogenous transporters and channels.

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S12 Translocation of *Bordetella pertussis* adenylate cyclase (CyaA) toxin across a tethered lipid bilayer.

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The adenylate cyclase toxin, CyaA, from *Bordetella pertussis*, is a member of the RTX cytolysin family. It displays the remarkable property of invading eukaryotic cells through a unique pathway that involves a direct translocation of its catalytic domain across the plasma membrane of the target cells. To characterize this original translocation process, we have designed an *in vitro* assay based on a novel biomimetic membrane model, in which a tethered lipid bilayer is assembled over a surface derivatized with calmodulin (CaM) on top of an SPR (Surface Plasmon Resonance) biosensor. The assembled bilayer forms a continuous and protein-impermeable boundary fully insulating the underlying CaM (trans side) from the above medium (cis side). SPR spectroscopy is used to monitor the membrane association of CyaA, while activation of CyaA catalytic activity by the tethered CaM serves as a highly sensitive reporter of toxin translocation across the bilayer. Translocation of the CyaA catalytic domain was shown to be strictly dependent upon the presence of calcium, and upon application of a negative trans-membrane potential, in agreement with prior studies done on eukaryotic cells. Our results demonstrate that CyaA does not require any specific eukaryotic components to translocate across a membrane - apart CaM - and suggest an electrophoretic transport of the toxin catalytic domain across the membrane driven by the electrical field. This work constitutes, to our knowledge, the first *in vitro* demonstration of toxin translocation across a tethered lipid bilayer. Such a biomimetic structure opens new opportunities to explore the molecular mechanisms of protein translocation across biological membranes in precisely defined *in vitro* conditions.

S13 Pore complex of ostreolysin A and a MACPF/CDC-like protein, pleurotolysin B, on cholesterol/sphingomyelin membranes

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Ostreolysin A (OlyA), a 15 kDa aegerolysin (Pfam 06355), and pleurotolysin B (PlyB), a 59 kDa MACPF/CDC-like protein, from *Pleurotus ostreatus*, are bi-component pore-forming proteins. Neither of the proteins alone is capable of membrane perforation, however, if combined they oligomerize on artificial lipid bilayers and cellular membranes rich in cholesterol and sphingomyelin. The resulting rosette-like pores with a ~4.9 nm inner diameter resemble those of other MACPF/CDC proteins. It has been shown that the cholesterol/sphingomyelin selectivity and recruitment of pleurotolysin B (PlyB) to these membranes is mediated by ostreolysin A (1, 2). However, mechanism of assembly of the proteins and their lateral distribution on membranes are not known. Here, we have addressed this question by using transmission electron (TEM) and fluorescence microscopy (FM) to visualize OlyA and PlyB on large unilamellar (LUVs) and giant unilamellar vesicles (GUVs), respectively. In TEM, hexahistidine-tagged OlyA or PlyB were labeled with 5 nm Ni-NTA Nanogold particles while OlyA and PlyB fused with fluorescent mCherry and eGFP, respectively, were imaged on GUVs using FM. Altogether, our results suggest that i) the C-termini of OlyA and N-termini of PlyB are located on the outer perimeter of the pore-complex formed on the LUVs membrane and are accessible for the gold particles, ii) pores on the GUVs may be progressively clustered, and iii) the bound proteins may induce shape changes of the GUVs from spherical to prolate, and appearance of membrane nanotubes.

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S14 Assembling of lysenin on sphingomyelin-containing membranes

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Lysenin is a pore-forming toxin known for its specific interaction with sphingomyelin (SM), which is one of the major lipids in the outer leaflet of the plasma membrane of mammalian cells. Previous studies suggest that lysenin oligomerizes after binding to SM and forms 3-nm diameter pores within a hexagonal close-packed (hcp) structure.[1,2] Recently, we revealed the mechanism underlying this hexagonal arrangement on SM/cholesterol membrane using high-speed atomic force microscopy (HS-AFM).[3] The HS-AFM images showed that the formation of the hcp structure took place within a few tens of seconds. Initially, both individual and small domains of lysenin oligomers formed randomly at different locations on the membrane. Although some of these domains grew continuously, most of them reorganized either by dissociating into monomers or by rapidly diffusing along the membrane in less than a second. After the membrane surface had been fully covered with close-packed oligomers, the hcp structure stabilized. Our results suggest that the assembling of lysenin on the membrane into an hcp structure is dependent on the rapid association/dissociation and diffusion of lysenin oligomers.

We also followed the assembling of lysenin oligomers on a ternary SM-containing membrane. The mixture of SM with cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) formed a phase-separated membrane, in which SM localizes with cholesterol as liquid-ordered domains within the fluidic DOPC phase. Oligomers firstly formed on SM/cholesterol-rich domains and gradually covered the entire membrane. Oligomers of lysenin possibly reduced the line tension between liquid-ordered and -disordered phases, resulting in phase merging or homogeneous mixing of two phases. During phase mixing the hcp assemblies of lysenin oligomers, which had formed on SM/cholesterol-rich domains, showed a continuous growth. The oligomers at the edges of the growing assemblies were highly dynamic. We could even follow the diffusion of an individual oligomer through the DOPC-rich phase between the assembly edges. The assembling of lysenin oligomers into an hcp structure both on SM/cholesterol and SM/cholesterol/DOPC membranes indicated that the full coverage of the membrane surface by the close-packed oligomers of lysenin did not depend on SM concentration in the membrane. The dynamic nature of the oligomers of a lipid-binding toxin during its assembling on a lipid membrane could be directly visualized for the first time using HS-AFM in this study.

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S15 Reconstitution of cholesterol dependent cytolysins into tethered bilayer membranes

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We present a comparative study on functional reconstitution of two cholesterol dependent cytolysins: vaginolysin (VLY) and intermedilysin (ILY) into artificial tethered phospholipid bilayers. Cholesterol dependent cytolysins (CDCs) comprise a large family of structurally similar pore-forming toxins (PFT) secreted by Gram positive bacteria. VLY secreted by a *Gardnerella vaginalis* pathogen is a virulent factor in bacterial vaginosis, a gynecological disease that has been linked to infertility and adverse pregnancy outcomes [1]. ILY is secreted by *Streptococcus intermedius,* which is commonly linked to the abscess formation in the liver and brain [2]. Up to 50 monomeric units of CDC oligomerize into membrane-spanning pores that may as large as 30 nm in diameter. The mechanism of reconstitution of CDCs that involves complex sequence of receptor mediated protein binding and oligomerization steps is not fully understood. In particular, both VLY and ILY being strictly cholesterol dependent are human specific, and do not affect other mammalian cells [1, 2]. The specificity is linked to a presence of the CD59 receptor in human plasma membranes [2].

In our study, utilizing minimalistic molecular compositions of tBLMs and extremely sensitive electrochemical impedance spectroscopy (EIS) technique, we detected functional reconstitution of both PFT into tBLMs containing cholesterol but not CD59. In both cases we observed irreversible pore-formation that manifested itself by an increased conductance of tBLMs as well as changes in EIS spectra, consistent with the presence of water filled pores in membrane. According to combined EIS/surface plasmon resonance data both toxins require cholesterol for anchoring to bilayers. Mutations in cholesterol binding domains totally inhibited membrane binding of both toxins Further oligomerization into pores was different for VLY and ILY. In the case of VLY reaching certain level of membrane cholesterol was enough to trigger fast oligomerization into pores. However, the initiation of ILY pore-formation was found to be sensitive not only to a level but also to a way of how cholesterol is introduced into the bilayer. This result suggests the membrane damage by CDCs may be dependent on the physical state of cholesterol in bilayer.

Our work reveals the potential utility of tBLMs in biomedical applications such as detection of pathogens. Fine-tuning of the tBLM composition may significantly modulate sensitivity of tBLM sensors and make it applicable for a particular PFT. Currently, using EIS we were able to detect membrane damaging activity of vaginolysin at concentrations as low as 0.5 nM, which is comparable to immunochemical methodologies albeit does not require expensive antibodies, and may be utilized directly in the media containing pathogen bacteria.

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S16 Diphteria toxin conformational switching at acidic pH

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Diphteria toxin (DT), the protein that causes the homonymous disease, like other bacterial toxins has to undergo a dramatic structural change in order to be internalized into the cytosol, where it finally performs its function. The mechanism of transit of the toxin across the membrane is not well known, but available experimental evidences indicate that one of the three domains of the toxin, called T domain, inserts into the lipid bilayer, so favoring the translocation of the catalytic domain C. This process is driven by the acidic pH of the endosomal lumen. We describe the crystal structure of DT at acidic pH and grown in the presence of bicelles. We were unable to freeze the moment of DT insertion into the lipid bilayer, but our crystal structure indicates the low pH causes the unfolding of α-helices TH2, TH3 and TH4. This causes the consequent exposure of a hydrophobic surface including α-helices TH5 and TH8 and the loop region connecting α-helices TH8 and TH9. Their exposure is probably favored by the presence of lipid bilayers in the crystallization solution, and they appear to be ready to insert into the membrane.

S17 Thioredoxin - Thioredoxin Reductase system is present on synaptic vesicles and its inhibition prevents the neuroparalysis induced by botulinum neurotoxins

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Botulinum neurotoxins (BoNTs) consist of a metalloprotease linked via a conserved interchain disulfide bond to a heavy chain responsible for neurospecific binding and translocation of the enzymatic domain in the nerve terminal cytosol. Once bound to the neurons plasma membrane, BoNTs are trafficked inside the nerve terminal through the recycling of synaptic vesicles whose lumen become acidic after endocytosis, triggering the translocation of the catalytic chain into the nerve terminal cytosol. The metalloprotease activity is enabled only upon disulfide reduction and causes the flaccid neuroparalysis typical of botulism by cleaving the SNARE proteins, i.e. the key components of the regulated neuroexocytosis machinery. We found that the Thioredoxin Reductase-Thioredoxin system is present on synaptic vesicles and it is responsible for the reduction of the interchain disulfide of botulinum neurotoxins serotype A, C and E. Specific inhibitors of Thioredoxin Reductase or Thioredoxin prevent intoxication of cultured neurons in a dose dependent manner and they are also very effective inhibitors of the paralysis of the neuromuscular junction. Remarkably, such inhibitors significantly reduce the lethality of BoNT/A, the most frequent causative agent of human botulism. This is the first group of inhibitors that act very effectively *in vivo,* thus representing good pharmaceutical lead for the prevention and therapy of botulism.

Poster (P)

P01 Pore-forming activity of adenylate cyclase toxin is not required for colonization capacity but contributes to virulence of *Bordetella pertussis*

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The adenylate cyclase toxin-hemolysin (CyaA, ACT or AC-Hly) plays a crucial role in virulence of the whooping cough agent *Bordetella pertussis.* The 1706 residue-long bi-functional leukotoxin targets cells expressing the complement receptor 3 (CR3, also known as the $\alpha_M\beta_2$ integrin, CD11b/CD18, or Mac-1). It delivers into their cytosol an N-terminal adenylyl cyclase (AC) enzyme domain (~400 residues) that upon activation by calmodulin catalyzes unregulated conversion of cytosolic ATP into the second messenger molecule cAMP, thereby ablating bactericidal functions of host phagocytes. In parallel, the ~1300 residue-long hemolysin moiety of CyaA forms cation-selective pores that permeabilize target cell membranes and account for the hemolytic (Hly) activity of *B. pertussis* on blood agar. While importance of the cAMP-elevating activity of CyaA for colonization and immunomodulatory capacities of *B. pertussis* has previously been established, the role of the pore-forming (hemolytic) activity could not be tested before. Here we constructed the first non-hemolytic *B. pertussis* mutant that produces a CyaA exhibiting an intact capacity to elevate cAMP in host phagocytes (AC^+Hly^-). Using the mouse respiratory challenge model we show that the pore-forming activity of CyaA is not required for mouse lung colonization by *B. pertussis* and immunomodulatory capacities of CyaA on mouse dendritic cells *in vitro.* The pore-forming capacity of CyaA, however, importantly contributes to the resulting lung pathology and appears to play a major role in virulence and harnessing of the host response that yields lethality of *B. pertussis* infection in mice.

P02 A nanopore sensor for a post-transcriptional RNA modification: the case of the alpha-hemolysin beta-barrel.

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Post-transcriptional modifications of the 3'-ends of RNA molecules have a profound impact on their stability and processing in the cell. Uridylation, the addition of uridines to 3'-ends, has recently been found to be an important regulatory signal to stabilize the tagged molecules or to direct them towards degradation. Simple and cost-effective methods for the detection of this post-transcriptional modification are not yet available. Here, we demonstrate the selective and transient binding of 3'-uridylated ssRNAs inside the beta-barrel of the staphylococcal alpha-hemolysin (αHL) nanopore, and investigate the molecular basis of uridine recognition by the pore. We show the discrimination of 3'-oligouridine tails on the basis of their lengths and propose the αHL nanopore as a useful sensor for this biologically relevant RNA modification.

P03 Functional characterization of StI W111C, a mutant of the pore-forming toxin sticholysin I, designed for immunotoxins construction.

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The use of pore-forming toxins in the construction of immunotoxins against tumour cells is an alternative for cancer therapy. In this protein family one of the most potent toxins are the actinoporins, cytolysins from sea anemones. We work on the construction of tumour proteinase-activated immunotoxins using sticholysin I (StI), an actinoporin isolated from the sea anemone *Stichodactyla helianthus*. To accomplish this objective StI with a mutation in the membrane binding region has been employed. In this work, it was evaluated the impact of mutating tryptophan 111 to cysteine on the toxin pore forming capability. StI W111C is still able to permeabilize erythrocytes and liposomes, but at ten-fold higher concentration than StI. This is due to its lower affinity for the membrane, which corroborates the importance of residue 111 for the binding of actinoporins to the lipid bilayer. In agreement, other functional characteristics not directly associated to the binding, are essentially the same for both variants, i.e.: pores have oligomeric structures with similar radii, conductance, cation-selectivity, and instantaneous current-voltage behavior. In addition, this work provides experimental evidences sustaining the toroidal protein-lipid actinoporins lytic structures, since the toxins provoke the trans-bilayer movement (flip–flop) of a pyrene-labeled analogue of phosphatidylcholine in liposomes, indicating the existence of continuity between the outer and the inner membrane leaflet. Finally, our planar lipid membranes results have also contributed to a better understanding of the actinoporin's pore assembly mechanism. After the toxin binding and the N-terminal insertion in the lipid membrane, the pore assembly occurs by passing through different transient conductance states. These states, usually 3 or 4, are due to the successive incorporation of N-terminal α-helices and lipid heads to the growing pores until a stable toroidal oligomeric structure is formed, which is mainly tetrameric.

P04 Insights into the structure/activity relationship of the *M. galloprovincialis* antimicrobial peptide myticin C

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One of the most intriguing AMPs of the marine mussel Mytilus galloprovincialis is Myticin C (MytC) because of its remarkable constitutive/induced expression levels, high transcript polymorphism and peculiar expression profiles in individual mussels. The conformation of both Myticin C precursor and mature peptide (40 aa, 4.377 kDa) is still unsolved but mainly depends on an evolutionary conserved array of eight cysteine residues.
Following stepwise and optimized protocols of chemical synthesis, we obtained the mature peptide and smaller fragments, useful to disclose secondary structure in different experimental conditions by circular dichroism spectroscopy and nuclear magnetic resonance.
Aiming to determine the antimicrobial properties of the chemically synthesized MytC peptides and to identify the minimal active fragment we evaluated their bacterial growth inhibition (MIC) and bactericidal potency (MBC) on bacterial and fungal strains. The preliminary results confirm the importance of the β-sheet peptide portion for antibacterial action. In order to better understand structure-activity relationship of this peptide, our next objectives will be to characterize the interaction between our AMP and bacterial membrane and to disclose its mode of action in cell killing.

P05 Effect of the membrane dipole modifiers on the channel-forming activity of polyene macrolide antibiotics

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The role of membrane components, sterols, phospholipids and sphingolipids, in the channel-forming activity of polyene macrolide antibiotics in planar lipid bilayers was studied. The dipole modifiers, flavonoids and styryl dyes, were used as a tool to study the molecular mechanisms of formation and functioning of ion-permeable nanopores formed by antifungal macrolides. It is showed that the introduction of phloretin in the cholesterol- or ergosterol-containing DPhPC-membrane bathing solutions accompanied by a decrease of the conductance of single amphotericin B channels. The quercetin reduced the conductance of single amphotericin B channels in the cholesterol-containing DPhPC-bilayers and did not influence on the single amphotericin B channels in the ergosterol-containing DPhPC-membranes. The introduction of styryl dyes, RH 421, RH 237 or RH 160, in the membrane bathing solutions led to increase the conductance of single amphotericin B channels independently of sterol composition of DPhPC-bilayers. It is shown that phloretin increased the steady-state amphotericin-induced transmembrane current through the DPhPC-bilayers containing cholesterol, 7-dehydrocholesterol or stigmasterol. Moreover, phloretin increased amphotericin-induced steady-state transmembrane current through bilayers made from binary mixtures of DPPC (DOPC) and ergosterol and ternary mixture of DPhPC, ergosterol and stearoylphytosphingosine. At the same times, RH 421 enhanced the steady-state amphotericin-induced transmembrane current only through ergosterol-containing DPhPC-bilayers. RH 421 increased the steady-state amphotericin B induced transmembrane current through membranes made from binary mixtures of DPhPC (DPhPS) and ergosterol and ternary mixture of DPhPS, ergosterol and stearoylphytosphingosine. The results of testing of various phospholipids, sphingolipids, sterols, polyene antifungals and dipole modifiing agents led to the conclusion that the influence of flavonoids and styryl dyes on the channel-forming activity of polyene antibiotics had a complex nature and was due to the superposition of several factors: the changes in the membrane dipole potential, the influence of dipole modifiers on the stability of polyene-sterol complexes, and/or the effect of dipole modifiers and polyenes on the properties of lipid ordered domains in the bilayer.

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P06 NPP binds to lipids from plasma membrane

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Necrosis and ethylene-inducing peptide 1 (Nep-1)-like proteins (NLP) form a superfamily of proteins which cause strong immune response in dicotyledonous which leads to cell death. They are secreted by various plant pathogens which originate from bacteria, fungi and oomycetes. The mechanism of NLP action is still unknown. Due to their great structural similarity with actinoporins it was suggested that they bind to a specific lipid molecule in plasma membrane.
Binding of NPP1 from *Phytophtora parasitica* to the lipids from tobacco plasma membranes was investigated. The lipid extracts were fractionated according to their density using gradient centrifugation on Percoll. Floatation assay with different fractions showed NPP1 binds to specific fraction only and thin layer chromatography was used to identify lipid class the fraction belong to.
The binding to the phosphatidylinositol class of lipids was tested using the surface plasmon resonance. The NPP1 was attached to the surface of a chip and different inositol phosphates were titrated using different conditions. There was a weak binding observed with D-myo-Inositol 1,3-bisphosphate and D-myo-Inositol 1,5-bisphosphate.

P07 High-throughput method for searching of binders and modifiers of pore forming toxins' activity

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Pathogenic bacteria often use pore forming cytolitic proteins as virulence factors. The basics of the pore forming mechanism are known, however for several different families of pore-forming toxins many details remain unclear. Novel insights might be unveiled by a new molecular biology technique, called ribosome display. It is an *in vitro* technique for directed evolution of protein scaffolds, where we can obtain highly specific binders for virtually any target of interest. The scaffold with exquisite binding characteristics used in our study was DARPin (Designed Ankyrin Repeat Protein). We designed it to contain 5 consensus repeats, forming a large binding surface, and randomized 20 aminoacid positions at the exposed sites of the protein. Upon binding of proteins, selected from deliberately designed gene library of DARPins, it is possible to identify the sites, important for different stages of pore formation, or utilize it for specific labeling of pore forming toxins. After several rounds of ribosome display selections, we obtained specific binders that bound with high affinity to Listeriolysin O (LLO), but not to other proteins. The binding of DARPins was confirmed by ELISA and micro-scale thermophoresis (MST) experiments. According to the sequencing results of 25 clones, the selection was succesful. Comparison of the aminoacid composition at randomized sites before and after selection showed statisticaly significant ($p < 0.005$) difference between them. We propose the ribosome display technique to be useful and inovative way of studying pore forming toxins.

P08 Structural consequences of the interaction of perfringolysin O and its derivative with liposomes – hydrogen-deuterium exchange study.

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Perfringolysin O (PFO) is a toxin secreted by *Clostridium perfringens*. PFO belongs to the cholesterol-dependent cytolysin (CDC) group. The interaction of perfringolysin O with cholesterol-rich membranes induces a series of structural changes which result in the formation of a ring-shaped-barrel pore [1]. The assembly of oligomeric pore is initiated by recognition of cholesterol by the tryptophan rich fragment of sequence localized in D4 domain of protein [2]. After that, PFO monomers that have been bound to the lipid membrane interact with one another through β1-β4 strands which leads to the formation of a pre-pore structure on the surface of the lipid bilayer. Finally, transmembrane β-hairpins are inserted into the lipid bilayer forming a large pore. Some of complex structural changes in perfringolysin O remain unrevealed.
In an effort to thoroughly understand the conformational transitions of PFO we compared the structural dynamics of wild-type perfringolysion O with its mutant derivative. Proteins have been analysed in two different states: a protein in aqueous buffer and a protein incorporated into the lipid bilayer. In perfringolysin O mutant's sequence the tryptophan residue was substituted with non-aromatic amino acid (W165T in D1 domain of perfringolysin O) which results in loss of hemolytic activity of protein. The dynamics of perfringolysin O structural changes were analyzed using hydrogen-deuterium exchange coupled with mass spectrometry (HDX MS). An incorporation of PFO into liposomes resulted in reduction of hydrogen-deuterium exchange especially in regions involved in oligomerization and binding to the lipid bilayer.
Our studies showed that disorder in D1 structure of PFO^{W165T} breaks the pathway of structural transitions in other domains, necessary for the formation of ring-shaped oligomers. That result gives a new insight into perfringolysin O structure and bring us closer to clarification of the role of particular domains in conformational changes of PFO during pore formation.

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P09 Properties of Listeriolsyin O transmembrane pores

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Listeriolysin O (LLO) from *Listeria monocytogenes* is one of the most important virulence factors of this food-borne intracellular pathogen. *L. monocytogenes* can be found in the soil, water, sewage, feces of wild and domesticated animals and also in human food. It causes listeriosis, a disease that mainly affects children, older people, other immunocompromised individuals and pregnant women.

LLO is a member of a protein family called Cholesterol-Dependent Cytolysins (CDC), including various bacterial Pore-Forming Toxins (PFTs). LLO is unique in respect to other CDCs because its stability and activity is highest at acidic pH. This property is crucial for the intracellular lifestyle of the pathogen since LLO is essential for the escape of *L. monocytogenes* from the acidic environment of phagosomes. By perforating the phagosomal membrane it enables the bacterial cell to enter the cytosol where it grows and then infects surrounding cells. LLO is expressed as a soluble monomer that later binds to cholesterol-rich membranes, where monomers oligomerize into circular complexes called prepores, which further dramatically change conformation to form β-barrel transmembrane pores. Pores are comprised of up to 50 monomers and have a diameter of about 30nm. These pores are structurally poorly characterized, as well as their lipid specificity and pH dependence.

We used 1-palmitoyl-sn-glycero-3-phosphocholine and cholesterol (ratio 1:1) planar membranes to study LLO pore-forming ability at pH 5.5 and 7.4. Surprisingly, the lipid planar membrane experiments showed a significantly different distribution of pore conductivity between the two pH values, despite the fact that atomic force microscopy (AFM) images indicated that the pH does not affect the physical size of the pore. We showed that by mutation of a single amino acid residue (His311), we can affect the conformational stability of the LLO monomer, the speed of pore formation and pore conductivity. Here we show for the first time the pH dependent plasticity of LLO pores, and how a single point mutation can affect LLO pore properties.

P10 Lipid model systems for interaction studies of listeriolysion O binding to lipid membranes

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More than 20 different pore-forming proteins that are expressed in various bacterial species form a family of cholesterol-dependent cytolysins (CDCs). The first step in pore formation is the CDCs monomers binding to host membranes. It only occurs if cholesterol content in target membrane is high, but the exact mechanism of cholesterol recognition is still unclear. For the studies of membrane-protein interactions of CDCs many membrane models were already successfully used, but the limitations exist, especially where the size of the models is in question. Cholesterol is insoluble molecule and has to be incorporated in larger lipid system or dissolved in organic solvents, such as chloroform, ethanol and others. We have prepared several small membrane sistems that have the potential to be used in interaction studies of CDCs with cholesterol. Our models were tested on listeriolysin O (LLO), a CDCs that is secreted by Gram-positive pathogen bacteria *Listeria monocytogenes*. We showed that cholesterol or its analog 5-cholesten-3-one dissolved in chloroform or ethanol inhibits haemolytical activity of LLO on red blood cells. However an analog cholesteryl acetate showed no impact on LLOs activity. We have also prepared bicelles and nanodiscs, lipid model membrane systems that are smaller than commonly used uni- or multilamellar vesicles and that could be used for interaction studies with methods such as solution state NMR. However, the amount of incorporated cholesterol was not high enough to allow stable binding of LLO and consequently inhibition of LLO's heamolytical activity. Bicelles and nanodiscs will need to be optimised for significant cholesterol incorporation, whereby mixtures of different phosphatidilcholin molecules could be tested.

P11 Perforin forms functional arc-like pores.

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Cytotoxic cells eliminate malignant or virally infected targets through interplay of pore forming protein Perforin (PFN) and serine proteases (Granzymes). How PFN assists transport of granzymes across the bilayer is unclear. Granzyme delivery can occur without evidence of PFN mediated cylindrical pore formation. Susceptibility to granzyme B (GzmB) induced death correlates with rapid PFN-induced phosphatidylserine externalization in non-permeabilized targets. These observations suggest that PFN may form limited membrane oligomers that encourage plasma membrane coalescence and flip-flop of anionic phospholipids from the inner leaflet, namely toroidal pores. Addition of increasing concentrations of a monoclonal antiperforin antibody (pf-80) to PFN treated targets rescues them from necrosis caused by cylindrical pores while increasing phosphatidylserine associated flip-flop and GzmB induced apoptosis. By atomic force microscopy (AFM), PFN can produce arc-like structures having measureable conductances in planar lipid bilayers, that are augmented by pf-80. These arcs represent toroidal pores that offer sites for granzyme translocation through the bilayer.

P12 The crystal structure of the C-terminal repeat blocks of adenylate cyclase toxin from *Bordetella pertussis*

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C-terminally located calcium-binding repeats-in-toxin (RTX) domains represent a structural hallmark of virulence factors secreted through the type I secretion system (T1SS) of Gram-negative bacteria. These RTX domains consist of tandemly repeated nonapeptide sequences that fold into characteristic parallel β-roll structures with two facing sheets of β-strands linked by calcium-loaded turns. The RTX domain of an adenylate cyclase toxin (CyaA), the 1706-residue long bifunctional toxin with a cell-invasive and pore-forming activity produced by *Bordetella pertussis*, is predicted to be organized into five successive blocks (I-V) separated by flexible linkers of variable lengths. Here, we show the X-ray structure of the last two C-terminal RTX blocks (IV-V) of CyaA solved to 1.8 Å resolution. Although the first 39 amino acids of the crystallized protein were missing in the crystal, the structure shows two blocks of regular RTX repeats assembled in a right-handed helix of parallel β-strands (β-roll). These are separated by several β-strands, which along with parallel β-strands of β-roll, form a continuous stack of β-strands along both sides of the molecule. Eight and seven calcium ions were unambiguously resolved in block V and block IV, each coordinated by aspartic side chain and by carbonyl groups of the polypeptide backbone chain of the tandem GGxGxDxxx motif. Determination of 3-D structure of RTX domain of CyaA paves the way for understanding the mechanism of interaction between CyaA and the cell surface receptor CD11b/CD18 and subsequent translocation of adenylate cyclase enzymatic domain through the plasma membrane of target cells.

P13 Interactions of perforin with ions and membrane

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Perforin (PFN) plays a vital role in the immune system. It is important for the elimination of malignant cells and cells infected with intracellular pathogens. Once released by killer cells, PFN binds to the target cell membranes in pH- and calcium- dependent manner, oligomerizes into a ring- or a half ring- (arc-) like structures and forms pores that induce death of the target cells. Initial ion-dependent membrane binding is regulated by a PFN's C-terminal C2 domain, whereas final pore formation is controlled by the N-terminal MACPF domain. In order to elucidate structural and dynamical changes of PFN upon calcium binding in solution, we performed two comparative all-atom molecular dynamics simulations of protein in the presence and absence of calcium ions. The simulation showed that the structure with calcium is overall more stable. Additionally, implicit molecular simulations of PFN in the presence of lipid membrane indicate the significant difference in orientation of PFN with respect to the lipid membrane. Simulation of PFN with all-atom membrane expectedly showed that calcium promotes stronger interaction with phospholipids. To address the biochemical features of PFN during the first steps of pore formation, we expressed fully-active recombinant PFN. Surface plasmon resonance (SPR) studies indicate that native human PFN strongly interacts with some divalent ions but hemolytic studies of recombinant PFN show that only calcium ions can promote its hemolytic activity, while strontium and nickel ions inhibit its hemolytic activity. Overall, we successfully constructed *in silico* model for studying PFN-membrane and PFN-ion interactions and *in vitro* approaches indicate that PFN activity can be inhibited with some of the metal ions.

P14 Equinatoxin II, an actinoporin from the sea anemone: pore formation mechanism, oligomer stoichiometry and lipid phase dependence

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Equinatoxin II (EqtII), isolated from the sea anemone *Actinia equina*, is an archetypal example of actinoporins, which belong to α-pore forming proteins (α-PFT). Isolated from the whole body extract, it probably plays a role in catching pray, defending against predators or even pray digestion. It is well established EqtII binds sphingomyelin (SM) in the target membrane, where it inserts N-terminal amphiphatic α-helix and forms cation selective pores. To form a functional pore assembly of several EqtII monomers on the membrane plane is necessary, however, due to pore complex instability the biochemical data regarding functional pore is insufficient. Firstly, it is not clear if a prepore complex is a prerequisite for pore formation. We used fluorescently labelled EqtII molecule in a series of kinetic experiments to show oligomerisation occurs simultaneously to α -helix movement from cis- to trans- membrane site, a process very different to most of the β-PFT with clear prepore formation. Secondly, there are two different models of pore structure. Biochemical data indicates a tetrameric pore with lipids present in the pore walls, whereas crystallographic data suggested a non-toroidal nonameric structure. With the use of Cy3B labelled EqtII and photobleaching technique on the supported bilayer we concluded the stoichiometry distribution is broad with the average of 3.3 monomers in one complex, directly questioning the validity of the actinoporin nonameric model. And thirdly, there is a conflicting evidence of the lipid phase separation role on EqtII activity. As EqtII binding depends on the SM, we hypothesized actinoporins form a functional pore in liquid-ordered membrane domains, where SM and cholesterol are enriched. Surprisingly, we observed single pore ionic flux across the artificial lipid membrane predominantely in the liquid-disordered membrane phase. Although EqtII is concentrated in the phase boundary as also shown previously, there are no pore opening events in this region.

P15 Biochemical and functional analyses of *Escherichia coli* ClyA

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E coli Cytolysin A (ClyA) belongs to the alpha group of pore forming toxins. It oligomerizes as a dodecamer and this switch in state is accompanied by large topological reorganization of its tertiary structure. The molecular mechanisms underlying pore formation are not completely understood. To explore this in greater detail, ClyA was cloned, expressed and purified to homogeneity and activity was estimated. Upon biochemical analysis, It was observed that a critical concentration (175 -200 ng) was required for lysis. A kinetic model was developed with the aim of quantifying lysis dynamics of ClyA using the Langmuir-Hinshelwood adsorption-desorption paradigm as framework for monomer binding and pore formation. Various oligomerization schemes were tested and the sequential irreversible mechanism was found to be in best agreement with experimental data.

The C-terminus of ClyA has been shown to be an essential for its activity. Deletion of residues from the C-terminus renders the protein inactive. This region appears as a solvent exposed loop in the monomeric structure and no electron densities are observed for the corresponding residues in the pore structure. RMSD trajectories of fully atomistic molecular dynamics simulations of the pore (lacking residues in C-terminus) in POPC and DMPC lipid membranes exhibit minimal deviation for ~ 100 ns and similar trends are observed for coarse grained martini simulations carried out for ~ 38 μs indicative of a stable structure. This implies that albeit pore formed by deletion mutants can remain stable, the rate limiting steps could precede pore formation. Pathway proposed by examination of X-ray structures of monomer and oligomer do not attribute any role for this region in pore formation and it is imperative to understand this in the context of pore formation considering obligate requirement of this region for activity. In order to test these results experimentally, a series of deletion mutants lacking residues from C-terminus were generated to investigate its role in pore formation. Membrane binding, oligomerization and pore geometry are key steps in the pathway that were assessed for the mutant proteins in order to establish the critical step that resulted in drastic loss of activity. In order to study these processes *in vitro,* artificial lipid bilayers were synthesized on glass substrates using the vesicle fusion method. The ability of ClyA to form pores on supported lipid bilayers were assessed by AFM.

P16 Lipid bilayer resistance to surfactin-induced leakage is provided by modification of its phospholipid composition

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Surfactin, a cyclic anionic lipoheptapeptide produced by *Bacillus subtilis*, is a promising antimicrobial compound. Surfactin inserts in lipid membrane and compromise its barrier function by forming pores and, with increasing concentration it completely disrupts the entire membrane. In our study we aimed to clarify the mechanism which *B. subtilis* employs to protect its membrane from being disrupted by otherwise toxic concentrations of its own product. First, we investigated surfactin's pore forming activity in vitro using BLM (Black Lipid Membranes). Surfactin at a concentration of 2 to 4 μM formed variety of pores with broad conductance distribution ranging from few pS to over 1 nS with Poisson-like distribution. Analysis of membrane composition of *B. subtilis* cultivated in sublethal concentration of surfactin showed significant differences both in fatty acid and polar head group region. Most interestingly, presence of surfactin resulted in reduction in the content of major membrane phospholipid PG from 44% down to 20% and increase of PE from 24% up to 40% accompanied with increase of acidic phospholipids such as CL and PA. Fatty acid analysis showed marked increase in high melting fatty acids at the same time. Liposome leakage of CF loaded vesicles mimicking the phospholipid composition of surfactin-adapted cells showed that the susceptibility to surfactin-induced leakage is strongly dependent on the composition of target bilayer. We concluded that the phospholipid modification of *B. subtilis* cells might provide self-tolerance to the membrane active surfactin.

P17 Characterization of extracellular vesicles induced by Ostreolysin A -mCherry in MDCK cell membranes

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Ostreolysin A –mCherry (OlyA-mCherry), a fluorescent recombinant protein developed recently in our laboratory, has shown to be a promising tool for labelling a specific pool of cholesterol/ sphingomyelin membrane domains in living and fixed cells [1]. However, when incubated with living Madin-Darby canine kidney (MDCK) epithelial cells for longer times (≥5 minutes), and/or in concentrations ≥1 mM, OlyA-mCherry induced the formation of relatively large (2-10 mm diameter) extracellular membrane vesicles in these cells. This process, however, did not lead to cell death; indeed, we demonstrated that the cells remained viable and metabolically active even after 24 h of exposition to 1 mM OlyA-mCherry. The number of these extracellular vesicles increased proportionally with increasing concentration of OlyA-mCherry and was temperature-independent. Although we could not detect their cholesterol content by NMR, LC-MS based lipidomic analyses of these vesicles identified 84 lipid molecular species. In the vesicles, enrichment in lysophospholipid and diacylglycerol species as well as variation in some other lipid classes (phosphatidylcholine and phosphatidylserine, etc) was noticed. Meanwhile, the proteome analysis indicated the presence of several membrane-associated and cytoplasmic proteins. These results suggest that OlyA-mCherry - induced extracellular vesicles could be used as tools for non-invasive sampling of both plasma membrane and cytosol of different cells. Membrane vesiculation has been previously observed in cholesterol/ SM 1:1 vesicles exposed to recombinant OlyA [2], as well as in fixed MDCK cells, and in several other fixed or living cells. Therefore, we suggest that the vesiculation is not a cell-regulated process but results from a direct physical effect of OlyA-mCherry on targeted membranes.

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[2] Ota et al. (2013) *Biochimie* 95:1855

P18 The influence of extracellular Ca^{2+} on the staphylococcal leukotoxins activity acting through C5aR

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Staphylococcus aureus is responsible for a high proportion of nosocomial infections that are mediated by a wide range of virulence factors. Two components, S-F, leukotoxins are included in these virulence factors, and Panton and Valentine Leukocidin LukS-PV/LukF-PV (PVL) and the γ-haemolysin HlgC/HlgB are two of their representatives. Both of these toxins target human cells, especially immune cells such as polymorphonuclear neutrophils or macrophages. Cells need to have the LukS-PV/HlgC class S component receptor C5aR, the natural receptor of the complement-derived protein C5a. With flow cytometry, immunocytochemistry and confocal microscopy, we evaluated the effect of these two toxins on human neutrophils in the presence of 1mM Ca^{2+} (physiological extracellular concentration) or not. The PVL and HlgC/HlgB in absence of extracellular calcium rather lead to a membrane damaging activity with cell death, probably activated by the same complex. The process of cell death is being characterized. The presence of Ca^{2+} in the medium triggers an internalization pathway and intracellular calcium release. In this way, PVL and HlgC/HlgB modify the intracellular Ca^{2+} homeostasis after binding the complement C5a receptor. But, the two toxins do not appear to activate a unique intracellular pathway. Actually, the F component of these leukotoxins seems to lead the activation of intracellular pathway and toxic effect.

P19 Characterization of a new class of scorpion metalloproteinases

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Scorpions develop complex venoms to capture prey and warn predators. In particular, animals that belong to *Buthidae* family i.e. *Tityus* species, including all the medically significant species, have a venom and toxins composition which to date has gone largely untapped.
Tityus venoms are able to induce exocytosis and, recently, it has been reported that a metalloprotease, called antarease, causes the specific proteolysis of the VAMP/synaptobrevin protein. Up to now, the clostridial toxins were unique in their ability to cleave SNAREs and this scorpion toxin could turn out to be a novel tool to study the SNARE apparatus.
We are characterizing the activity of antarease recombinant form and searching other scorpion metalloproteases with a similar activity, making a comparison with the well-known clostridial toxins. A rapid paralysis is detectable in *ex vivo* neuromuscular junction preparations of mammals and insects, which are the main scorpion targets.
To investigate on these scorpion toxins might be very important to uncover new aspects of intracellular trafficking events. Moreover, these proteins could be considered as target to develop more efficient anti-scorpion therapies.

P20 Functional and structural studies of trypanolytic pore formation by ApoL1

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Trypanolytic factor 1 (TLF1) is a human lipoprotein particle that protects against infection by *Trypanosoma brucei brucei*. It has been hypothesised that apolipoprotein L1 (ApoL1), a protein component of TLF1, is able to form pores in the lysosome of the parasite, causing cell lysis. The aim of this study is to characterise TLF1 and ApoL1, and determine the mechanism of parasite lysis. By assaying escape of fluorescent dye from liposomes, we have shown that TLF1 can form pores in lipid bilayers without affecting the integrity of the liposomes. The concentration dependence of dye leakage indicates that individual ApoL1 molecules may act alone in pore formation and remain in the targeted membranes once this has occurred. In addition we have obtained a cryo-EM reconstruction of the TLF1 particle to reveal a nanodisc-like assembly. Further work is needed to characterise the structure and selectivity of the ApoL1 pores and of the TLF1 particles from which they are generated.

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Notes

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